Studies on factors influencing viability after cryopreservation of excised zygotic embryos from recalcitrant seeds of two amaryllid species

by

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As the candidate's supervisor I have/have not approved this thesis/dissertation for submission.

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Abstract

Recalcitrant unlike orthodox seeds do not show a sharp border between maturation and germination and remain highly hydrated and desiccation-sensitive at all developmental and post-harvest stages. In contrast with recalcitrant seeds, orthodox types retain viability for predictably long periods in the dry state and hence can be stored under low relative humidity and temperature conditions. Storage of recalcitrant seeds under conditions allowing little to no water loss, at moderate temperatures, allows for short- to medium-term storage but only facilitates viability retention for a matter of a few weeks to months, at best, because the seeds are metabolically active and initiate germination while stored. Cryopreservation, i.e. storage at ultra-low temperatures (usually in liquid nitrogen [LN] at -196°C), is a promising option for the long-term germplasm conservation of recalcitrant-seeded species but their seeds present some unavoidable difficulties in terms of the amenability of their germplasm to cryopreservation. Pre-conditioning treatments can reduce the amount of 'free' water available for freezing and may increase the chances of cells or tissues surviving exposure to cryogenic temperatures. Such conditioning may be imposed by physical dehydration or cryoprotection, i.e. exposure to compounds that depress the kinetic freezing point of water and so reduce the likelihood of lethal ice-crystal formation during cooling (i.e. exposure to LN at -196°C or sub-cooled LN at -210°C) and subsequent thawing. Partial dehydration is presently a standard pre-treatment for the cryopreservation of recalcitrant zygotic germplasm and explant cryoprotection has been shown to improve postthaw survival in some recalcitrant-seeded species. However, there is a paucity of information on the physiological and biochemical basis of post-thaw survival or death in recalcitrant seeds, and this is the major focus of the current contribution. Additionally, in light of the lack of understanding on how cryo-related stresses imposed at the embryonic stage are translated or manifested during subsequent seedling growth, this study also investigated the effects of partial dehydration and the combination of partial dehydration and cooling of recalcitrant zygotic embryos on subsequent in and ex vitro seedling vigour. All studies were undertaken on the zygotic embryos of two recalcitrant-seeded members of the Amaryllidaceae, viz. Amaryllis belladonna (L.) and Haemanthus montanus (Baker); both of which are indigenous to South Africa.

Studies described in **Chapter 2** aimed to interpret the interactive effects of partial dehydration (rapidly to water contents > and <0.4 g g⁻¹), cryoprotection (with sucrose [Suc; non-penetrative] or glycerol [Gly; penetrative]) and cooling rate (rapid and slow) on subsequent zygotic embryo vigour and viability, using three stress markers: electrolyte leakage (an indicator of membrane integrity); spectrophotometric assessment of tetrazolium chloride-reduction (an

indicator of respiratory competence); and rate of protein synthesis (an indicator of biochemical competence). These studies showed that in recalcitrant *A. belladonna* and *H. montanus* zygotic embryos, stresses and lesions, metabolic and physical, induced at each stage of the cryopreservation protocol appear to be compounded, thus pre-disposing the tissues to further damage and/or viability loss with the progression of each step. Maximum post-thaw viability retention in both species appeared to be based on the balance between desiccation damage and freezing stress, and the mitigation of both of these via Gly cryoprotection. Post-thaw viabilities in both species were best when Gly cryoprotected + partially dried zygotic embryos were rapidly, as opposed to slowly, cooled. However, the rate at which water could be removed during rapid drying was higher in *A. belladonna* and this may explain why the optimum water content range for post-thaw survival was <0.40 g g⁻¹ for *A. belladonna* and >0.40 g g⁻¹ for *H. montanus*. These results suggest that to optimise cryopreservation protocols for recalcitrant zygotic germplasm, attention must be paid to pre-cooling dehydration stress, which appears to be the product of both the 'intensity' and 'duration' of the stress.

Cryoprotection and dehydration increased the chances of post-thaw survival in *A*. *belladonna* and *H. montanus* zygotic embryos. However, transmission electron microscopy studies on the root meristematic cells from the radicals of these embryos (described in **Chapter 3**) suggest that their practical benefits appear to have been realised only when damage to the sub-cellular matrix was minimised: when (a) pre-conditioning involved the combination of cryoprotection and partial dehydration; (b) the cryoprotectant was penetrating (Gly) as opposed to non-penetrating (Suc); and (c) embryos were rapidly cooled at water contents that minimised both dehydration and freezing damage.

The ability of *A. belladonna* and *H. montanus* embryos to tolerate the various components of cryopreservation in relation to changes in extracellular superoxide (O_2^{-1}) production and lipid peroxidation (a popular 'marker' for oxidative stress) was investigated in studies featured in **Chapter 4**. Pre-conditioning and freeze-thawing led to an increase in oxidative stress and the accompanying decline in viability suggests that oxidative stress was a major component of cryoinjury in the embryos presently investigated. Post-thaw viability retention in Gly cryoprotected + partially dried embryos was significantly higher than non-cryoprotected + partially dried embryos, possibly due to the relatively lower post-drying lipid peroxidation levels and relatively higher post-drying and post-thawing enzymic antioxidant activities in the former.

Exposure of certain plant tissues to low levels of oxidative or osmotic stress can improve their tolerance to a wide range of stresses. In contrast, exposure of *H. montanus* zygotic embryos to low levels of oxidative stress provoked by exogenously applied hydrogen peroxide (H₂O₂) or exposure of *A. belladonna* embryos to low levels of osmotic stress provoked by low water potential mannitol and polyethylene glycol solutions (in studies featured in **Chapter 5**) increased their sensitivity to subsequent dehydration and freeze-thaw stresses. Exposure of Gly cryoprotected and non-cryoprotected amaryllid embryos to such stress acclimation treatments may pre-dispose *A. belladonna* and *H. montanus* embryos to greater post-drying and post-thaw total antioxidant and viability loss than untreated embryos.

To assess the vigour of seedlings recovered from partially dried *H. montanus* embryos, seedlings recovered from fresh (F) and partially dried (D) embryos in vitro were hardened-off ex vitro, and subsequently subjected to either 42 days of watering or 42 days of water deficit (in studies described in Chapter 6). In a subsequent study (described in Chapter 7), seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) A. belladonna embryos were regenerated in vitro, hardened-off ex vitro and then exposed to 12 days of watering (W) or 8 days of water stress (S) followed by 3 days of re-watering. Results of these studies suggest that the metabolic and ultrastructural lesions inflicted on A. belladonna and H. montanus zygotic embryos during cryopreservation may compromise the vigour (e.g. development of persistent low leaf water and pressure potentials and reduced photosynthetic rates) and drought tolerance of recovered seedlings, compared with seedlings recovered from fresh embryos. While the adverse effects of freeze-thawing were carried through to the early ex vitro stage, certain adverse effects of partial drying were reversed during ex vitro growth (e.g. the increased relative growth rate of seedlings from partially dried embryos). The reduced vigour and drought tolerance of seedlings recovered from partially dried and cryopreserved embryos in the present work may therefore disappear with an extension in the period afforded to them for hardening-off under green-house conditions, and in the field.

The results presented in this thesis reinforce the notion that each successive manipulation involved in the cryopreservation of recalcitrant zygotic germplasm has the potential to inflict damage on tissues and post-thaw survival in such germplasm relies on the minimisation of structural and metabolic damage at each of the procedural steps involved in their cryopreservation. The results also highlight the need to design research programmes aimed not only at developing protocols for cryopreservation of plant genetic resources, but also at elucidating and understanding the fundamental basis of both successes and failures.

Preface

This research was undertaken at the Plant Germplasm Conservation Research Unit (PGCRU) in Durban, KwaZulu-Natal, South Africa during February, 2006 to March, 2010. PGCRU is based at the University of KwaZulu-Natal (UKZN), School of Biological and Conservation Sciences.

The supervisors of the research were:

Prof. Patricia Berjak, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, South Africa.

Prof. Norman Pammenter, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, South Africa.

Dr. James Wesley-Smith, Electron Microscopy Unit, University of KwaZulu-Natal, Durban, South Africa.

I would like to declare that the work reported in this thesis has never been submitted in any form to any other university. It therefore represents my original work except where due acknowledgments are made.

Sershen Naidoo

Signed: _____ Date: _____

Declaration 1 – Plagiarism

I, Sershen Naidoo, declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a. Their words have been re-written but the general information attributed to them has been referenced;

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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the Reference sections.

Sershen Naidoo

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Declaration 2 – Publications

1. Sershen, Berjak, P., Pammenter, N.W. 2010. Effects of cryopreservation of recalcitrant *Amaryllis belladonna* zygotic embryos on vigour of recovered seedlings: a case of stress 'hangover'? Physiologia Plantarum 139, 205-219.

2. Sershen, Berjak, P., Pammenter, N.W. 2011. Effects of partial dehydration of recalcitrant *Haemanthus montanus* zygotic embryos on vigour of recovered seedlings. South African Journal of Botany 77, 193-202.

3. Sershen, Berjak, P., Pammenter, N.W., Wesley-Smith, J. 2011. The effects of various parameters during processing for cryopreservation on the ultrastructure and viability of recalcitrant zygotic embryos of *Amaryllis belladonna*. Protoplasma (In press).

4. Sershen, Berjak, P., Pammenter, N.W., Wesley-Smith, J. 2011. Rate of dehydration, state of subcellular organisation and nature of cryoprotection are critical factors contributing to the variable success of cryopreservation: Studies on recalcitrant zygotic embryos of *Haemanthus montanus*. Protoplasma (In press).

Sershen Naidoo was responsible for the conceptual development, analysis, and writing publications 1-4. The contribution of the co-authors was providing comments during the analysis stage, as well as editing of the manuscripts.

Sershen Naidoo

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Acknowledgments and dedication

First and foremost, I would like to thank my supervisors, Professors Patricia Berjak and Norman Pammenter, for affording me the opportunity to learn from them, the encouragement to explore new ideas, the time to express my opinions and the freedom to make mistakes. All I can do to return the favour is promise to some day afford my students the very same. I would also like to acknowledge the assistance and support of a truly remarkable mentor, Dr. Boby Varghese, whose advice and opinions shaped much of the work presented here. My appreciation is also extended to the staff and students of the Plant Germplasm Conservation Research Unit (UKZN) and the staff of the Electron Microscopy Unit (UKZN), most especially Dr. James Wesley-Smith. This work was funded by the National Research Foundation (South Africa) and the Millennium Seed Bank Project.

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This thesis is dedicated to my grandparents...

I may not have known you for as long or as well as I would have liked to but I am a product of your hard-work and for that, I am eternally grateful!

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List of abbreviations

Ψ_{p}	pressure potential
Ψ_{s}	osmotic potential
$\Psi_{ m w}$	water potential
O_2^-	superoxide radical
$^{1}O_{2}$	singlet oxygen
³ H	tritiated
A	CO ₂ -assimilation rate
A@600: A@400	ratio between CO2-assimilation rate at Ca: 600 and Ca: 400 $\mu mol.~mol^{\text{-1}}$
ABA	abscisic acid
ANOVA	Analysis of Variance
AsPX	ascorbate peroxidase
BGCI	Botanic Gardens Conservation International
Ca	calcium
C _a	carbon dioxide concentration
CAT	catalase
chl	chlorophyll
СР	cryoprotected
dmb	dry mass basis
DNA	deoxyribose nucleic acid
DW	dry weight
FAO	Food and Agricultural Organisation
F _m	maximum fluorescence
Fo	fluorescence intensity at 50 µs
F_v	variable fluorescence
F_v/F_m	the ratio of variable to maximum fluorescence
Gb	golgi body
GI	germination index
Gly	Glycerol
GPX	guaiacol peroxidase

GR	glutathione reductase
H_2O_2	hydrogen peroxide
HODEs	hydroxyoctadecadienoic acids
IBPGR	International Board for Plant Genetic Resources
LEAs	late embryogenic abundant proteins
LN	liquid nitrogen
LOOHs	lipid hydroperoxides
LPI	leaf plastochron index
MA	molar absorbance
md	midday
MDA	malondialdehyde
Mg	magnesium
mRNA	messenger ribose nucleic acid
MTG	mean time to germinate
n	number of samples
NA	not applicable
NADPH	the reduced form of nicotinamide adenine dinucleotide phosphate
ОН	hydroxyl
OH∙	hydroxyl radical
р	significance
pd	predawn
pd-md	predawn value less midday value
r	correlation coefficient
RGR	relative growth rate
RH	relative humidity
ROS	reactive oxygen species
SD	standard deviation
SEM	Scanning Electron Microscope
SOD	superoxide dismutase
spp.	several species
Suc	sucrose

TBARS	thiobarbituric acid-reactive substances
TEM	transmission electron microscopy
UNCED	United Nations Conference on Environment and Development
v/v	volume per volume
viz.	namely
w/v	weight per volume
WC	water content
WSC	water soluble carbohydrates
WWF	World Wildlife Fund

Units of measurement

%	percent
°C	degree Celsius
μm	micrometres
μΜ	micromolar
μs	microsecond
μCi	microCurie
μl	microlitre
μmol	micromole
cm	centimetre
d	day
g	gram
g	G force
h	hour
М	molar
m	metre
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mm	millimetre
MPa	megapascal
mSiemen	milliSiemen
nm	nanometre
nmol	nanomole
S	seconds
W	watt

CHAPTER ONE: General Introduction and Study Rationale

1.1 Plant biodiversity

Biodiversity, defined as the sum of the taxonomic, ecological and genetic variety of all life on earth or within a particular habitat (Lovejoy, 1996), is under severe pressure from human activities (Paunescu, 2009). The Food and Agricultural Organisation (FAO, 2001) reported that with global forest cover shrinking at a rate of 9 million hectares per year, up to 100,000 plant species (more than one third of all the world's plant species) are currently threatened or face extinction (Botanic Gardens Conservation International [BGCI], 2005). This is a global phenomenon but the rate of loss of floral biodiversity appears to be particularly alarming in Africa; this has been variously attributed to one or more of: civil war; wild fires; invasive aliens; conversion of land for agriculture and silviculture; poor land management; urbanisation; nonsustainable harvesting for food; medicine; fuel and construction; overgrazing; displacement and loss of landraces; pests and diseases; pollution; and incomplete knowledge of the biology (e.g. reproductive and adaptive abilities) of many plant species (Eneobong, 1997; Geldenhuys, 2000). Also, pressure placed on wild plant populations by the global market for medicinal plants has lead to diminishing populations of numerous wild species (World Wildlife Fund, 2009). The World Wildlife Fund (WWF, 2009) has identified China and India as being two of the largest markets for medicinal plants but it is believed that the extent of the African medicinal plant trade is grossly under-estimated (van Wyk et al., 1997; von Ahlefeldt et al., 2003). Additionally, climate change over the past ~30 years has been associated with numerous shifts in the distribution and abundance of plant species (Thomas et al., 2004) and even been implicated in species-level extinction (Pounds et al., 1999). However, responsiveness of plant species to recent and past climatic changes suggests that anthropogenic activities could represent a major cause of extinctions in the near future, with the Earth set to become warmer than at any other period in the past 1 - 40 million years (Thomas et al., 2004).

However, a global strategy for plant conservation, *viz*. the Convention on Biological Diversity (1993), was developed to halt the loss of plant biodiversity systematically. This strategy involves understanding, documenting, conserving and generating awareness around plant biodiversity, implementing its sustainable use and building infrastructure and human capacity for the conservation of this diversity. Some of the targets identified by this strategy include: protection of 50% of the globe's priority areas of plant diversity; conservation of 70% of the genetic diversity of crop and other socio-economically important plant species; conservation of 60% of the world's threatened species *in situ*; and safeguarding 60% of

threatened species in accessible *ex situ* collections (of which 10% should be directed towards recovery and restoration programmes).

Germplasm is defined as the total genetic diversity of a species encompassing both cultivated and wild varieties (Ford-Lloyd and Jackson, 1991) and an increasing number of agencies have adopted germplasm conservation as part of their mandate (Ashmore and Drew, 2005). Additionally, biotechnology, i.e. the use of living organisms or parts thereof to manufacture or modify a product, develop microorganisms for specific uses, or improve plants or animals (Uyoh *et al.*, 2003), has become an important component of contemporary germplasm conservation efforts (Berjak and Pammenter, 2004a; Paunescu, 2009).

1.2 Plant germplasm conservation

Plant genetic resources may be conserved *in situ* or *ex situ*. *In situ* conservation encompasses the maintenance of plants in their native environments, e.g. in nature reserves or national parks, allowing for the evolution of species in their natural habitats, or in the case of domesticated species, in the surroundings where they have developed their distinctive properties (Ford-Lloyd and Jackson, 1991; Krøgstrup *et al.*, 1992; Shands, 1993; Meilleur and Hodgkin, 2004). In the context of *in situ* conservation there are a number of social and economic factors that need to be considered including the availability of a sufficient number of individuals to maintain viable populations, the availability of land, and maintenance costs of natural reserves (Berjak *et al.*, 1996). *In situ* conservation is perhaps the best way of biodiversity preservation but to allow plant material to be readily available for research, breeding and *in vitro* and long-term conservation, this must operate in tandem with *ex situ* conservation.

Ex situ conservation refers to the conservation of components of biological diversity outside their natural habitats and includes: field genebanks; arboreta and botanical gardens; seed, pollen and DNA storage; and *in vitro* storage. Some of these practices are labour- and/or land-intensive (Maxted *et al.*, 1997). A key requirement for this type of conservation is the acquisition of genetically representative populations or samples. Ideally, for successful *ex situ* storage the diversity within a species should be determined before germplasm collection is carried out, so that the sample size accommodates for this (Krøgstrup *et al.*, 1992; Berjak *et al.*, 1996; McFerson, 1998). For any given gene pool, a number of complementary approaches and methods are necessary for its efficient, cost-effective and safe conservation (Benson, 2008a). Most plant species are presently conserved using one or more of the five distinct germplasm types: (1) cultivars in current use; (2) obsolete cultivars; (3) special genetic stock such as those with induced mutations; (4) primitive varieties; and (5) related wild species (Wilkins and Dodds, 1983). While the method of preservation employed is dictated by the cellular and

metabolic nature of the tissue or organ selected, *ex situ* germplasm acquisitions are stored as either active or base collections (Krøgstrup *et al.*, 1992).

Active collections refer to those that are readily available for distribution and require methods of storage that retain the viability of samples for short (a few weeks) to moderate (several years) periods. Currently, most active collections around the world consist of orthodox seeds, which are dried and stored at temperatures above 0°C but below 15°C, and are periodically removed for regeneration, multiplication, characterisation, evaluation, or distribution (Krøgstrup *et al.*, 1992; Watt *et al.*, 2000). *Ex situ* collections of living plants growing under field or nursery conditions are often referred to as 'field gene banks'. These are generally placed within the category of active gene banks and are used for material which would be difficult to maintain as seed, e.g. species with desiccation-sensitive seeds (Guarino *et al.*, 1994).

A base collection functions for long-term (decades) conservation of germplasm which is not intended for extensive distribution, but serves as back up for active collections. Base collections have traditionally been comprised of accessions of orthodox (i.e. desiccation-tolerant [Roberts, 1973]) seeds dried to low water content (WC), sealed in airtight containers and stored at low temperatures (usually 0°C to -20°C), often for decades (Ford-Lloyd and Jackson, 1991; Krøgstrup *et al.*, 1992; Guarino *et al.*, 1994; Rao *et al.*, 2006). Accessions from base collections are removed only: (1) for regeneration, to assess whether their viability has declined to below an acceptable standard; (2) to provide material for an active collection for regeneration, when stocks held by the active collection are more than two or three regeneration cycles removed from the original material; and (3) when stocks of an accession can no longer be obtained from an active collection (Guarino *et al.*, 1994).

1.2.1 Seed storage

Seed storage is widely regarded as the most efficient and cost-effective means of *ex situ* plant germplasm conservation, being used for the majority of accessions maintained world-wide (International Board for Plant Genetic Resources [IBPGR], 1976; Withers, 1988; Vertucci and Roos, 1990; Hong and Ellis, 1996; Engels and Engelmann, 1998). Since each seed represents a genetically unique individual, stored seeds are ideal for re-establishing wild populations of threatened taxa, especially when the sample size and collection methods employed ensure a genetically representative sample (Frankel, 1990; McFerson, 1998). However, seed storage cannot be applied to all plant species with the same efficacy since post-harvest behaviour of seeds, which determines the most suitable method of conservation, differs. These differences are reflected in the categorisation of seeds as orthodox, recalcitrant (Roberts, 1973) and

intermediate (Ellis *et al.*, 1990a; 1991), based on their physiological responses to dehydration, and storage relative humidity (RH) and temperature, as determinants of longevity.

Based on their desiccation tolerance, orthodox seeds can generally be dried without damage to water (moisture) contents of *c*. 0.05 g H₂O per g dry mass (dmb; g g⁻¹) (Roberts, 1973; Chin and Roberts, 1980; Hong and Ellis, 1996), which is generally lower than those achieved in the field where WC of mature seeds equilibrates to ambient RH (Roberts, 1973; Bewley and Black, 1994). At low WCs and at temperatures of -18° C to -20° C (King and Roberts, 1980; Rao *et al.*, 2006), or even as low as -150° C to -196° C (Walters and Engels, 1998; reviewed by Pritchard and Nadarajan, 2008), high quality orthodox seeds can be stored for decades with little viability loss. Examples include: rice, millet and peanut (Hu *et al.*, 1998), sesame and soybean (Chai *et al.*, 1998), cucumber (Zeng *et al.*, 1998), wheat (Stefani *et al.*, 2000) and rye (Specht and Börner, 1998), all of which were stored at low but not cryogenic temperatures, and lettuce (Walters *et al.*, 2004, 2005) which was stored at cryogenic temperatures.

Ellis and Roberts (1980) suggested that the period for which high quality orthodox seeds can be stored without loss of viability is predictable from the storage temperature and seed moisture content, with storage longevity increasing logarithmically with decreasing water (moisture) content. However, there appear to be limits of dehydration below which no further advantage is gained (Ellis *et al.*, 1986, 1990b) and, in fact, if exceeded, may be damaging (Vertucci and Roos, 1990, 1993; Walters, 1998; Walters and Engels, 1998; Buitink *et al.*, 2000). (It must be noted though, that there is not unanimity about this [e.g. Hong *et al.*, 2005]). Deterioration of ultra-dry (0.01 - 0.05 g g⁻¹) stored seeds (manifested as a decline in vigour and viability) may be based on the fact that water is not only the suspension medium within cells, but also a structural component of proteins and other macromolecules (Bernal-Lugo and Leopold, 1992); for successful ultra-dry storage removal of this structure-implicated water should be avoided (Walters and Engels, 1998).

Although seeds of most commercial crop species exhibit orthodox post-harvest seed behaviour, conventional crops constitute less than 0.1% of all higher plants and as seed biology studies are extended, seed recalcitrance is encountered in an increasing number of species (Berjak and Pammenter, 2001). The phenomenon has been found to occur in hardwoods of tropical provenance (e.g. *Trichilia* spp. [Choinsky, 1990]; *Shorea* spp. [Krishna and Naithani, 1998]; *Artocarpus heterophyllus* [Chin and Roberts, 1980; Fu *et al.*, 1993; Wesley-Smith *et al.*, 2001a]) and temperate species (e.g. *Quercus* spp. [Connor and Sowa, 2002]); mangroves (e.g. *Avicennia marina* [Farrant *et al.*, 1989]); tropical crop plants (e.g. *Theobroma cacao* [Li and Sun, 1999]; *Persea americana* [Raja *et al.*, 2001]); *Hevea brasiliensis* [Berjak *et al.*, 1989]); various monocotyledonous species (e.g. *Cocos nucifera* [Chin, 1978]; *Zizania palustris* [Probert

and Longley, 1989], including several amaryllid species [Sershen *et al.*, 2008a]). Some gymnosperms (e.g. *Araucaria* spp. [Tompsett, 1984]; *Podocarpus henkelii* [Farrant *et al.*, 1989; Mbamezeli and Reynolds, 2002] and *Encephalartos* spp. [Woodenberg, 2009]) also produce recalcitrant propagules.

Recalcitrant seeds are sensitive to desiccation (Roberts, 1973; Chin and Roberts, 1980), freezing (for reviews see Walters et al. 2008; Berjak and Pammenter, 2008) and very often to chilling, in some cases at temperatures $\approx 15^{\circ}$ C (Berjak *et al.*, 1995). This precludes their maintenance under conventional storage conditions as developed for orthodox seeds (e.g. King and Roberts, 1980; Farrant et al., 1989; Kioko et al., 1993; Sershen et al., 2008b; Wen, 2009). At present, the only conditions under which recalcitrant seeds can be stored in the short- to medium-term is by maintenance of their WC as close to that at shedding, at ambient or slightly reduced temperatures (referred to as 'hydrated-storage' from here on [e.g. Berjak, 1989; Kioko et al., 1993; Eggers et al., 2007]). However, under these conditions recalcitrant seeds initiate germination-associated events (Berjak, 1989; Bonner, 1990; Kioko et al., 1993; Pammenter et al., 1994; Motete et al., 1997; Berjak and Pammenter, 2004a & b; Sershen et al., 2008b), culminating in intracellular changes, including extensive vacuolation, and the initiation of cell division (Berjak, 1989). At this stage additional water is required and as this is not supplied in storage, the seeds are exposed to an initially mild, but increasingly severe, water stress (Pammenter et al., 1994, 1997). The deleterious events associated with this stress, which include unbalanced metabolism and the consequential generation of damaging free-radicals, culminate in seed death (Dussert et al., 2006; Ratajczak and Pukacka, 2006). Storage at temperatures lower than ambient can postpone the onset of germination by slowing down metabolic rate in recalcitrant seeds (e.g. for Araucaria hunsteinii [Pritchard et al., 1995a]), provided that the seeds are not chilling-sensitive. However, even then, storage longevity generally ranges from a few weeks to months (e.g. for Scadoxus membranaceus and Landolphia kirkii [Farrant et al., 1989]; A. hunsteinii [Pritchard et al., 1995a]; Symphonia globulifera [Corbineau and Côme, 1986, 1988]; Azadirachta indica [Nayal et al., 2000; Neya et al., 2004]; Quercus spp. [Connor and Sowa, 2002]; and various amaryllid species [Sershen et al., 2008b]). Where seeds can be stored for more extended periods, it is most likely a consequence of their being shed relatively immature and continuing development before germination is initiated (Berjak et al., 1989; Woodenberg, 2009). Hydrated storage lifespan of recalcitrant seeds is often further curtailed by the proliferation of a spectrum of fungi (Pongapanich, 1990; Mycock and Berjak, 1990; Calistru et al., 2000; Sutherland et al., 2002), even when seeds are treated with fungicidal agents (Mycock and Berjak, 1995; Berjak, 1996).

In vitro storage, i.e. the employment of tissue culture, by which cells, tissues or organs are excised from parent plants, decontaminated and thereafter transferred to artificial growth media *in vitro* (Krøgstrup *et al.*, 1992; George, 1993; Mandal *et al.*, 2000), represents another short- to medium-term germplasm storage option for recalcitrant-seeded species. Media can be manipulated to produce different cultures such as unorganised, undifferentiated callus or organised tissues and organs that can be converted into plantlets in the next phase of regeneration. Storage of actively growing cultures, minimal-growth storage and cryopreservation are the three basic approaches to *in vitro* storage of plant germplasm, and their application to seeds and seed-derived germplasm is discussed below.

Minimal-growth storage, which involves exposing cultures to factors that limit growth, has been successfully applied to recalcitrant seed germplasm (e.g. Chin, 1996; Sershen et al., 2008b). Variations to impose minimal growth include reductions in nutrients (e.g. Schnapp and Preece, 1986) and/or sucrose concentration (e.g. Kartha and Engelmann, 1994) in the growth medium, alterations to the osmotic potential of the culture media (using osmotica such as mannitol [e.g. Zandvoort et al., 1994]), or the addition of plant growth regulators such as abscisic acid (ABA) (e.g. Jarret and Gawel, 1991; Taylor et al., 1996). Lowering the partial pressure of oxygen, with temperature (between 0°C and 10°C [e.g. Blakesley et al., 1996; Bonnier et al., 1997]) or light (e.g. Grout, 1995) to below optimum in the culture environment, can also limit *in vitro* growth. However, irrespective of whether germplasm is stored *in vitro* as actively growing cultures (e.g. Krøgstrup et al., 1992) or as minimal-growth cultures (e.g. Schnapp and Preece, 1986), such storage will at some stage require the transfer of material onto new media (Krøgstrup et al., 1992; Mandal et al., 2000, Mycock et al., 2004), introducing the risk of contamination. Surviving, uncontaminated material can potentially be rapidly micropropagated to bulk up reserves (Razdan and Cocking, 1997; Mandal et al., 2000) but such clonal propagation, apart from being labour intensive and expensive, limits biological diversity within the collection and can impose selection pressures and environmental stresses, resulting in plants with genetic modifications (Reed and Chang, 1997; Staritsky, 1997). Like all tissue culture techniques clonal propagation introduces the risk of somaclonal variation during culture (Ashmore, 1997; Panis and Lambardi, 2006) but the use of organised systems such as embryos, meristems and shoot tips could reduce this risk (Ford-Lloyd and Jackson, 1991; Krøgstrup et al., 1992; Berjak et al., 1996; Engelmann, 1997; Mandal et al., 2000).

Conventional *in vitro* storage and seed banking techniques are unsuitable for the long-term storage of the genetic resources of recalcitrant-seeded species and other methods of germplasm conservation are required. To date, the most promising method for the long-term storage of recalcitrant seed germplasm appears to be cryopreservation (Normah *et al.*, 1986; Withers,

1988; Berjak *et al.*, 1999a; Engelmann, 1999, 2004; Walters *et al.*, 2008). Cryopreservation involves the cooling of biological material to, and subsequent storage at, ultra-low temperatures - generally in liquid nitrogen (LN) at -196°C, the vapour above LN at *c*. -150°C or, less ideally, at some temperature below -80°C (Finkel and Ulrich, 1983; Withers, 1988; Kartha and Engelmann, 1994; Berjak *et al.*, 1999a). Cryopreservation is regarded as the ultimate long-term storage approach since it is believed to arrest metabolic activity and deterioration, thus minimising, if not precluding, genetic changes (Krøgstrup *et al.*, 1992; Krishnapillay, 2000; Lynch, 2000; Kioko *et al.*, 2003; Engelmann, 2004; reviewed by Harding, 2004).

The absence of water that readily freezes and causes irreversible damage in cells (Burke *et al.*, 1976; Levitt, 1980; Stanwood, 1985) has facilitated successful cryopreservation of the dry seeds of a number of orthodox-seeded species (Walters *et al.*, 2005; reviewed by Pritchard and Nadarajan, 2008). However, recalcitrant seed germplasm must remain partially hydrated, yet be stored under conditions that preclude ice-crystal formation: in terms of the well documented desiccation and freezing sensitivity of recalcitrant seeds, cryopreservation of zygotic embryos or embryonic axes from such seeds remains a challenge (Berjak *et al.*, 1996, 1999a; Dumet *et al.*, 1997; Engelmann, 2004, 2009). Nevertheless, considerable success has been achieved in terms of excised zygotic embryos and axes of a variety of amaryllid species (Sershen *et al.*, 2007). The current studies go further, to investigate factors influencing vigour and viability after cryopreservation of *whole* zygotic embryos (i.e. embryonic axis with intact cotyledon), excised from recalcitrant seeds of two members of the Amaryllidaceae, *viz. Amaryllis belladonna* (L.) and *Haemanthus montanus* (Baker). To contextualise the studies described in later chapters, at this juncture the post-harvest responses and desiccation tolerance/sensitivity associated with orthodox and non-orthodox seeds are reviewed.

1.3 Post-harvest seed behaviour

In orthodox seeds tolerance to desiccation is acquired during development (Berjak *et al*, 1989; Vertucci and Farrant, 1995; Pammenter and Berjak, 1999) which is characterised by three phases, *viz.*, histodifferentiation, reserve deposition and maturation drying (Bewley and Black, 1994; reviewed by Kermode and Finch-Savage, 2002). Undifferentiated cells divide during the phase of histodifferentiation, developing function-specific tissues. During the deposition phase, reserves in various forms are accumulated in the endosperm, or ultimately in the cotyledons, providing nutrients that serve to sustain seedling development (Bewley and Black, 1994). Dry and fresh mass of orthodox seeds increases during the first two phases and while the duration of the period of dry matter accumulation varies among species, it always ends when the vascular connection between the parent plant and seed are severed at physiological maturity (Kermode

and Finch-Savage, 2002). At this stage of physiologically maturity, which coincides with the attainment of maximum dry mass, the seeds undergo metabolic shutdown accompanied by a loss of water (and hence fresh mass) as part of the maturation drying phase (Vertucci and Farrant, 1995; Kermode and Finch-Savage, 2002). Orthodox seeds acquire desiccation tolerance during development prior to maturation drying and are shed from the parent plant usually upon completion of all three phases, remaining quiescent until water becomes available for germination (Farrant *et al.*, 1993; Vertucci and Farrant, 1995; Kermode and Finch-Savage, 2002). Metabolic events associated with germination are not triggered in the dry state, and non-dormant orthodox seeds will germinate only upon imbibition, providing that environmental conditions are favourable (Bewley and Black, 1994).

Recalcitrant-seeded species, which appear to be most common in tropical to sub-tropical regions, produce highly hydrated seeds that are intolerant to desiccation and may be sensitive to low temperatures (Roberts, 1973; Chin and Roberts, 1980; King and Roberts, 1980; Berjak, 1989; Pammenter and Berjak, 1999; Berjak and Pammenter, 2008; Wen, 2009). The final phase of orthodox seed development, maturation drying, is absent in recalcitrant types and these seeds are shed wet and metabolically active (Berjak *et al.*, 1989; Farnsworth, 2000; Kermode and Finch-Savage, 2002); they remain so throughout their development, although rate of metabolism may be lowest immediately prior to shedding (Berjak *et al.*, 1989; Farrant *et al.*, 1989). The WC of recalcitrant seeds at shedding has been found to be in the range of $0.3 - 4.0 \text{ g g}^{-1}$, varying by species, but also inter-seasonally (Chin and Roberts, 1980; Pritchard *et al.*, 1999; Berjak and Pammenter, 2004b).

1.3.1 Seed responses to desiccation

Desiccation tolerance is defined as the ability of biological material to dry to equilibrium with atmospheric RH and resume normal functioning upon rehydration (Alpert and Oliver, 2002; Phillips *et al.*, 2002; Berjak, 2006). Desiccation-tolerant organisms, or life-cycle stages of an organism, generally survive for protracted periods at tissue WCs in the range 0.05 - 0.15 g g⁻¹ (reviewed by Berjak, 2006; Berjak *et al.*, 2007). While response to desiccation is probably the major factor differentiating orthodox from recalcitrant seeds, the categories orthodox and recalcitrant (Roberts, 1973) have been suggested to account for only those species that display the extremes of post-harvest behaviour: the ability or inability to tolerate desiccation (Berjak and Pammenter, 1994; Kermode and Finch-Savage, 2002; Berjak *et al.*, 2007). This may explain why these categories were later augmented by a third seed category, described as being 'intermediate' between the extremes of recalcitrant and orthodox behaviour. Seeds so categorised are relatively desiccation tolerant (but not to the extent of orthodox seeds) and may

be chilling sensitive in the dry state, particularly if they are of tropical origin (Ellis *et al.*, 1990a; Hong and Ellis, 1996). This wide range in post-harvest responses suggests open-endedness to the three categories, such that post-harvest physiology may be considered as constituting an extended continuum of seed behaviour, which grades from extreme desiccation-sensitivity, through a range of responses, to seeds capable of extreme desiccation tolerance (Berjak and Pammenter, 1994; Kermode and Finch-Savage, 2002; Berjak *et al.*, 2007).

The acquisition and maintenance of desiccation tolerance in orthodox seeds involves *inter alia* the protection of cellular membranes and intracellular components from the consequences of drying and appears to be based on "the presence and interplay of a suite of mechanisms and processes expressed during development" (Pammenter and Berjak, 1999), under complex genetic control (which is not fully understood [Berjak *et al.*, 2007]). An appreciation of the physiological status of seeds at various water potentials and the properties of water at the various hydration levels corresponding to specified water potential ranges (see Vertucci and Farrant, 1995; Pammenter *et al.*, 2000; Walters *et al.*, 2001) has also led to suggestions that while desiccation tolerance in orthodox seeds is based on the operation of a suite of interactive protection mechanisms and/or repair processes, desiccation sensitivity is probably characterised by the absence or poor expression of at least one, but probably more, of these (Oliver and Bewley, 1997; Pammenter and Berjak, 1999; Berjak and Pammenter, 2001; Walters *et al.*, 2002a; Berjak *et al.*, 2007). The individual components of the suite, and their interactions, have been extensively reviewed and are outlined below (for reviews see Pammenter and Berjak, 1999; Kermode and Finch-Savage, 2002; Walters *et al.*, 2002a; Berjak and Pammenter, 2008).

Intracellular physical characteristics

The ability to withstand the mechanical stresses associated with volume reduction is a major requirement for desiccation tolerance in plants cells (Iljin, 1957). Features contributing to orderly responses to such stresses include minimisation of vacuolation, protection of the integrity of the DNA, and orderly dismantling of cytoskeletal elements (Pammenter and Berjak, 1999; Berjak and Pammenter, 2008).

In all orthodox and also some recalcitrant seeds, physical protection includes a marked minimisation of fluid-filled vacuoles, brought about by either their sub-division into smaller bodies and/or their filling with insoluble reserve material (Vertucci and Farrant, 1995; Pammenter and Berjak, 1999; Walters *et al.*, 2008). Among several unrelated species of desiccation-sensitive seeds there appears to be a relationship between the extent of vacuolation and accumulation of insoluble reserves (primarily starch, protein or lipid bodies [Vertucci and Farrant, 1995]) and the degree of desiccation sensitivity (Berjak *et al.*, 1989; Farrant *et al.*, 1989, 1997).

The microtubules and microfilaments comprising the plant cytoskeleton provide internal support to cells as well as imposing spatial organisation on the intracellular milieu (Hoffman and Vaughn, 1995). The cytoskeleton has been suggested to dissociate upon dehydration in orthodox seeds, and reassemble upon imbibition (Pammenter and Berjak, 1999). In support of this theory Faria *et al.* (2005) using an α -tubulin antibody in an immunocytochemical assay, showed that in the dry state of orthodox Medicago truncutula seeds, axes exhibited only disassociated tubulin granules, but radicles that had protruded by 1 mm showed well-established cortical arrays of microtubules. In contrast, extensive cortical microtubule arrays were present in embryo cells of fresh mature Inga vera seeds, which are recalcitrant (Faria et al., 2004); however, disassociation following injurious levels of dehydration, gave rise initially to tubulin granules, which disappeared on further dehydration. Further to this, upon re-hydration, the damaged cells appeared to have lost the capacity for microtubule reconstitution (Faria et al., 2004). These results complement other findings indicating the failure of the reconstitution of cytoskeletal microfilaments following injurious degrees of dehydration in recalcitrant embryonic axes of Quercus robur (Mycock et al., 2000) and Amaryllis belladonna (Naidoo et al., 2005).

Since certain cytomatrical enzyme systems are functional as multi-enzyme complexes based on the binding of their key or anchor enzymes to cytoskeletal elements (Masters, 1984, 1992; Shearwin and Masters, 1990), incomplete re-assembly of the cytoskeleton after dehydration would have structural as well as physiological consequences in the cells of desiccation-sensitive seed tissues (Pammenter and Berjak, 1999).

The maintenance of the integrity of genetic material during dehydration, in the dry state, and upon rehydration, with the ability for repair on rehydration, is a critical requirement for desiccation tolerance (Osborne and Boubriak, 1994; Pammenter and Berjak, 1999). While DNA in desiccation-sensitive seed tissue becomes degraded during dehydration as a result of non-repaired double-strand breaks (Boubriak *et al.* 2000), DNA in desiccation-tolerant tissue appears to be stable in the dry state and exhibits repair upon rehydration (Osborne and Boubriak, 1994). Also, while DNA conformational change and orderly reversible chromatin compaction are associated with desiccation tolerance, chromatin decondensation characterises the desiccation-sensitive state upon germination of orthodox seeds (Deltour, 1985; Pammenter and Berjak, 1999).

In contrast, once compacted as a response to injurious degrees of dehydration of recalcitrant axes/embryos, the chromatin does not decondense when water again becomes available (Berjak *et al.*, 1999b; Wesley-Smith *et al.*, 2001a; Kioko *et al.*, 2006). This suggests that whereas chromosome condensation and decondensation in orthodox axes/embryos are consequences of

controlled processes, although appearing grossly similar, condensation is an injury response which is ultimately irreversible, in recalcitrant axes/embryos.

Intracellular dedifferentiation

In orthodox seeds, intracellular organelles such as mitochondria and plastids dedifferentiate, i.e. their internal structure regresses at the onset of maturation drying (Vertucci and Farrant, 1995; Pammenter and Berjak, 1999), while re-differentiation accompanies water uptake during early germination (Galau *et al.*, 1991). Additionally, the endoplasmic reticulum becomes reduced while cisternae of Golgi bodies appear to dissociate during maturation drying (Pammenter and Berjak, 1999). The absence of such phenomena in developing recalcitrant seeds and the resultant retention of highly differentiated organelles during drying is believed to contribute towards their desiccation sensitivity (Farrant *et al.*, 1997; Berjak and Pammenter, 2008). In some recalcitrant seeds metabolic rate may be at its lowest at, or shortly before, the seeds are shed, but Farrant *et al.* (1989, 1997) showed for several species that mitochondria retain well-developed cristae indicating the potential for ongoing respiration. As discussed below, the respiratory process itself is considered to be a major source of 'escaped' free-radicals and hence potentially lethal metabolism-linked damage during dehydration of recalcitrant axes/embryos/seeds (Pammenter *et al.*, 1998; Walters *et al.*, 2001, 2002a).

Metabolic 'switch-off' and oxidative metabolism

Oxidative metabolism supplies life sustaining energy in all aerobic organisms and the freeradicals and reactive oxygen species (ROS) generated as a consequence of this metabolism are strong oxidising agents (Leprince et al., 1994, 2000; Bailly et al., 1998; reviewed by Bailly, 2004). Formed as a consequence of the transfer of high energy state electrons to molecular oxygen, ROS, which include singlet oxygen $({}^{1}O_{2})$, hydrogen peroxide $(H_{2}O_{2})$ and the superoxide ((O_2)) and hydroxyl (OH) radicals, have long been considered as toxic species that can cause indiscriminate oxidative damage to nucleic acids, lipids and proteins (e.g. Wilson and McDonald, 1986; Halliwell, 1987; Hendry, 1993, Scandalios, 1997; Suzuki and Mittler, 2006). Such damage, in turn, results in the generation of a variety of biochemical and physiological lesions, which can cause metabolic impairment and even cell death (Dat et al., 2000; Halliwell, 2006). However, aerobic organisms have evolved an impressive array of endogenous enzymic and non-enzymic antioxidants that minimise, or ideally, prevent the injurious consequences of escaped free-radicals and ROS in hydrated cells (Bailly et al., 2001; Buitink et al., 2002; Haslekås et al., 2003; Mayaba and Beckett, 2003; Kranner and Birtić, 2005). While the strict control of ROS must occur in hydrated cells, oxidative damage occurs in plants during exposure to a wide range of stresses, including freezing and dehydration (Doke, 1997; Mittler, 2002; Beck *et al.*, 2007). In a review of specific and non-specific responses of plants to cold and drought stress Beck *et al.* (2007) identified oxidative stress as principally associated with both primary and secondary damage. While primary damage occurs when there is a dislocation of electron transport control and redox metabolism (Benson, 1990; Benson and Bremner, 2004), secondary damage results from the impacts of cold and excessive light energy, which leads to uncontrolled damage, but also could be implicated in controlled (adaptive) responses (Beck *et al.*, 2007). Such responses may occur not only in the short-term but could possibly have long-term effects on morphology and plant growth habit as well (Beck *et al.*, 2007).

The possession and effective operation of a suite of both enzymic and non-enzymic antioxidants is therefore of prime importance during dehydration of orthodox seeds and desiccation-tolerant vegetative tissues, in the dry state, and again as soon as rehydration commences in the desiccated cells (Pammenter and Berjak, 1999; Kranner et al., 2002; Bailly, 2004; Kranner and Birtić, 2005; Berjak, 2006; Berjak et al., 2007). Different levels of tissue hydration allow for the operation of different biochemical and physiological processes (Vertucci and Leopold, 1986; Vertucci and Farrant, 1995; Pammenter et al., 2000). Although low level electron transport may occur in dehydrated orthodox seeds (Vertucci, 1993), metabolic activity, which includes respiration, membrane and protein synthesis and DNA processing, has been reported to cease upon dehydration in desiccation-tolerant material (reviewed by Bewley, 1979; Vertucci and Leopold, 1986; Vertucci and Farrant, 1995). This down-regulation and eventual shutdown of metabolism during maturation drying in orthodox seeds is held to play an important role in avoiding oxidative stress and/or accumulation of by-products of oxidative stress to toxic levels, during the last phase of orthodox seed developmental (Leprince et al., 2000). Also, although metabolism is 'switched-off' in orthodox seeds during maturation drying, certain components of their antioxidant systems remain active, facilitating safe dehydration, maintenance of the dry state and rehydration (Bailly et al., 2001, Bailly, 2004; Türkan et al., 2005; reviewed by Berjak, 2006), making maturation drying a biologically successful strategy. It is has been conjectured that certain antioxidants may be operative within localised regions of higher water activity within desiccated cells. Interestingly, there may be localised regions with water activity adequate to facilitate molecular mobility in the desiccated state (Rinne *et al.*, 1999; Leubner-Metzger, 2005). If such regions were to occur in the *milieu* of the chromatin, then it is possible that 1-cys-peroxiredoxin (localised to nuclei in imbibed, dormant barley embryos by Stacy et al. [1999]) could function to protect the genome against ROS in desiccated seeds, given that the cysteinyl residue (of 1-cys-peroxiredoxin) can be regenerated (Berjak, 2006) ultimately by electron donors such as thioredoxins and glutaredoxins (Dietz, 2003). In this regard, Leubner-Metzger (2005) has shown localised β -1,3-glucanase activity in the inner

testa of air-dried tobacco seeds and suggested it to be instrumental in after-ripening. Similarly, the activity of other enzymic and non-enzymic antioxidants (Bailly, 2004) may be maintained in localised regions of greater water activity within intracellular glasses in dehydrated seeds (discussed below).

In contrast to the situation in orthodox seeds, recalcitrant types do not undergo maturation drying during the latter stages of development and metabolism is sustained at measurable levels throughout development (Farrant *et al.*, 1989, 1993; reviewed by Kermode and Finch-Savage, 2002; Faria *et al.*, 2004). During dehydration, and especially when water loss proceeds slowly, metabolism is held to become unbalanced and the deleterious aqueous-based reactions associated with such metabolism can result in considerable intracellular damage (i.e. metabolism-linked damage) and death of seeds/embryos at surprisingly high WCs (Pammenter *et al.*, 1998; Walters *et al.*, 2001, 2002a). Deleterious aqueous-based reactions are most likely to be free-radical-mediated and oxidative stress is held to be a major injurious factor during partial dehydration in recalcitrant seed tissues (Hendry *et al.*, 1992, Hendry, 1993; Smith and Berjak, 1995; Vertucci and Farrant, 1995; Côme and Corbineau, 1996; Pammenter *et al.*, 1998; Walters *et al.*, 2001; Pukacka and Ratajczak, 2006; Berjak and Pammenter, 2008; Roach *et al.*, 2008; Whitaker *et al.*, 2010).

It is now widely accepted that recalcitrant seeds appear to lack some of the mechanisms that allow orthodox seeds to tolerate dehydration (Oliver and Bewley, 1997; Pammenter and Berjak, 1999; Berjak and Pammenter, 2001, 2008; Walters *et al.*, 2002a) and even though recalcitrant seeds must necessarily possess antioxidant mechanisms, these are held to become non-functional or otherwise unable to cope with the level of ROS generated as a consequence of water stress (Hendry *et al.*, 1992; Chaitanya and Naithani, 1994, 1998; Côme and Corbineau, 1996; Leprince *et al.*, 1999; Chaitanya *et al.*, 2000; Greggains *et al.*, 2001; Varghese and Naithani, 2002; Dussert *et al.*, 2006; Francini *et al.*, 2006). Francini *et al.* (2006) for example, showed a transient increase in antioxidant activity in recalcitrant *Araucaria bidwillii* embryos upon initial dehydration but, with further water loss, activity declined. Further to this, those authors showed this decline to be accompanied by an increase in free radicals and thiobarbituric acid-reactive substances (TBARS), the latter indicating increasing lipid peroxidation (and by implication, substantial membrane damage); this trend of dehydration-induced increase in lipid peroxidation has also been reported in other studies on recalcitrant seeds (e.g. Chaitanya and Naithani, 1994; Varghese and Naithani, 2001).

Lipid peroxidation is widely considered to be a major ultimate contributor to seed deterioration (Priestly, 1986; Wilson and McDonald, 1986; Gutteridge and Halliwell, 1990; Hendry, 1993; Bailly 2004) and occurs when any activated -CH₂- groups of unsaturated fatty

acids are attacked by ROS to generate lipid free-radicals, which react with oxygen to form lipid hydroperoxides (LOOHs). *In vivo* these peroxides are reduced to the corresponding hydroxy acids, the most common of which are hydroxyoctadecadienoic acids (HODEs). An alternate fate of LOOHs is decomposition and rearrangement into secondary reaction products. Some of these products, such as ethane and ethylene, are relatively innocuous whilst others like malondialdehyde (MDA), can be highly toxic and mutagenic (Esterbauer *et al.*, 1991). Membrane lipids are a primary target for free radical attack and oxidised fatty acid products consequential to such attack can serve as reliable 'markers' of oxidative stress (e.g. Apgar and Hultin, 1982; Benson 1990; Varghese and Naithani, 2008); these compounds include conjugated dienes, lipid peroxides, aldehyde breakdown products, volatile hydrocarbons, and Schiff's bases (formed when aldehydic reaction products of lipid peroxides cross-link with protein groups) (Benson and Bremner, 2004).

The control of ROS by the spectrum of antioxidants is vital, since when they are out of control, as a result of exposure to a stress, the destructive effects of ROS come to the fore (Kranner *et al.*, 2006; Roach *et al.*, 2008). However, apart from their role in stress-induced intracellular damage, ROS have been established to be agents of intracellular signalling in hydrated tissue and are likely to play this dual role in hydrated recalcitrant seeds (Laloi *et al.*, 2004; Bailly, 2004; Foyer and Noctor, 2005; Suzuki and Mittler, 2006; Bailly *et al.*, 2008; Oracz *et al.*, 2009). Reactive oxygen species are believed to act as second messengers in a variety of signal transduction cascades (Foyer and Noctor, 2005, and references therein), with H_2O_2 and O_2^- being singled out due to their implication in many plant developmental and growth processes.

Oxidative stress (primary and secondary) appears to be a major component of chilling and cryoinjury in a variety of plant cells and tissues (Tapell, 1966; Benson, 1990; Prasad *et al.*, 1994; Benson *et al.*, 1995; Doke 1997; Day *et al.*, 1998; Park *et al.*, 1998; Harding, 1999; Fleck *et al.*, 2000, 2003; Benson and Bremner, 2004; Blagojević, 2007; Johnston *et al.*, 2007). This is held to be true for non-orthodox seeds tissues as well (Dussert *et al.*, 2003; Varghese and Naithani, 2008; Walters *et al.*, 2008; Whitaker *et al.*, 2010) and with the focus of the present study being the cryopreservation of recalcitrant zygotic germplasm, the role of oxidative stress in cryoinury in seed tissues will be elaborated in Chapter 4.

The presence of protective molecules

Sucrose and late embryogenic abundant proteins (LEAs) are held to be essential for the acquisition and maintenance of desiccation tolerance in orthodox seeds (e.g. Buitink *et al.*, 2002; Kermode and Finch-Savage, 2002; Berjak, 2006; Berjak and Pammenter, 2008). A

number of studies have reviewed the categorisation and possible functions of LEAs (Close *et al.*, 1989; Cuming, 1999; Tunnacliffe and Wise, 2007). To date, six groups of LEAs have been identified on the basis of particular peptide motifs; these proteins generally lack cysteine residues and are composed predominantly of charged and uncharged polar amino acid residues (reviewed by Cuming, 1999). Except for those belonging to Group 5, LEAs are highly hydrophilic and heat stable (Cuming, 1999). It has been postulated that the amphipathic nature of LEAs allows for interaction with a wide range of macromolecules, thus counteracting their denaturation under dehydrating conditions (Blackman *et al.*, 1995; Stupnikova *et al.*, 2006). For instance, the hydrophilicity of LEAs may allow some of these protein groups to provide a protective hydration shell around intracellular structures and macromolecules (Berjak and Pammenter, 2008 and references therein).

Induction by abscisic acid and desiccation, salt and cold stress has become one of the expression hallmarks for LEA protein genes (Wise, 2003) and improved stress tolerance is often correlated with increased LEA gene expression (Tunnacliffe and Wise, 2007). Cuming (1999) indicated that the evidence for LEAs being involved in desiccation tolerance derives mainly from "correlative and circumstantial evidence rather than by direct experimental demonstration". However, according Berjak and Pammenter (2008), the basis of the evidence is convincing: the appearance of LEAs accompanies orthodox seed maturation, and the imposition of a variety of stresses causing dehydration stress in plant cells (Cuming, 1999). In support of the above, Buitink et al. (2006) demonstrated that 18 genes coding for LEAs and two heat shock proteins (HSPs) were upregulated and identified as being common to the acquisition of desiccation tolerance in *Medicago truncutula* seeds, and its experimental re-imposition in recovered seedlings. What is noteworthy, in terms of desiccation tolerance, is that dehydration (particularly in the presence of sucrose) induces at least some LEAs to assume the α -helical conformation (Wolkers et al., 2001), suggested to be the basis of the formation and maintenance of the intracellular glassy state in desiccated cells (Berjak, 2006; Berjak et al., 2007). A further link between LEAs and desiccation tolerance is the fact that their appearance is concomitant with abscisic acid (ABA) regulation of LEA gene transcription (reviewed by Bray, 1993; Cuming, 1999; Kermode and Finch-Savage, 2002; Berjak et al., 2007).

There has been much conjecture about the role of sucrose, particularly in the desiccated state of seed tissues (reviewed by Berjak *et al.*, 2007). Orthodox seeds accumulate sucrose and certain raffinose series oligosaccharides during maturation drying (Koster and Leopold, 1988; Leprince *et al.*, 1993; Obendorf, 1997) and upon dehydration these constituents contribute towards a highly viscous, supersaturated solution known as a glass (Leopold *et al.*, 1994). It must, however, be noted that although first proposed as being the consequence of a

supersaturated sugar solution (Koster and Leopold, 1988; Williams and Leopold, 1989), there are now suggestions that many other intracellular molecules, such as LEAs, may contribute to the glass (e.g. Walters, 1998; reviewed by Berjak, 2006). In fact, it has been proposed that intracellular glasses in seeds (Berjak, 2006; Manfre *et al.*, 2009) and pollen (Wolkers *et al.*, 2001) may be based on sucrose in interaction with coiled LEAs and residual water. In narrow intermembrane spaces, however, a sugar-based phase might occur (Bryant *et al.*, 2001), with LEAs being excluded on the basis of size (Berjak, 2006).

Glasses have been suggested to curtail molecular diffusion and so minimise the potential for, and extent of, unregulated metabolism during dehydration (Pammenter and Berjak, 1999; Berjak and Pammenter, 2001, 2004b; Hoekstra *et al.*, 2001; Kermode and Finch-Savage, 2002; Alpert, 2006; Berjak, 2006: Lehner *et al.*, 2006). Also, concentrated sugars within the aqueous phase have been postulated to hinder the close approach of membranes to one another, and hence preventing their intimate lateral proximity during water loss (Koster and Bryant, 2005; Halperin and Koster, 2006). Close proximity of membranes could occasion phase transition of some phospholipids and even the demixing of membrane components, which is associated with the exclusion of integral proteins (Cordova-Tellez and Burris, 2002; Walters *et al.*, 2002a). It is likely that the intracellular glassy state is a major factor in the extended life span of dry orthodox seeds and the ultimate breakdown of glasses may underlie seed deterioration in storage (Leopold *et al.*, 1994; Lehner *et al.*, 2006; Manfre *et al.*, 2009).

In showing that sugars from desiccation tolerant zygotic embryos form glasses at ambient temperatures, whereas those from embryos that do not tolerate desiccation only form glasses at sub-zero temperatures, Koster (1991) suggested that the formation of intracellular glass may help protect embryos from damage due to desiccation. Results of a wide-ranging survey of sucrose accumulation among both orthodox and non-orthodox seeds showed that a variety of recalcitrant seeds accumulate substantial quantities of sucrose relative to oligosaccharide (Steadman *et al.*, 1996). Other studies have similarly shown recalcitrant embryonic axes to accumulate substantial amounts of sugars (Farrant *et al.*, 1993; Pritchard *et al.*, 1995b; Connor and Sowa, 2003) and in some cases this accumulation was found to accompany dehydration (e.g. Berjak *et al.*, 1989). However, the formation of glasses in axes of these seeds seems to only occur at sub-zero temperatures and/or at WCs where viability has already been lost (Pammenter and Berjak, 1999; Berjak and Pammenter, 2001). That is, WCs of *c.* 0.3 g g⁻¹ coincide with a marked increase in cytoplasmic viscosity, indicative of glass formation (Buitink and Leprince, 2004), but under natural, slow, drying conditions recalcitrant seeds die at WCs well above those at which any protective benefits could be derived from the contribution of sucrose to

intracellular glasses, or counteracting lateral contact between membranes (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999; Walters *et al.*, 2001, 2002a; Berjak and Pammenter, 2008).

The situation regarding the occurrence of LEAs in recalcitrant seeds is unclear, as dehydrins have been found to occur in a range of species from different habitats while apparently being absent from others (reviewed by Kermode and Finch-Savage, 2002). Group 2 LEAs (i.e. dehydrins) have been identified in recalcitrant seeds of some temperate trees (Finch-Savage et al., 1994; Gee et al., 1994), other temperate species and some of tropical/sub-tropical provenance (Farrant et al., 1996), and in grasses (e.g. Porteresia coarctata, Zizania spp. and Spartina anglica [Gee et al., 1994]). However, no dehydrin-type LEAs were found to occur in seeds of ten tropical wetland species (Farrant et al., 1996). It must be noted though, that those investigations were confined to the analysis of dehydrin-type, Group 2 LEAs only, which have since been found to be expressed in a wide range of plant and animal tissues (reviewed by Berjak, 2006). Consequently, studies need to be extended to the other groups of LEAs, particularly those unique to seeds. It must be noted though that slowly dried recalcitrant seeds generally die as a consequence of metabolism-linked damage (Pammenter et al., 1998; Pammenter and Berjak, 1999; Walters et al., 2001, 2002a) at WCs much higher than the range at which few, if any, of the mechanisms of protection suggested for LEAs would be operative (Berjak and Pammenter, 2008).

Other factors that may contribute to desiccation tolerance

Lipid composition

Differences in lipid composition between orthodox and recalcitrant seeds, particularly the proportion of saturated fatty acids in membrane phospholipids (Nkang *et al.*, 2003; Liu *et al.*, 2006) and the relative abundance and behaviour of lipid storage bodies during dehydration (Smith and Berjak, 1995), could possibly contribute to desiccation tolerance in orthodox seeds and its absence in recalcitrant types. For instance, in terms of membrane lipid composition, the highly desiccation sensitive axes of *Artocarpus heterophyllus* have been shown to have a higher proportion of 16:0 and 18:0 saturated fatty acids, but lack stigmasterol. Stigmasterol is a major sterol component of orthodox seeds (e.g. *Adenthera pavonina* [Nigam *et al.*, 1973]) and less (desiccation) sensitive temperate recalcitrant axes of *Aesculus hippocastanum* (D. Govender, 2010, pers. comm.¹).

Lipid composition may also change in response to dehydration conditions. Nkang *et al.* (2003) for example, showed that agronomically mature seeds of *Telfairia occidentalis* were

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characterised by predominantly saturated fatty acids when total lipids were evaluated. However, while dehydration at 28°C increased accumulation of both mono- and polyunsaturated fatty acids and was accompanied by viability loss, dehydration at 5°C led to a retention of high levels of saturated fatty acids and a delay in the marked decline in viability. Lipid composition has also been shown to be associated with deterioration of seeds exhibiting 'intermediate' post-harvest physiology, particularly in terms of their chilling sensitivity. In *Cuphea carthagenensis* seeds for example, crystallisation of the predominantly saturated storage lipid occurs at both high and very low WCs, after maintenance at 5°C (Crane *et al.*, 2006). Those authors went on to show rehydration without a preceding melting of crystallised triacylglycerides to be lethal. Despite the information presently available it is not possible to clearly identify the exact effects of lipid composition, of both membranes and storage bodies, on desiccation sensitivity and tolerance in seeds.

Endogenous amphiphilic substances

It was proposed that certain endogenous amphiphilic molecules might migrate into membranes of desiccation-tolerant pollen and seed embryos during dehydration and be reversed upon rehydration (Hoekstra *et al.*, 1997; Golovina *et al.*, 1998). Flavinols (which exhibit strong antioxidant potential) were identified as amphiphilic molecules that could possibly fulfill this migratory role *in vivo* (Hoekstra *et al.*, 1997). These amphiphiles were subsequently suggested to maintain core fluidity of membranes in the dry state but even though these substances can fluidise membrane surface, this phenomenon could not be correlated with desiccation tolerance with any confidence (Golovina and Hoekstra, 2002). To date, no evidence supporting a migratory stabilising role for amphiphilic substances has been forthcoming from *in vivo* studies (E.A. Golovina, 2009, pers. comm.²).

<u>Oleosins</u>

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In plant cells, lipid droplet bodies are surrounded by a layer of unique proteins, oleosins (Huang, 1992). Oleosins, which probably maintain oil bodies as discrete entities, were suggested to be present in inadequate proportions (to oil bodies), or to be completely lacking in lipid-rich recalcitrant seeds such as *Theobroma cacao* (Leprince *et al.*, 1998). However, subsequent studies involving cloning and characterisation of the cDNA and peptide sequencing suggest that oleosins are present in mature *T. cacao* seeds (Guilloteau *et al.*, 2003). The role that oleosins may play in stabilising lipid bodies during dehydration and subsequent rehydration is presently unclear.

<u>Repair</u>

The hypothesis that the primitive mechanism of desiccation tolerance in vegetative tissues probably involves a low intensity but constitutively functioning protection mechanism that is coupled with active cellular repair has existed for some time now (Oliver *et al.*, 2000). Early studies suggested that vegetative desiccation tolerance may rely to some degree on repair mechanisms (Gaff, 1989; Bartels *et al.*, 1993) but recent evidence concerning desiccation tolerance of desiccation-tolerant plants show that they utilise preventive mechanisms that rely heavily on inducible cellular protection systems and extensive repair may not be a major part of their desiccation tolerance strategy (reviewed by Kranner *et al.*, 2008 and Toldi *et al.*, 2009).

Hypotheses used to explain desiccation tolerance in desiccation-tolerant seeds are based largely on protective mechanisms against damage (Bewley and Oliver, 1992). However, the success of desiccation tolerance as the survival mechanism of dry orthodox seeds relies also on the repair of damage incurred during drying, and after inbibition is initiated (Vertucci and Farrant, 1995), during the lag phase of water uptake before radicle protrusion (e.g. Osborne, 1983). At least some repair mechanisms appear to be either poorly expressed or absent in recalcitrant seeds; for example, while mechanisms involved in DNA repair have been observed in orthodox seeds (e.g. Boubriak et al., 1997), in recalcitrant A. marina seeds, DNA did not repair fully after only 8% water loss, and DNA related damage was irreparable after 22% water loss (Boubriak et al., 2000). In the context of repair also, Connor and Sowa (2003) showed that dehydration-induced viability loss in recalcitrant *Quercus alba* acorns was accompanied by a decline in the ability to reverse the gel to liquid crystalline phase in membranes. To withstand such extensive drying, orthodox seeds are presumably able to protect and repair cellular constituents and according to Vertucci and Farrant (1995) the ability to repair requires some level of structural integrity. Mechanisms that appear to stabilise the subcellular organisation of tissues in orthodox seeds during drying appear to be either absent or poorly expressed in recalcitrant types, with axes/embryos of such seeds generally losing cytomatrical organisation and/or organellar integrity as a consequence of dehydration (reviewed by Berjak and Pammenter, 2000). This does not appear to be repaired upon rehydration (e.g. cytoskeletal disassembly in *Inga vera* seeds [Faria et al., 2005]).

Drying rate

Recalcitrant seeds are generally large, highly hydrated, metabolically active structures (Pammenter and Berjak, 1999; Berjak and Pammenter, 2004b). After shedding such seeds lose water more or less rapidly, under similar conditions, the rate being species related (Farrant *et al.*, 1989; Pammenter *et al.*, 2002). Accompanying the progressive reduction in freezable water

upon dehydration (Vertucci and Farrant, 1995) recalcitrant seed metabolism continues but becomes increasingly unbalanced (Berjak et al., 1990; Pammenter et al., 1998; Pammenter and Berjak, 1999; Walters et al., 2001). The degree of damage incurred as a consequence has been shown to be related to the rate at which this water is removed (Pammenter et al., 1991, 1998; Walters et al., 2001, 2002a; Pammenter et al., 2002, 2003). Although loss of viability in Hydration Level III (0.45 - 0.25 g g⁻¹; Ψ = > -11 MPa) appears to typify slowly dried recalcitrant seeds across species, rapid dehydration, particularly flash drying (originally devised by Berjak et al., 1989), facilitates axis viability retention well into Hydration Level III, and occasionally just into Level II (0.25 - 0.08 g g⁻¹; Ψ = > -150 MPa) (Vertucci and Farrant, 1995). Flash drying does not render recalcitrant axes desiccation tolerant since they rapidly lose viability at ambient or refrigerator temperatures if allowed to remain at the low WCs attained (Walters *et al.*, 2001; Berjak and Pammenter, 2008). However, flash drying allows recalcitrant embryos/axes to tolerate dehydration transiently to considerably lower WCs than slow drying, by achieving the rapid passage through the intermediate WC ranges at which aqueous-based metabolism-linked damage occurs: i.e., the time during which unbalanced metabolism occurs and associated ROSmediated damage accumulates is curtailed (Pammenter et al., 1998; Pammenter and Berjak, 1999; Walters et al., 2001). The major benefit of flash drying is that it allows dehydration of recalcitrant zygotic germplasm to WCs sufficiently low for successful cryostorage (e.g. Wesley-Smith et al., 2001b, 2004a; Sershen et al., 2007), which is currently considered to be the only means by which the genetic resources of recalcitrant-seeded species can be conserved, in the long-term. The significance of partial dehydration to the cryopreservation procedure for recalcitrant seed germplasm is discussed in section 1.4.1.

Developmental status

A number of studies have shown desiccation sensitivity in recalcitrant seeds to be influenced by their state of development, before and after harvest (e.g. Finch-Savage, 1992; Farrant *et al.*, 1993; Vertucci *et al.*, 1994; Sershen *et al.*, 2008a). Recalcitrant seeds remain metabolically active after shedding and display a steadily changing metabolic status as they approach germination (Tompsett and Pritchard, 1993; Hong and Ellis, 1995; Berjak and Pammenter, 2004b). Metabolic rate has been shown to decrease with development (e.g. *Aesculus hippocastanum* [Tompsett and Pritchard, 1993]; *Coffea canephora* [Hong and Ellis, 1995]) and most recalcitrant seeds appear to be least desiccation-sensitive when their metabolic rate is lowest; which appears generally to be at, or just before natural shedding (Pammenter and Berjak, 1999). Desiccation sensitivity generally increases as germination progresses in relation

to the increase in rate and complexity of metabolism (e.g. as shown for *A. marina* [Farrant *et al.*, 1988]; *Landolphia kirkii* [Berjak *et al.*, 1992]; *Camellia sinensis* [Berjak *et al.*, 1993]).

1.3.1.1 Dehydration damage

The cellular damage associated with the removal of water may be explained by two major - and not mutually exclusive - mechanisms (reviewed by Vertucci and Farrant, 1995; Pammenter and Berjak 1999; Walters *et al.*, 2001). Damage *sensu stricto* results from mechanical stresses that perturb organelle structures at high moisture levels (reviewed by Levitt, 1980) or macromolecule structures at lower levels (reviewed by Wolfe and Bryant, 1999). Studies have also shown that when metabolically active cells are dehydrated to intermediate moisture levels such cells may continue to respire but may be incapable of scavenging toxic metabolic by-products that accumulate (Leprince *et al.*, 1990, 2000; reviewed by Hand and Hardewig, 1996; Leprince and Hoekstra, 1998) and cause free-radical-associated damage (McKersie *et al.*, 1988; Hendry *et al.*, 1992; Finch-Savage *et al.*, 1994; reviewed by Berjak *et al.*, 2007).

In recalcitrant seeds the nature of dehydration damage is dependent on whether water is removed above or below the minimum WC tolerated by that species (Pammenter *et al.*, 2000; Walters *et al.*, 2002a, 2008). While dehydration of recalcitrant seeds to WCs above the upper limit of desiccation sensitivity *sensu stricto* (generally taken to be ~0.25 g g⁻¹ [Vertucci and Farrant, 1995]) has been suggested to be accompanied by deleterious aqueous-based reactions that give rise to what is referred to as metabolism-linked damage, removal of water below the lower limit tolerated directly affects macromolecular structure and membrane and organellar integrity, i.e. desiccation damage *sensu stricto* (Pammenter *et al.*, 1998, 2000; Walters *et al.*, 2001; Kim *et al.*, 2005).

The loss of turgor pressure of cells is regarded as an early indication of desiccation stress and is a feature of hydrated tissue that is losing water (Iljin, 1957; Walters *et al.*, 2002a; Corbineau *et al.*, 2004). More severe dehydration can lead to loss of membrane integrity which has been suggested to involve fusion of intracellular membrane (Koster and Bryant, 2005), resulting in the exclusion of integral proteins and micelle formation between the membranes (Cordova-Tellez and Burris, 2002; Walters *et al.*, 2002a). Consequently, cellular contents generally leak from the cell especially upon rehydration (e.g. Sacandé *et al.*, 2001; Varghese and Naithani, 2002), presumably because membrane permeability has been severely compromised (Pammenter *et al.*, 2002). Separate lipid bilayers may be reformed upon rehydration but these are likely to be a combination of more than one type of membrane (Cordova-Tellez and Burris, 2002; Walters *et al.*, 2002a), and non-functional. Also, excluded proteins are likely to be denatured and, in any case, would not be re-incorporated (Connor and Sowa, 2003).

Irrespective of the nature of the consequences of the two types of damage discussed above, dehydration-induced vigour and viability loss in recalcitrant seeds may be exacerbated by the absence and/or poor expression of post-dehydration repair mechanisms (e.g. Boubriak *et al.*, 1997; Connor and Sowa, 2003). Since cellular dehydration leads to physical and physiological changes which, if not lethal, may be reversible upon rehydration, desiccation damage may not be inferred from the differences between the hydrated and dry state, but rather only by the resumption of normal activity upon rehydration (Walters *et al.*, 2002a). Intracellular changes induced by dehydration in recalcitrant seed tissues, which include a dramatic increase in the degree of vacuolation, the loss of cytomatrical organisation and organellar integrity (Berjak *et al.*, 1984; Farrant *et al.*, 1997; reviewed by Berjak and Pammenter, 2000; Wesley-Smith *et al.*, 2001a; Raja *et al.*, 2005; Kioko *et al.*, 2006), and cytoskeletal disassembly (Faria *et al.*, 2005), are seldom reversed and will be discussed further in Chapter 3.

1.4 Long-term conservation of the germplasm of recalcitrant-seeded species

Development of long-term storage strategies for desiccation-sensitive germplasm remains challenging, but imperative for conservation of the genetic resources of recalcitrant-seeded species (Berjak et al., 1996, 1999a; Dumet et al., 1997; Engelmann, 2004, 2009; Benson, 2008a; Walters et al., 2008). Cryopreservation, which involves the cooling of biological material and subsequent storage at ultra-low temperatures (between -140 and -196°C; above or in liquid nitrogen [e.g. Finkel and Ulrich, 1983; Withers, 1988; Kartha and Engelmann, 1994; Sakai, 1997]) has proved to be the most promising long-term storage option for germplasm of recalcitrant-seeded species (Dumet et al., 1997; Engelmann, 2000, 2004, 2009; Berjak et al., 2004a; Chaudhury and Malik, 2004; Panis and Lambardi, 2006; Benson, 2008a; Berjak and Pammenter, 2008; Normah and Makeen, 2008; Walters et al., 2008). According to Keller et al. (2008), even though a number of institutions utilising more traditional genebank approaches still prefer traditional in vitro storage techniques, cryopreservation is regarded as the tool to best preserve genetic integrity (reviewed by Harding, 2004). Arrest of cellular metabolic processes in plant tissue during maintenance at cryogenic temperatures has been suggested to minimise, if not preclude, genetic changes, allowing for germplasm to be theoretically stored at such temperatures for unlimited periods (Krøgstrup et al., 1992; Krishnapillay, 2000; Lynch, 2000; Kioko et al., 2003; Engelmann, 2004; reviewed by Harding, 2004). Noting that deleterious events can occur at cryogenic temperatures (reviewed by Benson and Bremner, 2004; Walters et al., 2004), cryostorage obviates problems of increased ploidy, decline or loss of morphogenic

and biosynthetic capacity and the production of undesirable phenotypes, usually associated with *in vitro* storage (Kartha, 1985; Blakesley *et al.*, 1996; Harding, 2004).

However, the successful application of cryostorage to recalcitrant seed germplasm is challenged by the sensitivity of such germplasm to desiccation, freezing and to chilling, in some cases at temperatures $<15^{\circ}$ C (Berjak *et al.*, 1995; for reviews see Walters *et al.* 2008; Berjak and Pammenter, 2008). Thus, even though cryopreservation offers the potential for recalcitrant seed germplasm conservation, the variable success obtained across and within different species (see Table 1.1) has been a matter of concern for some time and demands further investigation (Berjak *et al.*, 1999a; Engelmann, 2000; Pammenter *et al.*, 2010). The present study was designed to contribute to this investigation – aiming, among other things, to generate a more fundamental understanding of the physiological and biochemical basis of this variable success.

Over approximately the past 30 years, there have been over 100 peer-reviewed publications on cryopreservation of recalcitrant seeds and/or their zygotic germplasm (for reviews see Dumet *et al.*, 1997; Engelmann, 1997, 1999, 2000, 2004, 2009; Chaudhury and Malik, 2004; Walters *et al.*, 2008; Pammenter *et al.*, 2010; and see Table 1.1 for citations on experimental work). A common thread that runs through many of these publications is that the distinction between recalcitrant and orthodox seeds yields a useful dichotomy upon which to base cryopreservation procedures for hydrated and dry tissue, respectively. In many ways this dichotomy has influenced contemporary cryopreservation theory and practice, which is expanded on below.

1.4.1 Cryopreservation: guiding principles

With the exception of mature orthodox seeds (Pritchard and Nadarajan, 2008) and certain varieties of pollen (Ganeshan *et al.*, 2008) and spores (Ingram and Bartels, 1996), biological tissues almost always contain considerable cellular water. However, their successful cryopreservation is best achieved when lethal intracellular ice-crystal formation is avoided, as this can cause irreversible intracellular damage (Burke *et al.*, 1976; Stanwood, 1985; Wesley-Smith, 1992; Sakai, 1995; Mazur, 2004). Cryopreservation is generally considered in terms of the liquid and solid (ice) phases of water but it is also possible to cryopreserve plant material by the process of vitrification, i.e. the solidification of the liquid phase without ice-crystal formation (Sakai, 2004). This comprises a glassy state as the system is amorphous and lacks organised structure, yet is still characterised by the mechanical and physical properties of a solid (Taylor *et al.*, 2004). In biological systems, vitrification of water is dependent on increased cell viscosity, as cell solutes become concentrated. Even though the vitrified state is metastable (i.e. not in a thermodynamic state with the lowest free energy, which does not preclude its breakdown) it inhibits the association of water molecules to form ice. The phenomenon of

vitrification has allowed for the development of 'ice-free' cryopreservation which, even though pioneered for animal cells (Fahy *et al.*, 1984, 1986a), has been extensively applied to plant tissues (Sakai *et al.*, 1992; Steponkus *et al.*, 1992). However, freezing of plant tissues that inherently have high WCs will inevitably involve the conversion of at least some of the water to ice (Burke *et al.*, 1976; Wesley-Smith *et al.*, 1992).

While the bulk of the water in plant tissues is involved in solvation and is available for freezing (termed free water), water associated with macromolecular constituents (which has been termed bound/structure associated water) plays a structural and functional role and does not contribute to lethal ice-crystal formation (Lyons, 1973; Burke et al., 1976; Levitt, 1980). Earlier studies showed that the removal of a large amount of freezable water from plant tissues by dehydration, coupled with appropriate cooling and re-warming rates, could preclude formation of lethal intracellular ice-crystals (e.g. Mazur, 1984; Steponkus, 1985). In recent years it has become increasingly apparent that success of cryopreservation protocols for recalcitrant zygotic germplasm depends on the optimisation of cooling rates in conjunction with tissuehydration level, to eliminate or at least minimise nucleation of intracellular ice-crystals (Wesley-Smith et al., 2004a; Chaudhury and Malik, 2004; Sershen et al., 2007; Walters et al., 2008). Success in this regard has, however, been hampered by the following: (a) lethal freezing damage occurring when hydrated seeds/embryos/axes are exposed to LN (Wesley-Smith et al., 1992; Berjak et al., 1999b); and (b) drying to WCs precluding ice formation to the extent of desiccation damage, which generally culminates in loss of viability (Pammenter *et al.*, 1998, 2000; Walters et al., 2008; and other references cited in Table 1.1). In contrast, pollen (reviewed by Ganeshan et al. 2008) and seeds and somatic embryos of most desiccation-tolerant species (reviewed by Pritchard and Nadarajan, 2008) appear to be highly amenable to cryopreservation, shifting the focus for successful cryopreservation of hydrated plant germplasm from freezing tolerance to dehydration tolerance (Engelmann, 2004, 2009; Panis and Lambardi, 2006). However, it must be noted that even though cooling and dehydration are the greatest sources of failure, and under other circumstances, the greatest contributors to post-cryo survival, the success or failure of any plant cryopreservation protocol is a consequence of *all* the manipulations involved in the preparation of the tissue for cooling, and all the steps involved in the recovery of that tissue after cooling (Berjak et al., 1999b; Benson and Bremner, 2004; Pammenter et al., 2010). These manipulations generally include those featured in Figure 1.1 and some of the intricacies of their application, to seed germplasm in particular, are discussed below.

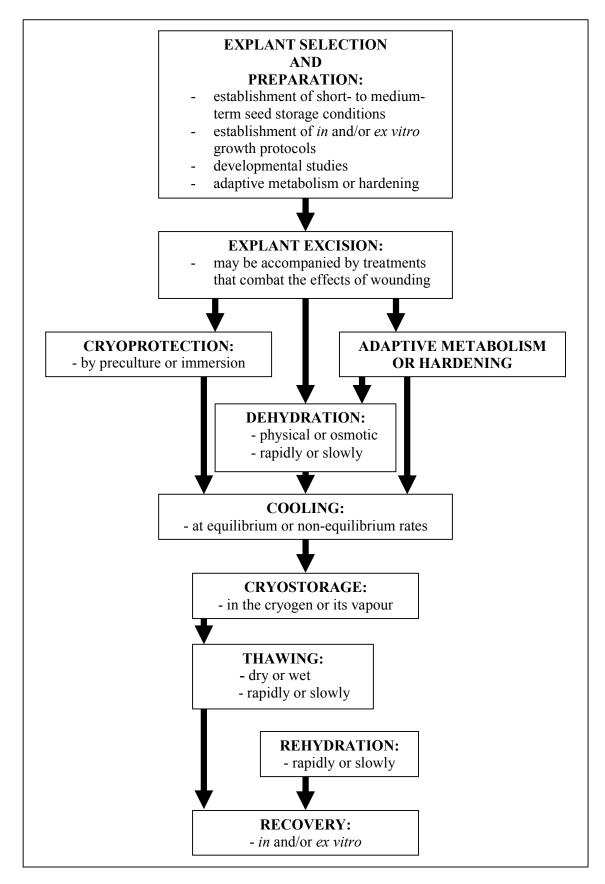


Figure 1.1 Generalised cryopreservation protocol for hydrated plant tissue.

Explant selection and excision

Explants that can be used for cryopreservation include buds, shoots, meristems, cell cultures, protoplast cultures, anthers, pollen, somatic and zygotic embryos, embryonic axes, callus, and whole seeds, if they are sufficiently small (Kartha, 1985; Benson, 2008a). A few studies have succeeded in cryopreserving whole non-orthodox seeds (e.g. Hor *et al.*, 1990; Chaudhury and Chandel, 1994; Hu *et al.*, 1994; Berjak and Dumet, 1996; Dussert *et al.*, 1997; Potts and Lumpkin, 2000; Kioko *et al.*, 2003), plant meristems (Chandel *et al.*, 1993; Matsumoto *et al.*, 1994; Dumet *et al.*, 2002; Varghese *et al.*, 2009) and somatic embryos of recalcitrant-seeded species (e.g. Mycock and Berjak, 1993; Hatanaka *et al.*, 1994; Mycock *et al.*, 1995; Dumet *et al.*, 1997). However, over approximately the past 25 years, embryonic axes and zygotic embryos have become the most popular choice of explant in cryopreservation studies involving non-orthodox-seeded species (see Table 1.1).

Zygotic embryos and embryonic axes constitute a much smaller volume of tissue than the usually bulky, highly hydrated, seeds from which they are excised, facilitating much faster drying and cooling rates (Wesley-Smith, 2001a; Pammenter et al., 2002). However, a prerequisite for post-thaw success appears to be selecting embryos at the most appropriate developmental stage (Chandel et al., 1995; Engelmann et al., 1995; Goveia et al., 2004). It seems reasonable to suppose that for any particular species there might be an optimal developmental stage that would facilitate successful cryopreservation which could well, be at full maturity (Vertucci et al., 1991; Dumet et al., 1997; Kim et al., 2002, 2005). Identifying this stage in recalcitrant seeds is challenging, however, since there are no clear-cut indications as to what constitutes full maturity; furthermore, seeds of the same species seem not invariably to be shed at exactly the same stage (Finch-Savage, 1992; Kermode and Finch-Savage, 2002). Irrespective of the developmental stage selected, however, excision most often involves severing the attachment of the axis to its cotyledon/s flush with the surface of the former, but which has been shown to have detrimental consequences (Goveia et al., 2004; Roach et al., 2008; Pammenter et al., 2010). Those authors indicated that excision results in an oxidative burst (i.e. an enhanced production of ROS) as part of a primary wounding response.

The oxidative damage associated with this wounding response is believed to pre-dispose the explants to further damage as they pass through the various manipulations involved in cryopreservation (Pammenter *et al.*, 2010). In some recalcitrant-seeded species, excising the cotyledons flush with the axis surface has been shown to damage the shoot apical meristem lethally, thus precluding shoot development, right at the outset of explant processing (Fig. 1.1) (Goveia *et al.*, 2004; Perán *et al.*, 2006). The genes controlling the development of meristematic tissue have been reported to be inhibited in response to wounding (Souer *et al.*, 1996) but the

exact nature of the damage response observed in shoot meristem of some recalcitrant axes remains to be resolved (e.g. Goveia *et al.*, 2004; Roach *et al.*, 2008; Pammenter *et al.*, 2010).

Cryoprotection

Natural Freeze tolerance

Seasonal sub-zero temperatures limit growth, development, and distribution of plants, and most tropical and sub-tropical species lack the ability to adapt to such temperatures, often being injured below 10°C (Xin and Browse, 2000). In contrast, many temperate, a few sub-tropical and all cold-adapted plant species will naturally withstand sub-zero temperatures; such plants are characterised by a range of mechanisms which are enhanced in response to a preceding period of low but non-freezing temperatures, subsequently facilitating survival of freezing temperatures (Hirsh, 1987; Pearce, 2004). This conditioning, which allows for survival of dormant tissue, is termed cold hardening or cold acclimation (Levitt, 1980), which is an extremely complex phenomenon, involving many biochemical and physiological changes, as well as the altered expression of hundreds of genes (Guy, 1990). One of these changes involves the synthesis of intracellular solutes which increase osmolality and reduce plasmolysis injury at freezing temperatures (Reed, 2008), although Meryman and Williams (1985) cautioned that such freeze avoidance mechanisms may permit tolerance only to temperatures around -15°C. However, based on evidence of the formation and stability of intracellular glasses at relatively high, sub-zero temperatures in plant tissues, it was proposed that proteins in conjunction with sugars may cause solutions to have glass transitions at temperatures lower than those of water and sugars alone (Hirsh, 1987). Since then a number of studies have suggested that cold acclimation may be used to pre-condition explants for exposure to cryogenic temperatures (reviewed by Reed, 1996, 2008; Benson, 2008b).

If plant tissues are amenable to cold acclimation, this can be induced in the laboratory by exposing the plants to low *in vitro* growth temperatures (1 - 6°C) generally in combination with shortened day-length and/or high-sugar pre-treatments (Reed, 1996, 2008). The responses of amenable plants after exposure to such conditions include: reduced hydration status; reduced growth rate; increased transcription; increased antioxidant production and/or activity; altered osmotic regulation; accumulation of sugars, polyols, betaine, proline; cell wall modification; and changes in lipid composition of membranes and hormone status (e.g. abscisic acid) (Xin and Browse, 2000).

The bio-molecular mechanisms that have been proposed to be involved in cold acclimation are very likely to be related to those facilitating dehydration tolerance (for reviews see Thomashow, 1999; Xin and Browse, 2000; Pearce, 2004). As partial dehydration is an integral step in cryopreservation protocols, this may explain why, in cases where cold acclimation has been successful in enhancing the ability of explants to withstand LN exposure, the species concerned has been of temperate provenance or naturally cold-hardy (Reed, 1996, 2008). It is probably relevant that most tropical plants tested do not respond to cold acclimation (Zhao *et al.*, 2008); however, some of the events occurring during cold acclimation of amenable plants could be achieved *in vitro* for explants of tropical species, e.g. culture on sucrose-enriched media which causes osmotic dehydration and accumulation of solutes and/or exposure to ABA which *inter alia* induces the *in vivo* synthesis of carbohydrates (Dumet *et al.*, 1993a; Xu and Bewley, 1993). Those observations, as well as successful attempts at increasing axis desiccation and freezing tolerance prior to cryopreservation in temperate species such as *Acer saccharinum* (Beardmore and Whittle, 2005) have generated much interest in the use of pre-conditioning treatments, other than cold hardening, to increase post-thaw survival of tropical plant germplasm. Some of these pre-conditioning treatments are described in Chapter 5.

Exogenous application of cryoprotectants

Any pre-conditioning that reduces the amount of 'free' water available for freezing may increase the chances of cells or tissues surviving exposure to cryogenic temperatures (Meryman and Williams, 1980; Reed, 1996). Such conditioning may be imposed by physical dehydration or the application of cryoprotectants (Meryman and Williams, 1980, 1985; Sakai, 1985; Kartha and Engelmann, 1994; Lynch, 2000; reviewed by Fuller, 2004).

The benefits of cryoprotection are best explained when they are related to the biophysical changes brought about by the transition of water to ice during cooling; this is the main cause of damage, rather than other effects of low temperatures (Mazur, 1990, 2004; Karlsson and Toner, 1996). The growth of extracellular ice-crystals gives rise to an effective osmotic stress as the solute concentration surrounding the cells is concentrated into an ever decreasing solvent volume. This 'freeze-dehydration' is one of the most harmful consequences of cryopreservation, with the potential to cause severe damage including changes in the ultrastructure of membranes, separation of membrane bilayers and organelle disruption (Lovelock, 1954; Mazur, 2004). At sufficiently low temperature (usually < -80°C) the remaining, highly viscous solution within and outside the cells transforms into a glassy matrix; this is the relatively stable form for long-term preservation.

The propagation of intracellular ice-crystals is the second major damaging event during cell freezing (Leibo *et al.*, 1978; Mazur, 2004) and ice-crystal-mediated damage includes membrane and organelle disruption and air bubble formation. The potential for intracellular ice formation increases if the osmotic potential inside the cell becomes dislocated from that in the surrounding medium on a kinetic basis (Mazur, 2004), e.g. during rapid cooling when there is insufficient

time for water to move down the chemical potential gradient from the relatively more dilute intracellular solution, to the concentrated extracellular medium. However, if cells are cooled under conditions that effectively inhibit ice-crystal formation down to the region of low temperature glass, then successful cryopreservation can be achieved (Fuller, 2004).

Cryoprotectants are a heterogeneous group of compounds that depress the kinetic freezing point of water (often referred to as the 'supercooling point' in biological solutions [Wilson *et al.*, 2003]), and so reduce the likelihood of lethal ice-crystal formation during cooling and subsequent thawing after cryostorage (Kartha and Engelmann, 1994; Santarius and Franks, 1998; reviewed by Fuller, 2004). Glycerol (Gly) was the first compound to be shown to have cryoprotective action (Lovelock, 1953) and since, a variety of natural and synthetic chemicals have been shown to confer cryoprotection (e.g. natural: methanol; ethanol; sorbitol; and trehalose [Morrisey and Baust, 1976] and synthetic: dimethylsulphoxide [DMSO]; dextran; and polyvinylpyrolidene [PVP] [Meryman and Williams, 1985]). Already in 1969, Karow recorded 56 solutes with cryoprotective activity; however, the large majority of these are not used in contemporary cryopreservation protocols because of their low efficiency. Also, most, if not all, cryoprotectants exhibit some degree of cytotoxicity (reviewed by Fuller, 2004).

Cryoprotectants may be used individually or in combination (see Table 1.1) and their application usually involves one of the following: (1) soaking explants in the cryoprotectant solution for a predetermined period prior to dehydration and cooling (e.g. Meryman and Williams, 1985; Mycock et al., 1995; Valladares et al., 2004); (2) immersing explants in the cryoprotectant solution during cooling (e.g. Sakai et al., 1992); or (3) pre-culturing the explants on a growth medium enriched with cryoprotectants, usually sucrose, before dehydration and cooling (e.g. Dumet et al., 1994; Thierry et al., 1997; Thammasiri, 1999; Lynch, 2000). Cryoprotectants are broadly categorised as either penetrating or non-penetrating, based on their ability/inability to move across biological membranes (Meryman and Williams, 1985). While penetrating cryoprotectants, e.g. DMSO and Gly, are able to diffuse through the plasma membrane and equilibrate in the cytoplasm, non-penetrating cryoprotectants (e.g. sucrose and mannitol), do not enter the cytoplasm but may accumulate apoplastically (Grout, 1995; Muldrew *et al.*, 2004). At this juncture it is worth mentioning that there is evidence that sucrose might be taken up by yeast cells with the aid of plasma membrane sucrose-binding proteins (SBP's) (Overvoorder et al., 1997). Proteins that share sequence and structural similarities with SBP's have been identified in seeds (e.g. those of maize), however, they were unable to mediate sucrose uptake when expressed in yeast (Overvoorder et al., 1997). While it is unlikely that sucrose diffuses through lipid bilayers to any significant extent during the short exposure periods required for the cryoprotection of explants in sucrose solutions, sucrose may well be taken up by plant cells after hydrolysis during extended periods of exposure, e.g. tissue culture. However, in the absence of concrete evidence for the rapid, active uptake of sucrose by plant cells sucrose is still widely regarded as a non-penetrating cryoprotectant in plant cryo-literature (e.g. Finkle *et al.*, 1985; Liu *et al.*, 2004; Winkelmann *et al.*, 2004) and will be referred to as such throughout this thesis.

Following the discovery by Polge *et al.* (1949) that Gly protects avian sperm cells against freezing some cryobiological research explored the mode of action of cryoprotectants in terms of colligative action (Lovelock, 1953; Meryman and Williams, 1980, 1985). The colligative property of a solution depends on the ratio of the number of particles of solute and solvent in the solution, and not the identity of the solute. The two fundamentals of the theory of colligative cryoprotection are: (1) cryoprotectants must be able to penetrate the cell, otherwise, they will cause osmotic dehydration, resulting in the very injury they are employed to avoid; and (2) cryoprotectants must be non-toxic to the cells at the concentrations required for their efficacy (reviewed by Benson, 2008b).

Based on the molar depression of freezing point associated with mixtures of solutes in solution, Lovelock (1953) proposed that at any given temperature below the ice transition during cooling, the increase in solute (salts) would be ameliorated by the presence of the penetrating cryoprotectant, glycerol. He went on to suggest that this would prevent critical damaging concentration of salts while the whole system was cooled sufficiently to achieve the glassy matrix state. In the words of Benson (2008b), "glycerol acts as antifreeze by reducing the concentration of extracellular salt and water loss due to osmosis". In general, penetrating cryoprotectants contribute to the overall osmolality of the cell and an additive such as Gly increases the initial osmolality of the cell colligatively prior to the initiation of the freezing process. As a result, the amount of water that needs to be frozen out to achieve osmotic equilibrium is far less and the extent of the freeze-induced dehydration that occurs is better tolerated (Lovelock, 1953; Meryman and Williams, 1985; Sakai, 2000). The added protective advantage is that this occurs at a lower temperature, as the additive depresses the freezing point. Arakawa and Timasheff (1982) have presented a series of arguments in which they suggest the modes of action of cryoprotectants to be related to inter-molecular interactions between these agents and biologically important macromolecules. In this regard, penetrating cryoprotectants such as DMSO and Gly have the ability to substitute reversibly for water in the hydration sheath of polysaccharides, proteins and nucleic acids, thereby altering their macromolecular structure (Chang and Simon, 1968; Barnett, 1972; Arakawa and Timasheff, 1982), which may render them less amenable to radiation-induced injury (Benson and Bremner, 2004), giving rise to the term 'radioprotectants'. Apart from the physiological mechanisms of protection described thus far, certain cryoprotectants, and especially radioprotectants such as Gly and DMSO may confer biochemical protection during dehydration and freezing, mainly by scavenging harmful free-radicals (Polge *et al.*, 1949; Benson and Bremner, 2004).

Non-penetrating cryoprotectants such as sucrose (Suc) withdraw water from cells by osmosis and, via this dehydrating action (together with their ability to form hydrogen-bonds with water), generally increase intracellular viscosity, arrest the molecular motion of water molecules and consequently reduce the amount of 'free' water available for lethal intracellular ice-crystal formation (Meryman and Williams, 1985; Kartha and Engelmann, 1994; reviewed by Fuller, 2004). The combination of these effects will promote vitrification during freezing (Fahy, 1986a; Dumet and Benson, 2000) but the use of non-penetrating cryoprotectants at high concentrations can result in osmotic injury, since they act by dehydrating cells before freezing (Leibo *et al.*, 1978; Muldrew *et al.*, 2004).

There has been much conjecture about the role of sucrose particularly in the desiccated state of seed tissues (reviewed by Berjak *et al.*, 2007). The disaccharide sugars, sucrose and trehalose, have also been suggested to stabilise membranes during hypertonic exposure as ice-crystals grow by interacting with polar head groups of phospholipids (Rudolph and Crowe, 1985; Strauss and Hauser, 1986). However, Bryant *et al.* (2001), Koster and Bryant (2005) and Halperin and Koster (2006) offer a more convincing argument for the role of sucrose, based on sound evidence: the role of sucrose is dynamic in hindering the close approach of membranes to one another, and hence preventing their lateral proximity. Such proximity would promote phase transition of some phospholipids and even the demixing of membrane components, accompanied by exclusion of integral proteins.

Some large polymer non-penetrating cryoprotectants, e.g. PVP and dextran, demonstrate cryoprotective action under certain conditions (Fuller, 2004). For example, at high concentration such cryoprotectants can exert appreciable effects on freezing point depression of the system (Connor and Ashwood-Smith, 1973) and are effective in red blood cell cryopreservation, but not for nucleated cells when used as a sole cryoprotectant (reviewed by Fuller, 2004). Fuller (2004) does however, suggest that such polymers may make a significant contribution to post-thaw survival when used in combination with other cryoprotectants.

The propensity for cryoprotectants to enhance viscosity of the cytoplasm to such an extent that intracellular ice-crystal formation is completely inhibited, i.e. the process of vitrification, is a very important aspect of their utilisation (Nash, 1966; Meryman and Williams, 1980, 1985). In this process extracellular freezing occurs but cells do not freeze intracellularly and are presumed to be preserved in a glassy matrix state. In this glassy state, the distribution of diffusible components and thus the potential interactions of these components is minimised (Lovelock,

1953; Buitink et al., 1998, 2000). Also, glasses may minimise cellular collapse during freezeinduced dehydration (Sakai, 2000). Increasing cell viscosity to the point at which ice nucleation is inhibited and water becomes vitrified on exposure to cryogenic temperatures is key to developing effective cryoprotective vitrification strategies (Benson, 2008b). Most plant vitrification protocols achieve this increase in cytoplasmic viscosity by the combination of cryoprotectants at high concentrations, followed by removal of water by evaporative dehydration. There is one drawback, however: in general, applied vitrification requires very high (>45% weight for weight) cryoprotectant concentrations (often in mixtures) and at such high concentrations cryoprotectants are often toxic (reviewed by Fuller, 2004). It must also be noted that there are biophysical implications, including mechanical stresses in the glassy matrix at low temperatures, which can give rise to ice-crystal growth during warming (Fuller, 2004). It is therefore recommended that mixtures of penetrating and non-penetrating cryoprotectants be used for this purpose, since this appears to reduce the toxicity of any single additive, limits the impacts of severe evaporative drying and helps to stabilise the glasses formed (Fahy et al., 1984, 1986b). For instance, polyols and sugars enhance the glass-forming tendency of aqueous solutions and when applied in combination with other cryoprotectants may reduce the cryoprotectant concentrations required to achieve a stable glass (reviewed by Fuller, 2004). However, this effect may also be affected by the steric conformation of the cryoprotectant employed, since the number and orientation of hydroxyl (OH) groups in sugar and polyol cryoprotectants influence the vitrification achieved. In this regard, Turner et al. (2001a) suggested that polyols like Gly with relatively more OH groups may be more efficient than sugars at promoting vitrification due to their increased ability to replace water and interact with membrane phospholipids.

When cryoprotectants have been applied alone and/or in combination with dehydration to recalcitrant zygotic germplasm the outcomes assessed by subsequent growth/performance have not been universally successful (see Table 1.1). In most cases, cryoprotectants alone do not provide the protection necessary to ensure post-thaw survival of tropical plant germplasm, but when combined with other pre-conditioning treatments such as partial dehydration (using air flow or desiccants) with or without prior preculture by incubation on media incorporating various sugars or other osmotically active substances, cryoprotection can greatly improve post-thaw survival of zygotic embryos (Pence, 1991; Sam and Hor, 1999), embryonic axes (Engelmann, 1997; Walters *et al.*, 2002b; Sershen *et al.*, 2007) and somatic embryos (Dumet *et al.*, 1994; Mycock *et al.*, 1995) of a range of non-orthodox-seeded species.

The mode of action of cryoprotectants is likely to be multi-factorial (Fuller, 2004) but minimising physical, and metabolic injury associated with cryopreservation is core to the principles of cryoprotection. In affecting tissue water concentration, and hence the range of temperatures at which water freezes in plant tissues, cryoprotectants offer the possibility of altering the relationship between WC and the required cooling rate in seed tissues (Volk and Walters, 2006; Benson, 2008b; Walters *et al.* 2008).

Partial dehydration

The high degree of hydration of embryonic axes/zygotic embryos from recalcitrant seeds contributes to a considerable thermal mass (Bachmann and Mayer, 1987) and also promotes lethal ice-crystal growth at sub-zero temperatures, making them unamenable to cryopreservation in their native state (Wesley-Smith et al., 1992, 2004a; Pritchard et al., 1995a; Walters et al., 2008). Consequently, partial dehydration is necessary to reduce the heat to be dissipated during cooling (Wesley-Smith, 2004a; Walters et al., 2008). Partial dehydration also increases cytoplasmic viscosity (Leprince et al., 1999; Wesley-Smith et al., 2001b; Walters et al., 2008) and so impedes intracellular ice-crystal growth during cooling in hydrated seed tissues (Stanwood, 1985; Steponkus, 1985; Wesley-Smith et al., 1992). Additionally, the lower freezing temperature (Pritchard et al., 1995a) and higher glass transition temperature (Wikefeldt, 1971) associated with lower WC, reduces the range of temperature at which icecrystal growth during cooling and warming is promoted. As discussed earlier, with an adequate reduction of water, ice-crystal formation may be avoided through glass formation (Steponkus et al., 1992; Sakai, 2000). The relative stability of the intracellular glassy state is thought to maintain viability (although not indefinitely) in dry orthodox seeds; however, intracellular glasses would not normally form in most recalcitrant seeds, as vitrification requires low water contents (≤ 0.3 g g⁻¹; reviewed by Berjak and Pammenter, 2008). According to Buitink and Leprince (2004) WCs of approximately 0.3 g g⁻¹ coincide with a marked increase in cytomatrical viscosity, indicative of glass formation, but under the slow drying conditions which would prevail in the field, recalcitrant seeds die at much higher WCs (Pammenter et al., 1993, 1998; Pritchard et al., 1995a; Pammenter and Berjak, 1999; Walters et al., 2001, 2002a). However, it is possible that intracellular glasses could be formed as a consequence of rapid dehydration which permits transient viability retention at suitably low WCs for cryopreservation in excised recalcitrant embryonic axes/zygotic embryos (Berjak et al., 1990; Berjak and Pammenter, 2008).

The potential of cryopreservation protocols for recalcitrant seed germplasm has therefore come to depend (apart from ice-crystal avoidance) on embryo/axis sensitivity to partial dehydration, which has become a standard pre-treatment for such germplasm (for reviews see Engelmann, 1999, 2000; Walters *et al.*, 2008; Berjak and Pammenter, 2008; also see Table 1.1).

It is most often achieved by one of the following methods: (a) the use of dehydrating agents such as silica gel (e.g. Dumet *et al.*, 1993b; Fu *et al.*, 1993); (b) drying in a laminar flow cabinet (e.g. Fu *et al.*, 1990; Pence, 1992); (c) drying over saturated salt solutions, i.e. drying under controlled humidity conditions (e.g. Pammenter *et al.*, 2002); or d) drying explants in a direct stream of dry air, referred to as flash drying (originally devised by Berjak *et al.*, 1989; Pammenter *et al.*, 1991; Vertucci *et al.*, 1991; Wesley-Smith *et al.*, 1992, 2001b).

The rapidity with which water is removed, and hence the shortening of the time during flash drying spent by the tissue at intermediate hydration levels (at which deleterious aqueous-based reactions occur [Pammenter *et al.*, 1998; Walters *et al.*, 2001]), has made flash drying the most efficient method for the partial dehydration of recalcitrant embryonic axes/zygotic embryos (Pammenter *et al.*, 2000, 2002; Engelmann, 2009). The success achieved with flash drying has spurred suggestions that the faster the drying rate, the lower the WC - within limits - to which recalcitrant embryonic axes/zygotic embryos can be dried without viability loss (Pammenter *et al.*, 2002; Walters *et al.*, 2001, 2002a). However, there is a *caveat* here: flash drying does not confer the property of desiccation tolerance and flash-dried axes will not survive for longer than a few hours, if not rehydrated or frozen at cryogenic temperatures (Walters *et al.*, 2001).

Cooling (i.e. exposure to cryogenic temperatures)

'Freeze-dehydration' and intracellular ice-crystal formation are the most harmful of the biophysical consequences of exposure to cryogenic temperatures and can cause a number of damaging events (Lovelock, 1954; Leibo *et al.*, 1978; Mazur, 2004). The attempts of Luyet and Gehenio (1940) to achieve the glassy state in biological systems by cooling sufficiently quickly to avoid ice-crystal formation on a kinetic basis, and the subsequent work of Luyet *et al.* did not achieve a robust protocol which facilitated recovery of living cells (reviewed by Fahy *et al.*, 1984). However, those studies were the precursors of later cryopreservation studies, including those on recalcitrant zygotic embryos/embryonic axes (e.g. Vertucci, 1989; Wesley-Smith *et al.*, 1992). According to those authors, the higher the final embryo/axis WC after drying, the more rapid the rate of cooling should be to restrict ice-crystallisation and associated freezing damage. Later work on the recalcitrant embryonic axes of *Poncirus trifoliata* (Wesley-Smith *et al.*, 2004a) showed that at low axis WCs intracellular viscosity was high, slowing down ice-crystal formation and making survival largely independent of cooling rate, while at high axis WCs, the intracellular viscosity was lower, facilitating rapid ice-crystal formation accompanied by viability loss.

Cooling rate is a function of the thermal mass of the material to be cooled, and the heat transfer properties of the cooling system (Bald, 1987). However, before reviewing the classical

and contemporary methods of cooling it must be noted that there exists some disparity in the literature with regards to the actual cooling rates termed 'rapid'. While some reports term rates of 3 - 10°C s⁻¹ rapid [e.g. Vertucci, 1989; Panis and Lambardi, 2006), others associate rapid cooling with rates of hundreds of °C s⁻¹ (e.g. Wesley-Smith et al., 2001b; 2004a & b). These disparities have been largely unavoidable, since cooling has been achieved using a variety of methods (see Table 1.1). For the purpose of this review, the term 'slow' will be used to describe cooling rates of between 0.5 and 2.0°C min⁻¹ (usually achieved using programmable freezing devices or an alcohol bath in a commercial -70°C freezer [Kartha, 1985; Poulsen et al., 1992]), while cooling rates achieved by enclosing explants within polypropylene cryovials or semen straws before plunging into LN, which generally result in cooling rates of c. 3 - 10°C s⁻¹ (e.g. Vertucci, 1989; Panis and Lambardi, 2006), will be referred to as 'intermediate'. The term, 'rapid', will be used to describe cooling rates achieved by: (a) droplet freezing, i.e. suspending explants in a droplet of cryoprotectant on aluminium foil strips before plunging into cryogens such as isopentane (-160°C), LN (-196°C) or sub-cooled nitrogen (-210°C; Echlin, 1992); (b) tumble-mixing naked (unenclosed) explants in isopentane. LN or sub-cooled nitrogen; and (c) plunging naked explants into isopentane, LN or sub-cooled nitrogen using a spring-loaded or compressed-air driven plunging device. These methods facilitate cooling rates in the range of >10 to hundreds of °C s⁻¹ and in some cases thousands of °C s⁻¹ (e.g. Walters *et al.*, 2002b; Wesley-Smith et al., 1992, 2004a & b).

With the above terminology in place, cryopreservation protocols based on what are termed classical methods (reviewed by Engelmann, 1997) generally involve explant chemical cryoprotection, followed by slow cooling (0.5 - 2.0°C min⁻¹) down to -30 to -40, or even -60°C (Krøgstrup et al., 1992). This controlled slow cooling (step 1) is said to encourage the formation of extracellular ice, progressively dehydrating the cells, as intracellular water is lost to exterior ice nucleation sites. This step is usually followed by immersion in LN (step 2). Optimally, the majority of cells are exposed to cryogenic temperatures (step 2) at a particular cytoplasmic concentration precluding lethal intracellular ice formation since most, if not all, intracellular freezable water is removed during the slow cooling step (Kartha and Engelmann, 1994; Engelmann, 1997). However, when freeze-induced dehydration during step 1 is too intense, various damaging events associated with the concentration of intracellular salts and changes in cellular membranes are possible (Mazur, 1990; Pritchard et al., 1995a). Also, some of the cells may fail to reach the optimum intracellular concentration and upon supercooling undergo lethal intracellular ice-crystal formation (Mazur, 1990). Thus, while slow cooling may retain the integrity of individual cells, it may be less efficient at retaining the tissue integrity necessary for the survival of complex tissues, e.g. meristems and embryos (Panis and Lambardi, 2006). Nevertheless, this 'two-step' cooling method, regarded as the first standard protocol developed for hydrated plant tissue (e.g. Withers and King, 1980), has not been completely abandoned and still finds application in the cooling of undifferentiated culture systems such as cell suspensions and calli (e.g. Withers and Engelmann, 1997), and even differentiated structures such as the shoot apices of cold-tolerant species (e.g. Reed and Chang, 1997) and embryonic axes/zygotic embryos of a few recalcitrant-seeded species, e.g. *Ilex* spp. (Mroginski *et al.*, 2008).

While classical cryopreservation protocols employed freeze-induced dehydration, modern protocols are predominantly vitrification-based (e.g. Fu *et al.*, 1990, 1993; Pence, 1992; reviewed by Engelmann, 1997, 2000, 2009). Here, cell dehydration to increase cytoplasmic viscosity precedes cooling, promotes the formation of glasses and avoids the factors that encourage ice-crystal formation - before exposure to the cryogen (e.g. Stanwood, 1985; Wesley-Smith *et al.*, 1992, 2004a; reviewed by Chaudhury and Malik, 2004; Sershen *et al.*, 2007; reviewed by Walters *et al.*, 2008). Cooling rates typically used to cool embryonic axes/zygotic embryos in these modern protocols, range from *c.* 10°C min⁻¹ (e.g. Vertucci, 1989; Sershen *et al.*, 2007) to hundreds of °C s⁻¹ (e.g. Wesley-Smith *et al.*, 2001b, 2004a & b; Walters *et al.*, 2002b; Sershen *et al.*, 2007).

The rate of cooling influences the number, size and location of ice-crystals formed within cells and tissues (Wesley-Smith et al., 1992, 2004a). Traditional views on the freezing process have favoured slow (i.e. 0.5 and 2.0°C min⁻¹) over rapid cooling rates since the former encourage the formation of relatively few, large extracellular ice-crystals (Mazur, 1990; Kartha and Engelmann, 1994). However, at cooling rates of ≥ 1.6 °C s⁻¹ exosmosis can still occur and since this is usually at a rate much slower than the rate of formation of extracellular ice-crystals, the cytoplasm becomes increasingly supercooled, pre-disposing the cells to intracellular icecrystal formation (Acker and Croteau, 2004). If samples are cooled at rates of $\geq 16^{\circ}$ C s⁻¹ the icecrystals that form may be very small and therefore relatively innocuous (Muldrew et al., 2004) but the amount of ice formed in cells and solutions at physiological concentration appears to be best limited by increasing cooling rates above 100°C s⁻¹ (Luvet et al., 1962). Such rapid rates can be achieved by various methods originally developed for the preparation of biological tissue for cryo-microscopy (see Bald, 1987; Ryan and Purse, 1985) but it is the rapid non-equilibrium cooling methods (pioneered by Luyet et al., 1962), in particular, that have proved to be most useful for cryopreservation of recalcitrant zygotic germplasm (e.g. Walters et al., 2002b; Wesley-Smith et al., 2004a & b; Sershen et al., 2007).

Rapid non-equilibrium cooling methods demand direct contact between the explant and the cryogen (see Luyet *et al.*, 1962); however, when naked specimens at room temperature are plunged into LN to intensify cooling rates, many bubbles appear suddenly on the surface of the

sample, quickly enclosing them in a vapour film and so preventing direct contact with the LN (see Han *et al.*, 1995). This vapour film acts as thermal insulation between LN and the sample, restricting heat transfer and greatly reducing the cooling rate within the sample (referred to as the Leidenfrost phenomenon; Bald, 1987). The only way of counteracting this and intensifying the cooling rate is to keep the vapour film from being stable and complete; this is generally achieved by employing forced convection (Han *et al.*, 1995; reviewed by Walters *et al.*, 2008), or using sub-cooled nitrogen (i.e. nitrogen slush at -210°C [Echlin, 1992]). However, since recalcitrant embryonic axes/zygotic embryos almost always exceed the upper size limit (0.1 mm linear dimensions [Bailey and Zasadzinski, 1991]) required to surpass the benchmark cooling rate of $10^{4\circ}$ C s⁻¹, which is thought to preclude freezing artefacts in cryo-fixation for electron microscopy (Moor, 1971), the complete avoidance of intracellular freezing may be unattainable in such explants (Wesley-Smith *et al.*, 2004a). Those authors suggest that in recalcitrant embryonic axes/zygotic embryos rapid cooling may, at best, only limit the amount of ice formed intracellularly.

A common feature of all contemporary plant cryopreservation protocols is that the critical step to achieve post-thaw viability is dehydration and not cooling, as in classical protocols. The relationships among WC, cooling rate and post-thaw survival in recalcitrant embryonic axes/zygotic embryos will be discussed in greater detail in Chapter 2 but, it must be noted that while the lower limit of the 'optimal hydration window' in recalcitrant embryonic axes/zygotic embryos is constrained by desiccation sensitivity of the tissue, its upper limit may be constrained by freezing injury due to intracellular ice formation at relatively high WCs (Becwar *et al.*, 1983; Pritchard and Prendergast, 1986; Pence, 1992; Wesley-Smith *et al.*, 1992; Sun, 1999; reviewed by Walters *et al.*, 2008). Thus, if samples to be frozen using dehydration-based techniques are amenable to desiccation to appropriately low WCs with little or no viability loss, then there is likely to be little to no post-thaw viability loss. Partial dehydration, in minimising or even precluding ice formation during cooling (Stanwood, 1985; Wesley-Smith *et al.*, 1992), makes dehydration-based protocols less operationally complex than the classical methods.

Thawing and rehydration

Once the explants have been introduced into the cryogen, they can theoretically be stored at these ultra-low temperatures (at or near -196°C) for considerable periods (Kartha and Engelmann, 1994; Berjak *et al.*, 1999a; Walters *et al.*, 2004; Benson, 2008a). However, non-injurious retrieval from cryostorage is critical to the ultimate success of any cryopreservation protocol, necessitating the optimisation of thawing and rehydration procedures. In partially dehydrated explants which have been cryopreserved, water uptake during thawing and

subsequent rehydration can be damaging (Dussert *et al.*, 2003). Water content, temperature of the rehydration medium and rate of water uptake, all influence the sensitivity of partially dried seed tissue to imbibitional injury (Hoekstra *et al.*, 1999; Perán *et al.*, 2004); possibly because these factors affect the structure and organisation of intracellular membranes and conformation of DNA (Osborne *et al.*, 2002).

During warming there is the danger that small ice-crystals might coalesce into larger, damaging ones (Mazur, 1984), but rapid warming should obviate (re)crystallisation (Wesley-Smith *et al.*, 2004a; Panis and Lambardi, 2006). For best results, rapid non-equilibrium thawing, which usually involves immersing cryotubes or naked explants into a thawing medium held at 40 - 45°C is preferred (Dumet *et al.*, 2002; Sershen *et al.*, 2007). Interestingly though, while rapid cooling followed by slow warming is almost invariably detrimental, slow warming was found to be less damaging to slowly-cooled recalcitrant embryonic axes of *Poncirus trifoliata* (Wesley-Smith *et al.*, 2004a).

For rehydration after thawing, the explants can be simply transferred to the same medium (as used for thawing) held at ambient temperature, but the medium used appears to be critical to its success (Sershen *et al.*, 2007). There are suggestions that for partially dried recalcitrant embryonic axes/zygotic embryos, the addition of Ca^{2+} and Mg^{2+} cations to the rehydration medium has positive effects on subsequent survival (Sershen *et al.*, 2008a) and regeneration (Berjak and Mycock, 2004). This appears to be at least partly because Ca^{2+} and Mg^{2+} cations facilitate the reconstitution of cytoskeletal elements in the partially dried axis tissues upon rehydration and also normalise starch accumulation (Berjak and Mycock, 2004). Also, for recalcitrant embryonic axes/zygotic embryos, rapid rehydration by direct immersion into the medium has been demonstrated to be far superior to slow equilibration with saturated relative humidity, probably because slow rehydration extends the time spent at undesirable intermediate WCs (Péran *et al.*, 2004).

Recovery

Given the variety of factors that influence post-thaw viability, specific *in vitro* conditions must be optimised before cryopreservation can be attempted. Unless involving small, intact desiccation-tolerant seeds, which can be directly planted out after thawing (e.g. Walters *et al.*, 2005), cryopreservation requires *in vitro* methods of recovery to assess viability of the explants before, and following cryostorage, and to generate plants after cryopreservation (Mycock *et al.*, 2004; reviewed by Reed, 2008). Successful *in vitro* germination demands aseptic conditions and an efficient, non-injurious explant decontamination protocol, as micro-organisms degrade explant tissues rapidly, resulting in viability loss (Reed *et al.*, 2004). This is especially true for

zygotic embryos/embryonic axes excised from recalcitrant seeds, since they frequently harbour a wide spectrum of fungal and bacterial contaminants (Mycock and Berjak, 1995; Berjak 1996; Sutherland *et al.*, 2002; Sershen *et al.*, 2008b).

The culture medium used for recovery should ideally contain macro- and micronutrients, essential minerals, a carbon source, and perhaps the plant growth regulators that would have originally been supplied by the seed storage tissues. The appropriate combination of nutrients, minerals and vitamins in the medium are likely to be species-specific and, depending on the morphogenic result required, the medium can be manipulated by the addition of plant growth regulators (e.g. auxins and/or cytokinins) to stimulate either root production, shoot production, or callus growth (Turner *et al.*, 2001b; Renau-Morata *et al.*, 2005), or by the alteration of environmental conditions such as temperature and light (Reed *et al.*, 2004). For some species, even minor modifications in the hormonal balance of the culture medium can lead to dramatic improvements in post-thaw viability and onwards development (e.g. Normah and Vengadasalam, 1992; Perán *et al.*, 2006).

Environmental factors such as light and temperature are equally important since these influence *in vitro* development and morphogenesis (Amirato, 1989). *In vitro* cultures are generally not photoautotrophic, which implies that their light requirements are different from those required for photosynthesis, but light may be critical for photomorphogenic and phototropic responses (Thorpe, 1980). High light intensities should be avoided immediately after recovery from LN, since these can promote oxidative stress in frozen-thawed embryonic axes/zygotic embryos and, in some cases, an interim dark period immediately after recovery from cryostorage is essential before introduction to full light conditions (e.g. for *Zizania palustris* [Touchell and Walters, 2000]; and various amaryllids [Sershen *et al.*, 2007]). The *in vitro* effect of temperature on plant growth is based on its *in vivo* effects (Wayne *et al.*, 1998), and for recalcitrant embryonic axes/zygotic embryos cultures temperatures of ~25°C are usually favoured for *in vitro* recovery (e.g. Berjak *et al.*, 1999b; Wesley-Smith *et al.*, 2001b).

The ultimate aim of all plant cryopreservation protocols is recovery and *ex vitro* establishment of normal, vigorous seedlings. This implies that the most rigorous means of gauging the success of any cryopreservation protocol is to quantify the number of frozen-thawed explants that produce normal seedlings capable of surviving *ex vitro* establishment. However, except for the requirement that seedlings developed from cryopreserved explants *in vitro* should be hardened-off in a misthouse and probably require to be treated with systemic fungicides before transfer to *ex vitro* conditions (unpublished data; Plant Germplasm Conservation Unit, University of KwaZulu-Natal, Durban, South Africa), there is at present a paucity of information on the precautions that should be taken during the *ex vitro* recovery step.

1.5 Rationale for the present study and thesis outline

Cryopreservation of recalcitrant seed germplasm is extremely challenging (see Berjak et al., 1999b; Walters et al., 2008; Engelmann, 2009; Pammenter et al., 2010). One of the basic problems is that recalcitrant seeds do not undergo maturation drying and associated metabolic arrest, and are always desiccation-sensitive (reviewed by Kermode and Finch-Savage, 2002; Faria et al., 2004; reviewed by Berjak and Pammenter, 2008). Furthermore, successful cryopreservation of hydrated tissue requires the largest possible volume:mass ratio to facilitate rapid dehydration and cooling, generally precluding the use of whole recalcitrant seeds. This has prompted the preferential selection of zygotic embryos and embryonic axes as explants for cryopreservation of recalcitrant seed germplasm, since they constitute a much smaller volume of tissue than the usually bulky, highly hydrated, seeds from which they are excised. However, in some cases zygotic embryos are too large, making them impossible to dry or cool sufficiently rapidly without lethal consequences (e.g. Theobroma cacao and Crinum bulbispermum: >1.5 cm in length [unpublished data; Plant Germplasm Conservation Unit, University of KwaZulu-Natal, Durban, South Africa]). For some species, the size of the zygotic explant can be reduced by severing the attachment of the axis to its cotyledon/s flush with the surface of the former; however, this has recently been shown to have detrimental consequences in a number of cases (Goveia et al., 2004; Perán et al., 2006; Roach et al., 2008; Pammenter et al., 2010). Nevertheless, manipulation of the combination of dehydration techniques and cooling rates, and in some cases the application of cryoprotectants, has resulted in the successful cryopreservation of the zygotic germplasm of a number of recalcitrant-seeded species (see Table 1.1). However, the extensive list of species given in Table 1.1 belies the difficulties in the quantitative and qualitative achievements of seedling and plant production from cryopreserved zygotic explants, particularly because it is frequently not possible to ascertain exactly what is meant by 'successful' cryopreservation. This is mainly because explanations as to how post-cryo viability was assessed are not provided - i.e. whether, in fact, 'success' simply indicates that explants did not die, or at the opposite extreme, retained the full capacity to produce both roots and shoots.

In reviewing the studies listed in Table 1.1, it was evident that many of them reported postcryo viability as indicated by explant greening, elongation, expansion or callus growth (e.g. Chandel *et al.*, 1995; Berjak and Dumet, 1996; Pence, 2004) rather than actual seedling regeneration. Additionally, where root and shoot production were reported, survival percentages were seldom as high as those achieved with frozen-thawed somatic embryos (e.g. Bertand-Desbrunais *et al.*, 1992; Dumet *et al.*, 1993b; Mycock and Berjak, 1993; Hatanaka *et al.*, 1994; Mycock *et al.*, 1995), nucellar and pollen embryos (reviewed by Ganeshan *et al.*, 2008), cell suspension cultures (e.g. Aguilar *et al.*, 1993), shoot tips (e.g. Demeulemeester *et al.*, 1993; Harding and Benson, 1994), or plant meristems (Chandel *et al.*, 1993; Varghese *et al.*, 2009). Also in some studies reviewed in Table 1.1 and elsewhere (e.g. Engelmann, 1997, 2000; Pammenter *et al.*, 2010) the low seedling recovery rates following cryopreservation of recalcitrant zygotic explants reflect a high incidence of abnormal embryo/axis growth. A prevailing problem is that while zygotic explants, particularly of tropical and sub-tropical species, survive to form roots and/or callus - shoot production often fails to occur (e.g. Wesley-Smith *et al.*, 2001b; Perán *et al.*, 2006; Sershen *et al.*, 2007; Pammenter *et al.*, 2010). In rare cases, shoots are formed but roots fail to develop (e.g. Pence, 1992).

The reporting of post-thaw survival is further complicated for certain species, as the seeds were initially considered as recalcitrant, but have emerged as not being so. For instance, the seeds of oil-palm were originally classified as being recalcitrant (Chin and Roberts, 1980) but are now regarded as showing intermediate storage behaviour (Ellis *et al.*, 1991), while the seeds of *Azadirachta indica* show post-shedding responses resulting in their being categorised as orthodox (e.g. Tompsett and Kemp, 1996), intermediate (e.g. Varghese and Naithani, 2002) or recalcitrant (Berjak *et al.*, 1995), which may well be related to provenance.

Apart from the inconsistencies in the categorisation of seeds (in terms of post-harvest behaviour) and assessment of post-cryo viability, the major hindrance to the wider application of recalcitrant seed germplasm cryopreservation is the unavailability of protocols that will produce reproducible results across species (irrespective of provenance). This is of particular concern in Africa since many indigenous species are now emerging as producing short-lived, recalcitrant seeds (Sacandé et al., 2004). Reviewing the literature suggested that irrespective of the species concerned or treatment combination selected, the success and reproducibility of any plant germplasm cryopreservation protocol demands the optimisation of all the manipulations involved in the preparation of the explant for cooling, the actual cooling, and its thawing and recovery after cooling, as illustrated in Figure 1.1 (Grout, 1986; Hor et al., 1990; Mycock et al., 1991; Hu et al., 1994; Kartha and Engelmann, 1994; Engelmann et al., 1995; Berjak et al., 1999b; Engelmann, 2000; Benson and Bremner, 2004; Benson, 2008a & b; Normah and Makeen, 2008; Walters et al., 2008). It is also clear that the optimisation of any of these manipulations is best achieved when the process is informed by a fundamental understanding of the physiological and biochemical consequences of the manipulation concerned on the tissues composing the explant.

Therein lies the problem: while there is some understanding of the physical factors (e.g. ice formation and intracellular dehydration) associated with freezing and desiccation sensitivity of recalcitrant seed tissue (see Wesley-Smith *et al.*, 1992, 2001b, 2004a; Pritchard *et al.*, 1995a; Walters *et al.*, 2008), there is a paucity of information on the physiological and biochemical

basis of post-cryo viability and death in recalcitrant seed germplasm. Consequently, the present study investigates some of the morphological, physiological, biochemical, and ultrastructural consequences of cryoprotection, partial dehydration and freeze-thawing on whole *Amaryllis belladonna* (L.) and *Haemanthus montanus* (Baker) zygotic embryos, both of which are recalcitrant-seeded amaryllids (Sershen *et al.*, 2008a). In doing so, the present study aimed to understand the fundamental basis of the successes and failures of conventional recalcitrant seed germplasm cryo-protocols and assess the potential for the use of selected markers of cryo-related stress to optimise cryo-protocols for such germplasm.

Figure 1.1 shows that cryopreservation of hydrated germplasm involves a number of preand post-cooling manipulations: each successive manipulation, *viz.* excision; decontamination; cryoprotection; partial dehydration; exposure to the cryogen; thawing; rehydration; and *in vitro* regeneration, has the potential to impose potentially lethal damage on critical tissues of the explant (Berjak *et al.*, 1999b; Benson and Bremner, 2004; Pammenter *et al.*, 2010). As the current investigation aimed to inform future cryopreservation protocols for recalcitrant seed germplasm, the experimental design had to allow for an appreciation of the independent and interactive physiological and biochemical effects of the various procedures. In an attempt to achieve this, experiments in this study were designed to trace the 'metabolic/stress history' of *A. belladonna* and *H. montanus* zygotic embryos consequential to the various procedural steps involved in their cryopreservation (e.g. cryoprotection, partial drying, freeze-thawing). This involved in their cryopreservation (e.g. cryoprotection, partial drying, respiratory activity, rate of protein synthesis and electrolyte leakage after individual and various combinations of these procedural steps. Results of these studies are featured in **Chapter 2**.

Membranous sub-structure and intracellular organisation are essential to proper cell functioning and damage to this sub-structure during dehydration (Berjak *et al.*, 1989; Walters *et al.*, 2001; Faria *et al.*, 2005) and cryopreservation (Wesley-Smith *et al.*, 1992; Naidoo *et al.*, 2005) can affect subsequent explant regeneration (Mycock, 1999). Hence, electron microscopy was used to observe the ultrastructural responses of *A. belladonna* and *H. montanus* zygotic embryos to the various procedures of cryopreservation (in particular, cryoprotection, partial dehydration and freeze-thawing). Results of these studies are presented in **Chapter 3**.

Reactive oxygen species are produced during periods of stress (Oliver *et al.*, 2001) and oxidative stress (primary and secondary) has been identified as a major component of chilling and cryoinjury in plant tissues (Levitt, 1980; Benson, 1990; Benson *et al.*, 1995; Fleck *et al.*, 2003; Johnston *et al.*, 2007). In light of suggestions that oxidative stress metabolism may be a major determinant of post-thaw survival in recalcitrant seed tissues (Normah and Makeen, 2008; Varghese and Naithani, 2008; Walters *et al.*, 2008; Pammenter *et al.*, 2010; Whitaker *et al.*,

2010), the present study considered the oxidative stress metabolism, in particular, lipid peroxidation, extracellular superoxide production and enzymic antioxidant status, following the various procedures involved in the cryopreservation of *A. belladonna* and *H. montanus* zygotic embryos (in particular, cryoprotection, partial dehydration and thawing after cryogenic exposure). **Chapter 4** features the results of these studies.

Based on their desiccation- and/or chilling sensitivity (particularly if they are of tropical origin [Roberts, 1973; Ellis et al., 1990a; Hong and Ellis, 1996]), recalcitrant seeds and their zygotic explants are not ideal candidates for cold acclimation applications (described in section 1.4.1). However, many of the responses observed during cold acclimation are strikingly similar to responses to other stresses, e.g. drought and salinity, suggesting the existence of a web of overlapping signals in stress response pathways (Gazzarrini and McCourt, 2001). Also, the induction of tolerance to one particular stress may result in acquired tolerance to other stresses, a phenomenon referred to as cross-tolerance (Xiong et al., 2002). In this regard, the exogenous application of H_2O_2 , an active oxygen species and signal molecule involved in stress transduction (Azevedo Neto et al., 2005; Hung et al., 2005; Wahid et al., 2007), and the application of non-lethal osmotic stress (Guan and Scandalios, 1998; Guan et al., 2000) represent two potential methods of improving plant tolerance to a wide range of stresses, including chilling (Murphy et al., 2002). Enhanced stress tolerance is thought to be related to the direct expression of a number of genes, some of which are involved in plant defence (Kovtun et al., 2000), antioxidants and cell rescue/defence protein expression (Robert and David, 2004; Hung et al., 2005). Hence, studies described in Chapter 5 investigated whether the exogenous application of H_2O_2 to A. belladonna zygotic embryos or exposure of H. montanus zygotic embryos to a non-lethal osmotic stress, prior to cryopreservation, could enhance cryo-tolerance. Also, since elevated antioxidant status has been implicated in cryotolerance in plant tissues (Dussert et al., 2003; Johnston et al., 2007; Varghese and Naithani, 2008), the study related post-thaw viabilities to antioxidant activities after thawing.

In some cases seedlings recovered from cryopreserved zygotic explants have been reported to exhibit morphological abnormalities (e.g. Pence, 1992; Kioko *et al.*, 1998; Wesley-Smith *et al.*, 2001b; Sershen *et al.*, 2007). Apart from cooling, the manipulation of these explants prior to freezing, especially partial dehydration, can lead to physico-chemical damage, which could explain the post-thaw growth abnormalities observed when recalcitrant zygotic explants of a number of species have been set to germinate (e.g. Dumet *et al.*, 1997; Wesley-Smith *et al.*, 2001b; Péran *et al.*, 2004; Sershen *et al.*, 2007). This requires urgent investigation for future remediation, as the ultimate aim of cryopreservation of zygotic explants is the *ex vitro* establishment of normal, vigorous plants. In light of this, the current contribution assessed the

effects of zygotic embryo dehydration (in *H. montanus* [Chapter 6]) and dehydration and cooling (in *A. belladonna* [Chapter 7]) on subsequent *in* and *ex vitro* seedling vigour.

Results for each chapter are discussed independently but the salient points from all studies are drawn together in **Chapter 8**. In evaluating the extent to which this study has answered the research questions posed here, **Chapter 8** also presents suggestions for future studies and highlights the contradictions and problems that emerged during the experimental phase of this thesis.

Species & explant type	Dehydration rate/method	Explant water content* & cryoprotection	Cooling rate/method	Highest post-cryo viability (%)	Method of viability assessment	Post-drying & post-cryo growth	Reference
Araucaria hunsteinii K. Schum. (EA)	Slow; LF	0.25 g g ⁻¹ ; no CP	Slow; CV	<u>0.25 g g⁻¹</u> =80%	Root meristem survival	High incidence of abnormal growth after decontamination, dehydration and freezing.	Pritchard and Prendergast, 1986
Camellia sinensis [L.] O. Kuntze (EA)	Slow; LF	1.05, 0.33, 0.15, & 0.12 g g ⁻¹ ; no CP	Slow; CV	<u>0.15 g g⁻¹=95%</u>	Root+shoot	Post-cryo seedling phenotype (<i>in vitro</i>) similar to control.	Chaudhury et al., 1991
Juglans regia [L.] CV Franquette (EA)	No physical dehydration	0.05 & 0.25 g g ⁻¹ +1,2- propanediol+Suc CP	Slow; CV	0.05 g g ⁻¹ +CP=61% & <u>0.25 g g⁻¹+CP</u> =75%	Shoot	Toxic effects of cryoprotectants disappeared after freezing. Freezing induced a 2 month delay in growth.	de Boucaud <i>et al.</i> , 1991
Theobroma cacao (immature ZE)	No physical dehydration	Suc-DMSO CP	Two-step cooling: 0.4°C min ⁻¹ down to -40°C then into LN	Suc-DMSO CP=<20%	Somatic embryos or callus	No roots or shoots after freezing.	Pence, 1991
Landolphia kirkii Dyer (EA)	Rapid; stream of nitrogen gas	1.77-0.60, 0.59- 0.45, 0.44-0.30 & <0.29 g g ⁻¹ ; no CP	Slow: 16°C min ⁻¹ down to -70°C within DSC pans in chest freezer	<u>0.44-0.30 g g⁻¹</u> =93%	Greening+root	Freezing induced a 4 week delay in growth.	Vertucci <i>et al.</i> , 1991
Musa acuminata & Musa balbisiana (ZE)	Slow; LF	0.12-0.18 g g ⁻¹ for <i>M. acuminate</i> ; 0.12-0.16 g g ⁻¹ for <i>M. balbisiana</i> ; no CP	Slow; CV	0.12-0.18 g g ⁻¹ for <u>M.</u> <u>acuminata</u> =80-83% & 0.12-0.16 g g ⁻¹ for <u>M.</u> <u>balbisiana</u> =80-92%	Root+shoot	Post-cryo seedling phenotype (<i>in vitro</i>) similar to control.	Abdelnour- Esquivel <i>et al.</i> , 1992a
Coffea arabica; Coffea canephora & Coffea arabusta (ZE)	Slow; LF	0.19 g g ⁻¹ for C. arabica; 0.41 g g ⁻¹ for C. canephora; 0.38 g g ⁻¹ for C. arabusta; no CP	Rapid; foil envelopes plunged into LN	$\frac{0.19 \text{ g g}^{-1} \text{ for } C.}{arabica} = 96\%;$ $\frac{0.41 \text{ g g}^{-1} \text{ for } C.}{canephora} = 42\% \&$ $\frac{0.38 \text{ g g}^{-1} \text{ for } C.}{arabusta} = 84\%$	Root+shoot	Development of frozen embryos delayed compared to control.	Abdelnour- Esquivel <i>et al.</i> , 1992b

 Table 1.1 Review of selected cryopreservation studies on embryonic axes/zygotic embryos from non-orthodox seeds.

LF=laminar flow; FD=flash drying; CV=cryovials; SL=nitrogen slush; EA=embryonic axes; ZE=zygotic embryos; CP=cryoprotection and ng=not given. g g^{-1} = g H₂O per g dry mass.

Species & explant type	Dehydration rate/method	Explant water content* & cryoprotection	Cooling rate/method	Highest post-cryo viability (%)	Method of viability assessment	Post-drying & post-cryo growth	Reference
Cocos nucifera (ZE)	No physical dehydration	Gly & Sorbitol CP	Slow; CV	<u>Gly CP</u> =1 embryo	Root+shoot	Freezing induced a 6 week delay in growth.	Assy-Bah and Engelmann, 1992a
Cocos nucifera; 4 varieties (EA)	Slow; LF	0.07-0.08 g g ⁻¹ +Gly-Glucose CP	Slow; CV	$\frac{0.07 \text{ g g}^{-1}=93\% \text{ for variety}}{1;}$ $\frac{0.08 \text{ g g}^{-1}=73\% \text{ for variety 2;}}{0.08 \text{ g g}^{-1}=86\% \text{ for variety}}$ $\frac{0.08 \text{ g g}^{-1}=86\% \text{ for variety}}{3 \text{ \& }}$	Root+shoot	Post-cryo seedling phenotype (<i>in vitro</i>) similar to control but growth of haustorium suppressed and delayed by 8 weeks. CP slowed growth.	Assy-Bah and Engelmann, 1992b
<i>Coffea liberica</i> Bull. Ex Hiern	Slow; LF	0.11, 0.12, 0.18, 0.24, 0.26, 0.39, 0.89 & 1.52 g g ⁻¹ ; no CP	Slow; CV	$0.18 \text{ g g}^{-1} = c.51\%$	Root+shoot	Growth hormones required for root and shoot production.	Normah and Vengadasala, 1992
Quercus robur (EA)	Slow; LF	0.33 g g ⁻¹ ; no CP	Two-step cooling: -1°C min ⁻¹ down to -38°C then into LN	0%	Root+shoot	Drying was more detrimental to shoots than roots.	Poulsen, 1992
Camellia sinensis (EA)	Rapid; FD	>1.6 1.6-1.1, 1.1-0.7, 0.7-0.4 & 0.4-0.3 g g ⁻¹ ; no CP	10°C min ⁻¹ ; 200°C min ⁻¹ & 1000's of °C s ⁻¹	$\frac{1.6-1.1 \text{ g g}^{-1}+1000\text{'s}}{0.7-0.4 \text{ g g}^{-1}+200^{\circ}\text{C min}^{-1}}$ =60% & <u>0.4-0.3 g g}^{-1}+10^{\circ}\text{C min}^{-1} 10°C min}^{-1}=14%</u>	Root	No shoot production.	Wesley-Smith <i>et</i> <i>al.</i> , 1992
Euphoria longan Lour. (EA)	Slow; silica gel	0.22 g g ⁻¹ ; no CP	Slow; glass tubes plunged into LN or held for 30 min at -18°C then into LN	$\frac{0.22 \text{ g g}^{-1} + \text{directly into}}{\text{LN} = 25\% \&}$ $\frac{0.22 \text{ g g}^{-1} + -18^{\circ}\text{C and}}{\text{then into LN} = 50\%}$	Root+shoot	Plumule growth inhibited by dehydration. Post-cryo growth retarded.	Fu <i>et al.</i> , 1993
Camellia sinensis [L.] O. Kuntze (EA) Theobroma	Slow; LF	0.15 & 0.11 g g ⁻¹ ; no CP	Slow; CV	$\frac{0.15 \text{ g g}^{-1}}{0\%} = 95\%$	Elongation of radicle and plumule		Chandel et al., 1995
cacoa (EA)		g ⁻¹ ; no CP					

Table 1.1 continued...Review of selected cryopreservation studies on embryonic axes/zygotic embryos from non-orthodox seeds.

LF=laminar flow; FD=flash drying; CV=cryovials; SL=nitrogen slush; EA=embryonic axes; ZE=zygotic embryos; CP=cryoprotection and ng=not given. g g⁻¹= g H₂O per g dry mass.

Species & explant type	Dehydration rate/method	Explant water content* & cryoprotection	Cooling rate/method	Highest post-cryo viability (%)	Method of viability assessment	Post-drying & post-cryo growth	Reference
Artocarpus heterophyllus (EA)	Slow; LF	0.16 g g ⁻¹ ; no CP	Slow; CV	<u>0.16 g g⁻¹</u> =30%	Elongation of radicle and plumule		Chandel <i>et al.</i> , 1995
Araucaria hunsteinii (ZE)	Slow; saturated salt solutions+LF	0.18-3.00 g g ⁻¹ ; no CP	Slow; CV above LN	0%	Root+shoot	Callus from radicle only. Dehydration stunted growth.	Pritchard <i>et al.</i> , 1995a
Azadirachta indica (EA)	Slow; silica gel	0.23 & 0.19 g g ⁻¹ ; no CP	Slow; CV	$\frac{0.23 \text{ g g}^{-1}}{0.19 \text{ g g}^{-1}} = 100\% \text{ \&}$	Expansion		Berjak and Dumet, 1996
Prunus persica [L.] Battsch (EA)	Slow; LF	4.00, 0.54, 0.43, 0.14, 0.10, & 0.08 g g ⁻¹ ; 0.54 g g ⁻¹ +1,2-propanediol- Suc CP & 35%+PVS2 CP	Slow; CV	$\frac{0.54 \text{ g g}^{-1}=95\%;}{0.43 \text{ g g}^{-1}=96\%;}$ $\frac{0.54 \text{ g g}^{-1}=96\%;}{0.54 \text{ g g}^{-1}+1.2-}$ propanediol-Suc=95% & $\frac{0.54 \text{ g g}^{-1}+\text{modified}}{\text{PVS2}=85\%}$	Shoot		de Boucaud <i>et al.</i> , 1996
Camellia japonica (EA)	Slow; LF	ng	Slow; CV	8.3-13.3%	Root+shoot		Janeiro <i>et al.</i> , 1996
Citrus hystrix (EA) Citrus halimii (EA)	Slow; LF	0.65, 0.18, 0.12, 0.10 & 0.06 g g ⁻¹ ; no CP 1.61, 0.20, 0.10, 0.07 & 0.06 g g ⁻¹ ; no CP	Rapid; foil envelopes plunged into LN	$\frac{0.12 \text{ g g}^{-1}}{0.20 \text{ g g}^{-1}} = 100\%$	Root+shoot		Normah <i>et al.</i> , 1997
Trichilia dregeana (EA)	Rapid; FD	0.16 g g ⁻¹ +Gly- DMSO CP & 0.16 g g ⁻¹ +Gly-Suc CP	Slow; CV Rapid; SL	$\frac{0.16 \text{ g g}^{-1}+\text{Gly-DMSO}}{+\text{slow}} = 49\% \text{ \&}$ $\frac{0.16 \text{ g g}^{-1}+\text{Gly-Suc}}{+\text{slow}} = 40\%$	Root	Shoots failed to develop with and without drying and cooling.	Kioko <i>et al.</i> , 1998
Quercus rubra (EA)	Slow; LF	0.6-1.7 g g ⁻¹ ; no CP	$\leq 10^{\circ}$ C min ⁻¹ then in LN; 100°C min ⁻¹ then into LN	0 %	Root+shoot		Sun, 1999
Artocarpus heterophyllus Lamk. cv. (EA)	Slow; LF	Suc-Gly CP+ vitrification & 3.00-0.14 g g ⁻¹	Slow; CV	Suc-Gly CP+vitrification= 50%	Root+shoot	Post-cryo seedling phenotype (<i>in vitro</i>) similar to control.	Thammasiri, 1999

Table 1.1 continued...Review of selected cryopreservation studies on embryonic axes/zygotic embryos from non-orthodox seeds.

LF=laminar flow; FD=flash drying; CV=cryovials; SL=nitrogen slush; EA=embryonic axes; ZE=zygotic embryos; CP=cryoprotection and ng=not given. g g^{-1} = g H_2O per g dry mass.

Species & explant type	Dehydration rate/method	Explant water content* & cryoprotection	Cooling rate/method	Highest post-cryo viability (%)	Method of viability assessment	Post-drying & post-cryo growth	Reference
Zizania palustris (ZE)	Rapid; stream of nitrogen gas	0.56, 0.36 & 0.30 g g ⁻¹ ; no CP	Rapid (250°C s ⁻¹); SL	$\frac{0.56 \text{ g g}^{-1}=50-60\% \text{ \&}}{0.36 \text{ g g}^{-1}=50-60\%}$	Coleoptile length		Touchell and Walters, 2000
Aesculus hippo- castanum (EA)	Rapid; FD	>1, 1-0.8, 0.8-0.5, 1.0-0.5, <0.5 & <0.4 g g ⁻¹ ; no CP	Rapid: EAs plunged into SL & isopentane (40-850°C s ⁻¹)	$\frac{\text{Isopentane:}}{0.8-0.5 \text{ g g}^{-1}=80\%}$ & 0.5-0.3 g g^{-1}=100% SL: 0.5-0.3 g g^{-1}=100\%	Root+shoot	Freezing induced abnormal growth; absence of roots, presence of callus and stunted growth.	Wesley-Smith <i>et al.</i> , 2001b
Sechium edule Jacq. Sw.; 2 cultivars (ZE)	Slow; LF	0.30 & 0.23 g g ⁻¹ ; no CP	Slow; CV	$\frac{0.30 \text{ g g}^{-1}=10\% \text{ for}}{\text{cultivar 1 \&}}$ $\frac{0.23 \text{ g g}^{-1}=20-30\% \text{ for}}{\text{cultivar 2}}$	ng	Desiccation reduced viability (by 80% at 0.30 g g ⁻¹ in cultivar 1 and by 30% g g ⁻¹ at 0.23 g g ⁻¹ in cultivar 2). Excision of cotyledons affected viability	Abdelnour- Esquivel and Engelmann, 2002
Quercus suber (EA)	Slow; LF	0.51 & 0.22 g g ⁻¹ ; no CP	Slow (28°C s ⁻¹); CV & Rapid (45°C s ⁻¹); SL	0.22 g g ⁻¹ +slowly cooled=<10%	Shoot	Desiccation reduced viability (by 40% at 0.51 g g^{-1} and by 30% at 0.22 g g^{-1})	González-Benito et al., 2002
Quercus ilex (EA)		0.28 & 0.15 g g ⁻¹ ; no CP	Slow (28°C s ⁻¹); CV	$0.15 \text{ gg}^{-1} = <20\%$	_	Many radicles elongated but turned necrotic later. Desiccation reduced viability (by 60% at 0.28 $g g^{-1}$ and by 80% at	
		1.16, 0.34 & 0.22 g g ⁻¹ ; no CP	Rapid (45°C s ⁻¹); SL	$0.22 \text{ g g}^{-1} = <20\%$		0.15 g g^{-1})	
Camellia sinensis [L.] (EA & EA+ cotyledons)	Slow; LF	0.23 g g ⁻¹ for EA+cotyledons & 0.22 g g ⁻¹ for EA; no CP	Slow; CV	$\frac{0.23 \text{ g g}^{-1} \text{ for EA+}}{\frac{\text{cotyledons}=71.7\% \text{ \&}}{0.22 \text{ g g}^{-1} \text{ for EA}=}$ 56.2%	Root+shoot	Desiccation reduced viability.	Kim et al., 2002
Zizania texana (ZE)	Rapid; stream of nitrogen gas	0.3 & 0.6 g g ⁻¹ +CP with mixture of sugars	Rapid (250°C s ⁻¹); SL	$\frac{-\text{CP: } 0.3 \text{ g g}^{-1}}{+\text{CP: } 0.6 \text{ g g}^{-1}} = 70\%$	Coleoptile length		Walters <i>et al.</i> , 2002b

 Table 1.1 continued...Review of selected cryopreservation studies on embryonic axes/zygotic embryos from non-orthodox seeds.

LF=laminar flow; FD=flash drying; CV=cryovials; SL=nitrogen slush; EA=embryonic axes; ZE=zygotic embryos; CP=cryoprotection and ng=not given. g g^{-1} = g H_2O per g dry mass.

Species & explant type	Dehydration rate/method	Explant water content* & cryoprotection	Cooling rate/method	Highest post-cryo viability (%)	Method of viability assessment	Post-drying & post-cryo growth	Reference
Citrus sinensis [L.] OSB (EA)	Slow; silica gel	0.50-0.12 g g ⁻¹ +Suc CP	Slow (78°C min ⁻¹); CV	$\frac{0.50-0.12 \text{ g s}^{-1}+\text{Suc}}{\text{CP}=80-93\%}$	Root+shoot	Desiccation reduced viability. Post-cryo seedling phenotype (<i>in vitro</i>) similar to control.	Santos and Stushnoff, 2003
Castanea sativa (EA)	Slow; LF	1.94, 1.04, 0.54, 0.410,0.32, 0.25 & 0.22 gg ⁻¹ ; no CP	Slow; CV	$0.25 \text{ g g}^{-1}=63\%$	Root+shoot		Corredoira <i>et al.</i> , 2004
Aesculus hippocastanum (EA) Aesculus glabra (EA)	Slow; LF	>0.67 g g ⁻¹ ; no CP 0.41 g g ⁻¹ ; no CP	Slow; CV	$\frac{>0.67 \text{ g g}^{-1}=90\%}{0.41 \text{ g g}^{-1}=70\%}$	Greening, swelling root or shoot production		Pence, 2004
Quercus palustris (EA)		ng; no CP		0%			
Poncirus trifoliata (EA)	Rapid; FD	1.7, 0.8 & 0.26 g g ⁻¹ ; no CP	Ultra-rapid(400- 1300°C s ⁻¹); Rapid (200-500°C s ⁻¹); Slow(80-90°C s ⁻¹); Slow-programmed (10, 200°C min ⁻¹)	$\frac{1.7 \text{ g g}^{-1}+0.17^{\circ}\text{C s}^{-1}}{0.8 \text{ g g}^{-1}+686^{\circ}\text{C s}^{-1}} \ge 70\%$ & $\frac{0.26 \text{ g g}^{-1}+0.17}{1300^{\circ}\text{C s}^{-1}} \ge 70\%$	Root+shoot		Wesley-Smith <i>et</i> <i>al.</i> , 2004a
Acer saccharinum [L.] (EA)	Slow; over sodium bromide (58% RH) + calcium chloride (31% RH)	0.11 g g ⁻¹ ; 0.11 g g ⁻¹ +20 μM ABA+tetcyclacis & 10%+60 μM ABA+tetcyclacis	Two-step cooling: 0 to -40°C at -0.33°C m ⁻¹ then plunged within CVs into LN	<u>0.11 g g⁻¹+60 μM ABA</u> <u>+tetcyclacis</u> =55%	Root+shoot	Cryopreservation had a more detrimental effect on shoots than roots.	Beardmore and Whittle, 2005
Citrus suhuiensis (EA)	Slow; LF	0.15 g g ⁻¹ ; no CP	Slow; CV	<u>0.15 g g⁻¹=83%</u>	Root+shoot		Makeen <i>et al.</i> , 2005
Ekebergia capensis, Sparrm. (EA)	Rapid; FD	0.4 g g ⁻¹ ; no CP	Rapid; plunged into LN within plastic net envelopes	<u>0.40 g g⁻¹=50%</u>	Root+shoot	80% post-cryo root production only; growth hormones required for shoot production.	Perán <i>et al.</i> , 2006

 Table 1.1 continued...Review of selected cryopreservation studies on embryonic axes/zygotic embryos from non-orthodox seeds.

LF=laminar flow; FD=flash drying; CV=cryovials; SL=nitrogen slush; EA=embryonic axes; ZE=zygotic embryos; CP=cryoprotection; RH=relative humidity and ng=not given. g g⁻¹= g H₂O per g dry mass.

Species & explant type	Dehydration rate/method	Explant water content* & cryoprotection	Cooling rate/method	Highest post-cryo viability (%)	Method of viability assessment	Post-drying & post-cryo growth	Reference
Brunsvigia gregaria (ZE) Amaryllis	Rapid; FD	Dried to ≤ 0.4 g g ⁻¹ ±Suc, Gly, Suc-Gly, Suc-Gly- DMSO, dextran,	Slow; CV & Rapid; SL	$\frac{\text{Rapid: } 0.24 \text{ g g}^{-1}+\text{Gly}=}{60\% \&}$ $\frac{\text{Slow: Vitrification}=15\%}{\text{Rapid: } 0.17 \text{ g g}^{-1}+\text{Gly}=}$	Root+shoot	Stunted growth after cooling.	Sershen <i>et al.</i> , 2007
belladonna (ZE)		PVP CP & vitrification with PVS2		75% & Slow: 0.21 g g ⁻¹ +Suc= 40%			
Strumaria discifera (ZE)				$\frac{\text{Rapid: } 0.20 \text{ g g}^{-1}+\text{Gly}}{65\% \&}$ $\frac{\text{Slow: } 0.20 \text{ g g}^{-1}+\text{Gly}}{45\%}$			
Haemanthus humulus				$\frac{45\%}{\text{Rapid: } 0.24 \text{ g g}^{-1} + \text{Gly}}=$ 70% &			
humulus (ZE) Brunsvigia				$\frac{\text{Slow: 0.24 g g}^{-1}+\text{Gly}}{70\%}$ Rapid: 0.22 g g ⁻¹ + Gly=			
orientalis (ZE) Nerine				55% & <u>Slow: 0.30 g g⁻¹=25%</u> Rapid: 0.21 g g ⁻¹ +Suc=			
humulus (ZE)				$\frac{40\% \&}{\text{Slow: } 0.21 \text{ g g}^{-1} + \text{Suc}} = 20\%; \ 0.24 \text{ g g}^{-1} + \frac{1}{20\%}$			
Nerine huttoniae				$\frac{\text{Suc-Gly-DMSO}=20\%}{\text{Rapid: } 0.23 \text{ g g}^{-1}+\text{Suc}=}$ 40% &			
(ZE) Nerine bowdenii (ZE)				$\frac{\text{Slow: } 0.32 \text{ g g}^{-1}=20\%}{\text{Rapid: } 0.31 \text{ g g}^{-1}=35\% \text{ \&}}$ $\frac{\text{Slow: } 0.31 \text{ g g}^{-1}=35\% \text{ \&}}{\text{Slow: } 0.31 \text{ g g}^{-1}=35\% }$			

Table 1.1 continued...Review of selected cryopreservation studies on embryonic axes/zygotic embryos from non-orthodox seeds.

LF=laminar flow; FD=flash drying; CV=cryovials; SL=nitrogen slush; EA=embryonic axes; ZE=zygotic embryos; CP=cryoprotection; PVS2=plant vitrification solution-2 and ng=not given. g g^{-1} = g H₂O per g dry mass.

Species & explant type	Dehydration rate/method	Explant water content* & cryoprotection	Cooling rate/method	Highest post-cryo viability (%)	Method of viability assessment	Post-drying & post-cryo growth	Reference
Boophane disticha (ZE) Haemanthus bakerae (ZE) Haemanthus coccineus (ZE) Crinum bulbispermum (ZE) Scadoxus puniceus (ZE) Nerine	Rapid; FD	Dried to ≤0.4 g g ⁻¹ ±Suc, Gly, Suc-Gly, Suc-Gly- DMSO, dextran, PVP CP & vitrification with PVS2	Slow; CV & Rapid; SL	$\begin{array}{r} \hline \text{Rapid: } 0.30 \text{ g g}^{-1} = 30\% \& \\ \hline \text{Slow: } = 0\% \\ \hline \\ \hline \\ \hline \text{Rapid: } 0.18 \text{ g g}^{-1} + \text{Suc} = \\ 30\% \& \\ \hline \\ \text{Slow: } 0.20 \text{ g g}^{-1} + \text{Suc} - \text{Gly} \\ = 10\% \\ \hline \\ $	Root+shoot	Stunted growth after cooling.	Sershen <i>et al.</i> , 2007
filifolia (ZE) Haemanthus deformis (ZE)				$\frac{\text{Rapid: 0.26 g g}^{-1}}{50\% \&} = \frac{1}{30\%}$ $\frac{\text{Rapid: 0.14+Gly}}{10\% \&} = 40\% \& \frac{10\% \& 0.14+Gly}{10\% \&} = 20\%;$ $\frac{\text{Slow: 0.14+Gly}}{10\% \&} = 20\%$			
Bactris gasipaes (EA)	Slow; LF	0.25 g g ⁻¹ ; no CP	Slow; CV	$0.25 \text{ g g}^{-1} = 29\%$	Root+shoot	Stunted growth after drying and cooling. <i>Ex vitro</i> seedling mortality in seedlings recovered from cryopreserved EAs higher than control.	Steinmacher <i>et al.</i> , 2007

Table 1.1 continued...Review of selected cryopreservation studies on embryonic axes/zygotic embryos from non-orthodox seeds.

LF=laminar flow; FD=flash drying; CV=cryovials; SL=nitrogen slush; EA=embryonic axes; ZE=zygotic embryos; CP=cryoprotection; PVS2=plant vitrification solution-2 and ng=not given. $g g^{-1} = g H_2O$ per g dry mass.

Species & explant type	Dehydration rate/method	Explant water content* & cryoprotection	Cooling rate/method	Highest post-cryo viability (%)	Method of viability assessment	Post-drying & post-cryo growth	Reference
Ilex brasiliensis (ZE) Ilex brevicuspis (ZE)	Slow; alginate beads dried in silica gel	0.33 g g ⁻¹ ±Suc CP	Slow; CV & two-step cooling: 1°C min ⁻¹ down to -30°C then plunged within	$\frac{\text{Two-step: } 0.33 \text{ g g}^{-1}}{\frac{+\text{Suc}}{-37\% \&}}$ $\frac{\text{Slow: } 0.33 \text{ g g}^{-1}=67\%}{\frac{\text{Two-step:} 0.33 \text{ g g}^{-1}}{+\text{Suc}=3\%;}$	Root+shoot		Mroginski <i>et al.</i> , 2008
			CVs into LN.	$\frac{\text{Two-step: } 0.33 \text{ g g}^{-1}=3\%}{\& \text{ Slow: } 0.33 \text{ g g}^{-1}=15\%}$			
Ilex dumosa (ZE)				$\frac{\text{Two-step: } 0.33 \text{ g s}^{-1}}{\frac{+\text{Suc}}{83\% \&}}$ $\frac{\text{Slow: } 0.33 \text{ g s}^{-1} + \text{Suc}}{67\%}$			
Ilex pseudoboxus (ZE)	•			$\frac{\text{Two-step: } 0.33 \text{ g g}^{-1}}{27\% \&} = \frac{1}{27\% \&}$			
Ilex theezans (ZE)				$\frac{\text{Two-step: } 0.33 \text{ g s}^{-1} + \text{Suc}^{-2} 20\% \text{ \&}}{\frac{\text{Slow: } 0.33 \text{ g s}^{-1} + \text{Suc}^{-1} + \text{Suc}^$			
Ilex intergerrima (ZE)				$\frac{\text{Two-step: } 0.33 \text{ g s}^{-1}}{\frac{+\text{Suc}}{2}=14\% \text{ \&}}$ $\frac{\text{Slow: } 0.33 \text{ g s}^{-1}+\text{Suc}}{50\%}=$			
Ilex paraguariensis (ZE)				$\frac{\text{Two-step: } 0.33 \text{ g s}^{-1}}{+\text{Suc}} = 50\% \text{ \&}$ Slow: 0.33 g s^{-1} = 57\%			
Ilex tauberiana (ZE)				$\frac{\text{Two-step: } 0.33 \text{ g s}^{-1} + \text{Suc}^{-1} + \text{Suc}$			
Trichilia dregeana Sond. (ZE)	Rapid; FD	0.27 & 0.64 g g ⁻¹ ; no CP	Slow; CV	0%	Assessed for root and shoot after cryo but not stated for control.	7% of explants dried to 0.27 g g ⁻¹ callused.	Whitaker et al., 2010

Table 1.1 continued...Review of selected cryopreservation studies on embryonic axes/zygotic embryos from non-orthodox seeds.

LF=laminar flow; FD=flash drying; CV=cryovials; SL=nitrogen slush; EA=embryonic axes; ZE=zygotic embryos; CP=cryoprotection and ng=not given. g g^{-1} = g H₂O per g dry mass.

1.6 Choice and details of species studied

Little to nothing is known of the post-harvest behaviour of the spectrum of African tree and shrub species that produce, or are suspected to produce, recalcitrant seeds (Sacandé *et al.*, 2004). Even where basic information on seed storage behaviour is available, tissue culture protocols for zygotic germplasm culture have generally not been developed, so that cryopreservation studies of germplasm of such species must start with these aspects. It was therefore logical to undertake the present studies on species, for which short- to medium-term seed storage techniques and tissue culture protocols were already in place. Selected members of the Amaryllidaceae provided ideal candidates, since previous studies on 15 species had yielded a sound understanding of the post-harvest behaviour of these recalcitrant amaryllid seeds (Sershen *et al.*, 2008a & b).

The family Amaryllidaceae is made up of 59 genera and about 850 species worldwide (Snijman, 2000), some of which produce recalcitrant seeds (Sershen *et al.*, 2008a). South America hosting 28 genera and South Africa, which hosts 18, are the major centres of amaryllid diversity, while the Mediterranean has eight genera and Australia just three (Snijman, 1984). Southern Africa as a whole boasts 210 indigenous amaryllid species, 77% of which are endemic (Snijman, 2000). The reasons for the specific selection of *Haemanthus montanus* and *Amaryllis belladonna* (Fig. 1.2C, D) for use in this study were four-fold: (1) both are endemic to South Africa; (2) the locations of wild populations of these species had already been established; (3) plants comprising these specific populations (Fig. 1.2A, B) seldom failed to set seed; and (4) unlike the zygotic embryos/embryonic axes of many other tropical recalcitrant-seeded species (Engelmann, 2000), the zygotic embryos excised from *H. montanus* and *A. belladonna* seeds (see Fig. 1.2E) could be easily generated into seedlings under *in vitro* conditions.

Plants of a number of amaryllid species are consistently utilised for traditional medicinal preparations; the bulbs and leaves are used as poultices and decoctions for treating sores and digestive disorders (Gericke *et al.*, 2002; von Ahlefeldt *et al.*, 2003). Harvesting of entire amaryllid bulbs (i.e. plants), which is common practice across Africa, effectively removes potential seed-bearing individuals from a population, threatening the existence of geophytes like the amaryllids, which take years to reach maturity, let alone set seed. Aside from the curtailed seed storage life span (Sershen *et al.*, 2008b) and non-sustainable harvesting of many amaryllid species, predation of amaryllid plants and seeds by the amaryllis caterpillar as well as habitat loss further threaten the survival of some species of the Amaryllidaceae. With dwindling wild populations (von Ahlefeldt *et al.*, 2003) and poor recruitment rates, 59 amaryllid species were listed as endangered or vulnerable in South Africa about 10 years ago, while a further 58 were categorised as near-threatened at that time (Snijman, 2000; Victor, 2002). According to the 2009

version of "The Red Data List of South African Plant Taxa" (South African National Biodiversity Institute, 2009), some amaryllid species that were previously categorised as near-threatened are now listed as endangered, but the number of species that are listed as near-threatened remains comparable to that quoted almost 10 years ago (Snijman, 2000; Victor, 2002). The ephemeral nature of amaryllid seeds is also believed to have contributed to the endangered or near-threatened status of over half of the 210 amaryllid species indigenous to southern Africa (Snijman, 2000). Seasonal gathering of plant components and governmental legislation have failed to ensure sustainable harvesting of amaryllids (Snijman, 2000) and the plants of several amaryllid species are presently being sold within and across the borders of South Africa, with little or no restriction (author's unpublished information). All of this validates the choice of *A. belladonna* and *H. montanus* as the species of interest in the present study, the outcomes of which will hopefully improve prospects for germplasm conservation and ultimate sustainability of the Amaryllidaceae.

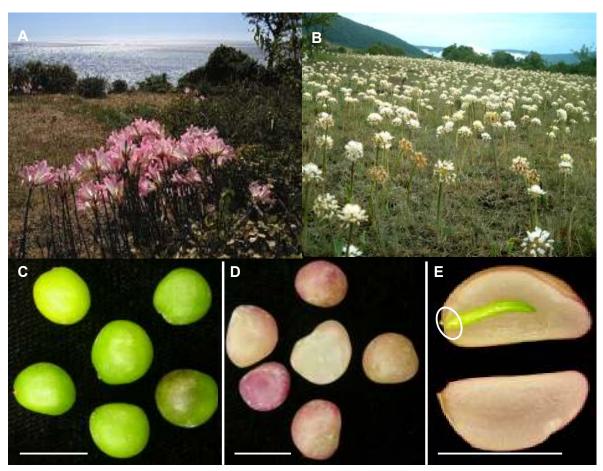


Figure 1.2 [A] *Amaryllis belladonna* and [B] *Haemanthus montanus* populations sampled in this study; [C] *H. montanus seeds*; [D] *A. belladonna* seeds; and [E] an *A. belladonna* zygotic embryo, lying within the endosperm. The structure visible is principally comprised of the single cotyledon with the small (2-3 mm) axis attached at the broader end (encircled). Bar = 10 mm. (Image B courtesy of C. McMaster [African Bulbs, Stutterheim, South Africa]).

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CHAPTER TWO: Interpreting the effects of cryopreservation on recalcitrant *Amaryllis* belladonna and Haemanthus montanus zygotic embryos using selected stress markers

Abstract

A study on cryopreservation of the zygotic embryos of two recalcitrant-seeded amaryllid species, viz. Amaryllis belladonna (L.) and Haemanthus montanus (Baker) is reported. This study aimed to interpret the interactive effects of water content (WC), cryoprotection and cooling rate on subsequent zygotic embryo vigour and viability, using three stress markers: electrolyte leakage; spectrophotometric assessment of tetrazolium chloride-reduction; and rate of protein synthesis. Cryopreservation studies involved cooling partially dried (rapidly; to WCs > and <0.4 g g⁻¹) and fully hydrated embryos, with and without prior sucrose (non-penetrative) and glycerol (penetrative) cryoprotection, at rapid and slow cooling rates. Zygotic embryos of both species lost some viability at relatively high WCs upon partial dehydration, with this loss being more severe at WCs <0.40 g g⁻¹. Zygotic embryos of both species could nevertheless be dried to WCs between 0.29 and 0.33 g g⁻¹, while retaining \geq 70% viability. However, the rate at which this water could be removed was higher in A. belladonna. Partial dehydration generally led to enhanced protein synthesis, an increase in electrolyte leakage and a decline in respiratory activity and vigour. Sucrose (Suc) cryoprotection induced greater viability loss than glycerol (Gly) cryoprotection. Cryoprotection + partial dehydration decreased vigour and often depressed protein synthesis and respiratory activity more than partial dehydration alone. Cooling led to the greatest over-all decline in viability, respiratory activity, protein synthesis and vigour. Glycerol was superior to the non-penetrating cryoprotectant, Suc, at enhancing postthaw viability retention. Post-thaw viabilities for both species were best when Gly cryoprotected + partially dried embryos were rapidly, as opposed to slowly, cooled, but the optimum WC range for post-thaw viability differed between species. The stresses and lesions, metabolic and physical, induced at each stage of the cryopreservation protocol appear to be compounded, thus pre-disposing the tissues to further damage and/or viability loss with the progression of each step. The results also suggest that to optimise cryopreservation protocols for recalcitrant zygotic germplasm, attention must be paid to pre-cooling dehydration stress, which appears to be the product of both the 'intensity' and 'duration' of the stress. The stress markers employed here were useful in differentiating among the effects of the various treatments, however, the mixture of living, weakened and dead cells in frozen-thawed embryos appears to have compromised the accuracy with which some of the markers (e.g. electrolyte leakage and tetrazolium chloridereduction) forecasted the post-thaw viability associated with these treatments.

2.1 Introduction

Essentially, successful cryopreservation of hydrated tissues demands the application of stresses (which include desiccation and ice formation) faster than the resulting potential damage can accumulate. Post-thaw viability of hydrated seed tissues is generally improved when partial dehydration is used as a pre-treatment (e.g. Pritchard and Prendergast, 1986; Pence, 1992; Kioko *et al.*, 1998; Wesley-Smith *et al.*, 2001, 2004a; Sershen *et al.*, 2007) since it reduces the heat to be dissipated during cooling (Wesley-Smith, 2004b; Walters *et al.*, 2008), increases cytoplasmic viscosity (Leprince *et al.*, 1999; Wesley-Smith *et al.*, 2001; Walters *et al.*, 2008), and slows down intracellular ice-crystal growth during cooling (Stanwood, 1985; Wesley-Smith *et al.*, 1992). When the removal of a large amount of water from tissues by desiccation is accompanied by appropriate cooling and re-warming rates, lethal intracellular ice-crystal formation can be avoided (Mazur, 1984; Steponkus, 1985). Also, there have been suggestions that the exogenous application of cryoprotectants may improve survival in recalcitrant zygotic germplasm of tropical provenance (Engelmann, 1997; Normah and Makeen, 2008; Walters *et al.*, 2008) and their benefits appear to maximised when they are applied in combination with optimum embryo/axis water contents (WCs) and cooling rates (Sershen *et al.*, 2007).

Sensitivity to desiccation stress (physical and osmotic) is therefore a fundamental physiological feature dictating cryopreservation strategy in recalcitrant (Roberts, 1973) seed germplasm. The moderately dense cytoplasm of mature recalcitrant embryos/axes and their ability to tolerate moderate dehydration allow the achievement of lower cellular water potentials, easing the stringent requirements for the exogenous application of cryoprotectants and super-fast cooling (reviewed by Walters et al., 2008). However, the success of most cryopreservation protocols involving recalcitrant seed germplasm is often hampered by the fact that ice-crystal formation invariably occurs when hydrated embryos/axes are exposed to liquid nitrogen (LN) (e.g. Wesley-Smith et al., 1992), while drying to WCs precluding ice formation generally leads to lethal desiccation damage (King and Roberts, 1980; Walters et al., 2008). This may explain why 'seedling' recovery in cryopreservation studies involving recalcitrant zygotic germplasm is often very low (0-30%; e.g. Poulsen, 1992; Assy-Bah and Engelmann, 1992a; Abdelnour-Esquivel and Engelmann, 2002; González-Benito et al., 2002; Steinmacher et al., 2007; some species in Sershen et al., 2007), and/or accompanied by a high incidence of 'abnormal' growth (i.e. no roots, no shoots or callus; e.g. de Boucaud et al., 1991; Pence, 1992; Poulsen, 1992 ; Kioko et al., 1998; Wesley-Smith et al., 2001; Perán et al., 2006 ; Steinmacher et al., 2007; Sershen et al., 2007). Of late, a number of studies have suggested that each successive manipulation involved in the cryopreservation of plant germplasm; excision, decontamination, partial dehydration (which may or may not be preceded by cryoprotection),

exposure to the cryogen, thawing and rehydration, and *in vitro* recovery, has the potential to inflict lethal damage on tissues (Berjak *et al.*, 1996; Dumet *et al.*, 1997; Mycock, 1999; Benson and Bremner, 2004; Péran *et al.*, 2004; Sershen *et al.*, 2007; Pammenter *et al.*, 2010). The empirical approach adopted by many cryopreservation studies involving recalcitrant zygotic germplasm has placed great limitations on the conclusions drawn though, and the development of improved methodologies demands a more fundamental understanding of the type and degree of physiological and biochemical damage associated with the different components of cryopreservation.

The present study aimed to interpret the interactive effects of WC, cryoprotection (penetrative and non-penetrative) and cooling rate (rapid and slow) on subsequent embryo vigour and viability, using three stress markers: (1) electrolyte leakage, which has been correlated with membrane integrity (Bramlage *et al.*, 1978, Mckersie and Tomes, 1980); (2) spectrophotometric assessment of tetrazolium chloride-reduction, which is an indication of respiratory competence (Harding and Benson, 1995; Verleysen *et al.*, 2004); and (3) rate of protein synthesis, which is an indicator of biochemical competence (Motete *et al.*, 1997). These studies were undertaken on zygotic embryos excised from the recalcitrant seeds of *Amaryllis belladonna* (L.) and *Haemanthus montanus* (Baker), two wild geophytes indigenous to South Africa.

For all the studies described below embryos were dried to WCs slightly above and below 0.4 g g⁻¹. This was based on the findings of a previous study on the cryopreservation of the zygotic embryos of 15 amaryllids species (Sershen *et al.*, 2007) which showed embryo WCs <0.4 g g⁻¹ to be superior to those >0.4 g g⁻¹, in promoting post-thaw viability. So, in cryopreserving embryos at WCs above and below 0.4 g g⁻¹ this study measured the selected viability indicators at WCs that were presumably either 'sufficiently low' or 'deleteriously high' for amaryllid zygotic embryo cryopreservation. To assess whether the stresses and lesions induced at each stage of the cryopreservation protocol are compounded, thus pre-disposing the tissue to further damage and/or viability loss with the progression of each step, the selected stress indicators were measured after cryoprotection, dehydration, cooling and after all possible combinations of these procedures.

In desiccation-sensitive tissue, the effect of a stress, particularly a mild stress, is unlikely to be instantaneous. In fact, if a stress induces a metabolic disorder, it takes time for the damage consequent upon that disorder to accumulate (Walters *et al*, 2001). In most cases the damage incurred may be evident only after the system has rehydrated fully, and metabolism has been re-initiated. Additionally, recovery from cryostorage involves a series of complex events and while severely damaged tissues undergo degradative processes that lead to cell death, less damaged

tissues can stabilise, recover and return to normal metabolic status (Benson and Noronha-Dutra, 1988). Based on the above, protein synthesis, respiratory activity, vigour and viability were measured on rehydrated embryos after an *in vitro* recovery period. These measurements are therefore presumed to reflect the damage caused by the treatment as well as the damage that accumulated or declined during recovery. Electrolyte leakage was the only parameter measured immediately after rehydration (in cooled and non-cooled embryos), since the avoidance of desiccation damage requires not only that membranes be stabilised when dry, but also that they remain intact during dehydration and rehydration (Halperin and Koster, 2006). The importance of measuring electrolyte leakage immediately after any particular treatment was highlighted by results of a pilot study which showed leakage values to peak shortly after rehydration but to decline dramatically after the embryos were introduced to tissue culture. The electrolyte leakage measurements carried out here are therefore presumed to reflect the damage caused by the treatment and not the damage that accumulated or declined during recovery.

2.2 Materials and Methods

Plant material

In two consecutive years, mature fruits were harvested directly from parent plants and transported in plastic bags to the laboratory with minimum delay (1-2 d) or water loss. Upon arrival, the external fruit covering was removed and seeds were decontaminated for 10 min in 1% aqueous sodium hypochlorite (3:1 dilution of commercial bleach) and left to dry back to their original mass on paper towel, at ambient temperature. The seeds were then dusted with Benlate, a surfactant fungicide, (active ingredient: benomyl [benzimidazole], Dupont, USA), and stored 'moist' (i.e. in a monolayer on a grid suspended approximately 200 mm above sterile, moistened paper towel that lined the base of individual buckets which sealed with lids), at 6°C (after Sershen *et al.*, 2008).

Embryo pre-treatment

Zygotic embryos were excised with the entire cotyledonary body attached (see Fig. A1 [Appendix A] for a description of amaryllid zygotic embryo morphology) and collected within closed Petri dishes on filter paper moistened with sterile calcium-magnesium solution (CaMg solution: 0.5μ M CaCl₂.2H₂O and 0.5 mM MgCl₂.6H₂O [Mycock, 1999]). In order to minimise the potential variation in drying and/or cooling rate as a function of embryo size, only embryos of between 4-6 mm in length were used for all the experiments described below. Excised embryos were rapidly dehydrated via flash drying (devised by Berjak *et al.*, 1990) to: (a) WCs between 0.40 and 0.25 g g⁻¹ (referred to as '<0.4 g g⁻¹, from here on); (b) WCs between 0.60

and 0.40 g g⁻¹ (referred to as '>0.4 g g⁻¹, from here on); and (c) WCs > and <0.40 g g⁻¹ after cryoprotection (CP) with either aqueous glycerol (Gly) or sucrose (Suc). The WC ranges used in the present study were defined by constructing WC and viability vs. drying time curves for each species (see Fig. A2, Appendix A). For CP, freshly excised embryos were immersed in a 5% solution of Gly (v/v) or a 0.5 M solution of Suc for 1 h, and thereafter transferred to a 10% Gly (v/v) or 1 M Suc solution for a further hour. The selection of the cryoprotectants and concentrations used here was based on the results of a previous study which showed Suc and Gly (as opposed to dimethylsulphoxide, dextran, polyvinylpyrolidene and the combination of Suc and Gly) to be least harmful to the zygotic embryos of more than ten amaryllid species (including the two investigated in the present study), at the concentrations of the Suc and Gly cryoprotectant solutions used in this study were very similar (e.g. 0.5 M for sucrose and 0.55 M for 5% glycerol), implying that their water potentials would have been comparable.

Partially dried embryos (with and without CP) were subsequently cooled at: (a) rapid, nonequilibrium (*c*. 200°C s⁻¹), cooling rates by direct immersion of naked embryos in nitrogen slush (LN sub-cooled to -210°C [Echlin, 1992]); or (b) slow, equilibrium cooling rates (1°C min⁻¹ in an isopropanol bath [Mr Frosty®] within a -70°C freezer) down to -40°C followed by direct immersion in nitrogen slush. Freshly excised embryos subjected to no dehydration or CP, as well as embryos exposed to CP but no dehydration, were also subjected to both cooling rates. After cooling in nitrogen slush embryos were transferred under LN into LN-containing cryovials (GreinerTM), mounted on aluminium cryo-canes (10 embryos per vial) and immersed in LN for no longer than a week before use; LN entered the cryovials. Upon retrieval from LN, embryos were rapidly thawed by direct immersion in sterile CaMg solution at 40°C for 2 min, rehydrated in CaMg solution at ambient temperature for 30 min in the dark, and recovered *in vitro*. Freshly excised embryos exposed to none of the treatments described above were also recovered *in vitro* to serve as a control.

Water content determination

Immediately after each of excision (referred to as 'fresh' from here on), partial dehydration (D), CP and CP+D, 10 embryos from each of the non-cooled treatment combinations were weighed individually using a 6-place balance (Mettler, MT5; Germany) and dried in an oven at 80°C for 48 h before being re-weighed to determine the dry mass. Water content was expressed on a dry mass basis (dmb; g H₂O per g dry matter [g g⁻¹]).

In vitro recovery and vigour and viability assessment

For *in vitro* vigour and viability assessments, 10 or 15 embryos from each of the 27 treatment combinations (see Table 2.1), were decontaminated after rehydration (and in the case of fresh embryos, immediately after excision) with 1% (w/v) aqueous calcium hypochlorite for 3 min, washed with sterile CaMg solution (3 times) and then set to germinate with five embryos per Petri dish on full-strength Murashige and Skoog medium (Murashige and Skoog, 1962), containing 3% (w/v) sucrose. All Petri dishes were initially placed in the dark, and transferred upon signs of root and shoot development to a growth room with cool fluorescent lights (52 μ E s⁻¹ m⁻²) and a 16 h photoperiod, at ~25°C. Embryos directed towards protein synthesis and respiratory activity studies were also recovered *in vitro* as described above.

Root and shoot production was scored daily across all 27 treatments for a period of 60 d. Embryos were regarded as having germinated upon callus free root and shoot production and from here on germinated embryos will be referred to as being 'viable'. Vigour, or speed of germination as it is often referred to, was assessed by using the daily germination records to calculate two commonly used indices of germination speed via Equations 1 and 2:

Mean time to germinate (d) =
$$\Sigma(Dn) / \Sigma n$$
 [1]

where n is the number of propagules that germinated on day D and D is the number of days from the beginning of the germination test (Ellis and Roberts, 1981) and;

Germination Index = (Maximum of % germination / D) × (Total % germination / D) [2] where D is the number of days from the beginning of the germination test (Czabator, 1962). (Maximum of % germination / D) is commonly referred to as the 'peak value'. While viability was measured for years 1 and 2, vigour was measured for year 1 only.

Electrolyte leakage

Electrolyte leakage from each of seven embryos across all 27 treatments was measured using a CM100 multi-cell conductivity meter (Reid and Associates, Durban, South Africa). Conductivity of individual embryos immersed in 2 ml distilled water was measured after a 16 h equilibration period. Fresh embryos were measured immediately after excision, dried embryos, immediately after drying, cryoprotected embryos, immediately after incubation at the highest concentration of the cryoprotectant, and cooled embryos immediately after thawing. After the final reading, embryos were dried in an oven at 80°C for 48 h and weighed using a 6-place balance (Mettler, MT5; Germany) to determine the DW. Leakage was expressed as total conductivity after 16 h, less the average conductivity of the distilled water blanks, in units of mSiemens m⁻¹ g⁻¹ DW. This parameter was measured for year 1 only.

Rate of protein synthesis

Protein synthesis was measured using a protocol modified after Farrant *et al.* (1985). The incorporation of [³H] amino acids was measured on three replicates of five embryos each, by incubating each batch of five embryos in 200 μ l of an aqueous solution of 0.5 μ Ci ml⁻¹ [³H] amino acid cocktail (specific activity: 9.25 MBq mmol⁻¹, 250 μ Ci mmol⁻¹ [Amersham International, UK]) after 48 h *in vitro* growth. After incubation in the precursor solution for 4 h at room temperature (*c.* 24°C) in the dark, embryos were rapidly rinsed in 2 × 1 ml changes of distilled water, ground in LN and suspended in 1 ml 0.1 M Tris-HCl buffer (pH 7.0). Samples were then gently centrifuged for 5 min, after which 200 μ l of the supernatant was spotted onto three 15-mm-diameter discs of Whatmann 3MM filter paper. The discs were further processed according to Mans and Novelli (1960) to remove all material except protein; for this, disks were immersed in a cold aqueous solution of 10% trichloroacetic acid (TCA; w/v) for 1 h, and then washed in 5% TCA (w/v) at 90°C for 30 min to remove lipids. De-fatted discs were then washed in a 1:1 solution of ethanol and di-ethyl ether at 37°C for 30 min and dehydrated in 99.7% di-ethyl ether for 30 min at room temperature.

For determination of [³H] amino acid incorporation into protein, the ether dehydrated discs for each replicate (i.e. 3 disks for each of 3 replicates) were immersed in 10 ml of Beckman liquid scintillation cocktail and the radioactivity was measured using a Beckman LS 6000IC scintillation counter. The counts generated were used to calculate the degradations min⁻¹ g⁻¹ DW; indicative of the incorporation of amino acids, and hence of protein synthesis. This parameter was measured for year 1 only.

2,3,5-triphenyl tetrazolium chloride spectrophotometric test

The reduction of colourless tetrazolium chloride (TTC) to insoluble pink/red triphenyl formazan was taken as a measure of respiratory activity. The assumption here is that TTC is reduced by components of the mitochondrial electron transport chain (Moore, 1962), however, it must be noted that TTC can be reduced by dehydrogenase enzymes in the presence of a reducing agent, which may or may not be mitochondrial (Schatz *et al.*, 1956). Using a protocol modified after Harding and Benson, (1995) and Verleysen *et al.* (2004), five embryos from each of the 27 treatments were individually incubated in 300 µl of aqueous TTC-solution (2% [w/v] of TTC in Tris-HCl buffer [0.05 M, pH 7.5]) and 1.2 ml Tris-HCl buffer (0.05 M, pH 7.5), after 48 h *in vitro* growth. Embryos were incubated in the TTC-solution for 12 h in the dark at room temperature. Embryos were then cut in half longitudinally, and incubated for 12 h in 3 ml of 95% ethanol at room temperature. After gentle centrifugation, the absorbance of 1 ml of the supernatant was read at 500 nm in a UV-Vis Spectrophotometer (Cary 50 Conc UV Vis

spectrophotometer, Varian). Embryos were thereafter dried in an oven at 80°C for 48 h and weighed individually using a 6-place balance (Mettler, MT5; Germany) to determine the DW, which was thereafter used to express respiratory activity in units of μ mol formazan g⁻¹ DW. This parameter was measured for year 1 only.

Data interpretation and analysis

Inter-treatment differences in respiratory activity, protein synthesis and electrolyte leakage were tested for by Analysis of Variance (ANOVA; SPSS, Version 15). Multiple comparisons were then made using a Duncan's mean separation test. Respiratory activity and protein synthesis data were not normally-distributed (p < 0.05; Komolgirov-Smirnov test) and had to be transformed to conform data to parametric assumptions. With transformation, inter-treatment differences within cooled and non-cooled treatment groups were poorly resolved (by mean separation). However, when respiratory activity, protein synthesis and electrolyte leakage data for cooled and non-cooled treatments were separated, with 'fresh' as the common control group, these separated datasets did not require transformation for normality (p > 0.05; Komolgirov-Smirnov test) and inter-treatment differences within cooled and non-cooled treatment groups were better resolved, statistically (by mean separation). So, in order to better identify intertreatment differences in electrolyte leakage, respiratory activity and protein synthesis within the cooled and non-cooled treatment groups by ANOVA, data for cooled and non-cooled treatments were analysed separately, with 'fresh' as the common control group. Correlations between viability and MTG, GI, electrolyte leakage, respiratory activity and rate of protein synthesis were tested for using a Pearson correlation test (SPSS, Version 15). Data for cooled and noncooled treatments were pooled (and transformed where necessary) for these analyses and viability percentages were arcsin transformed to conform data to parametric test assumptions. Root, shoot and viability data were tested for significant inter-treatment differences using nullmodel chi-squared analyses (specifically designed to assess non-parametric data) (EcoSim Version 7.72 [developed by Gotelli and Entsminger, 2009]). Water content data were tested for significant differences using a Mann-Whitney-U test (SPSS, Version 15). All statistical tests were performed at the 0.05 level of significance.

2.3 Results

Embryo water content, root and shoot production, and viability were measured for both species, for years 1 and 2, and unless otherwise stated, the trends reported for these data are applicable to both species and both years. Where it was necessary to discuss these parameters in terms of actual values, values for both species and both years are quoted. Vigour (i.e. mean time to

germinate and germination index), electrolyte leakage, respiratory activity and protein synthesis were measured for both species, but for year 1 only.

Drying characteristics

The embryos of both species were shed highly hydrated (A. belladonna: c. 4.67 and 5.71 g g⁻¹; *H. montanus*: c. 4.66 and 5.05 g g⁻¹ [Table 2.1A, B]). Freshly excised embryos lost viability at relatively high WCs upon rapid dehydration (A. belladonna: c. 0.53 g g⁻¹; H. montanus: c. 0.43 and 0.55 g g^{-1} [Table 2.1A, B]), with this loss being significantly more severe at WCs <0.4 g g⁻¹. A description of the vigour and viability responses of *H. montanus* and *A. belladonna* embryos to a broader range of WCs is given in Appendix A (Table A1 and Fig. A2). Even though embryos could be dried to WCs between 0.29 and 0.33 g g⁻¹ while retaining \geq 70% viability, the rate at which this water was lost differed between species, i.e. there were interspecies differences in drying kinetics. For instance, while embryos of A. belladonna could be dried to WCs of 0.42±0.09 and 0.53±0.09 g g⁻¹ in 15 and 5 min, respectively, H. montanus embryos took 180 and 240 min to reach 0.52±0.15 and 0.43±0.13 g g⁻¹, respectively. Also, embryos of A. belladonna could be dried to WCs of 0.32±0.10 and 0.29±0.09 g g⁻¹ in 30 and 15 min, respectively, while H. montanus embryos took 300 and 240 min to reach 0.34±0.09 and 0.33±0.13 g g⁻¹, respectively (see Table A1 and Fig. A2, Appendix A). Except for the fact that embryo dehydration to 0.42 g g^{-1} stimulated germination rate in A. belladonna (i.e. decline in MTG and increase in GI) compared to fresh embryos, partial dehydration generally increased MTG and decreased GI relative to fresh embryos (Table 2.1A, B). The decline in GI in both species was based largely on a decline in total germination, rather than peak value.

Vigour and viability

Cryoprotection and partial dehydration

Glycerol CP led to a significant reduction in embryo WC relative to fresh embryos and in keeping with its non-penetrative nature, the dehydrative effect of Suc CP was significantly greater (Table 2.1A, B). Cryoprotection had no adverse effect on viability in *A. belladonna*, but led to a slight decrease in viability in *H. montanus* (10% for Suc and Gly in year 1 and 10% for Suc in year 2) (Table 2.1A, B). Cryoprotection did not lead to any dramatic changes in MTG and except for the fact that Gly CP increased GI relative to fresh embryos in *A. belladonna*, CP always depressed GI (based on a reduction in peak value; Table 1A, B).

Unlike CP, partial dehydration of *A. belladonna* embryos to WCs >0.4 g g⁻¹ and <0.4 g g⁻¹, led to significant declines in viability, being slightly more severe at WCs <0.4 g g⁻¹ (Table

2.1A). In *H. montanus*, partial dehydration was more detrimental than CP, leading to viability losses of 10 and 30% at WCs >0.4 g g⁻¹ and 30% at WCs <0.4 g g⁻¹ (Table 2.1B).

When partial dehydration was preceded by CP (CP+D) post-drying viabilities were significantly lower in Suc CP embryos, irrespective of whether they were dried to WCs > or <0.4 g g⁻¹ (Table 2.1A, B). Viabilities after Suc CP+D were also significantly lower than those associated with non-CP embryos dehydrated to comparable WCs (i.e. >0.4D and <0.4D). Viabilities after Gly CP+D were either marginally higher or similar to those associated with non-CP embryos dehydrated to comparable WCs.

In *A. belladonna*, MTG in >0.4D-Gly and <0.4D-Gly embryos decreased while GI declined due to a decline in viability (Table 2.1A). In *A. belladonna* >0.4D-Suc and <0.4D-Suc embryos, MTG increased slightly while the marked decrease in viability led to GIs much lower than fresh and Gly CP+D embryos. In *H. montanus*, CP+D generally led to an increase in MTG (being slightly higher for Gly CP+D embryos) while GI in CP+D embryos was always lower than fresh embryos, owing to the combined decline of peak value and total germination (Table 2.1B). Between species, partial dehydration, CP and CP+D appeared to be more detrimental, in terms of a decline in viability, in *H. montanus* (Table 2.1A, B).

While root meristems were more sensitive to dehydration (with and without prior CP) than those of shoots in *A. belladonna* (most especially for year 1), root meristems were relatively less sensitive to desiccation in *H. montanus* (Table 2.1A, B). Seedlings recovered from partially dried embryos of both species appeared to be less vigorous than those from fresh embryos, even after 2 months of *in vitro* growth. These observations were, however, purely qualitative (Fig. 2.1).

Cooling

Cooling most often led to a dramatic reduction in viability, relative to fresh, CP and partially dehydrated embryos and when post-thaw viability was observed, this was generally confined to treatments that involved CP+D (Table 2.1A, B). Except for Suc-rapid (20% post-thaw viability in *H. montanus*), fully hydrated and CP embryos did not survive cryopreservation.

Of the six treatments in which post-thaw viability was observed in *A. belladonna*, two (>0.4D-slow and <0.4-Suc-slow) resulted in 7% viability (but for year 1 only) while four (>0.4D-Gly-rapid, >0.4D-Gly-slow, <0.4D-Gly-rapid and <0.4D-Gly-slow) involved Gly CP+D and resulted in 10-80% viability (Table 2.1A). Of the four favourable Gly CP+D treatments, post-thaw viability was highest (73 and 80%) when embryos were rapidly cooled at WCs <0.4 g g⁻¹, lower (47 and 40%) when embryos were slowly cooled at WCs <0.4 g g⁻¹, and lowest (20

and 10%) when embryos were cooled at WCs >0.4 g g⁻¹. In frozen-thawed *A. belladonna* embryos, vigour was greatly reduced relative to fresh and partially dried embryos; while post-thaw GI declined due to the combined reduction of total germination and peak value, post-thaw MTG was higher than fresh embryos in four of the six treatments in which post-thaw viability was observed (Table 2.1A).

Of the eight treatments in which post-thaw viability was observed in *H. montanus*, two (>0.4D-rapid, >0.4D-slow) involved non-CP+D embryos and resulted in 10% viability (but for year 1 only), three (Suc-rapid [for year 1 only], <0.4D-Suc-rapid and <0.4D-Suc-slow [for year 2 only]) involved Suc CP and resulted in 10-20% viability, while three involving Gly CP+D embryos (>0.4D-Gly-rapid, >0.4D-Gly-slow and <0.4D-Gly-rapid) resulted in 10-60% viability (Table 2.1B). Within these eight treatments, post-thaw viability was highest (50 and 60%) when Gly CP embryos were rapidly cooled at WCs >0.4 g g⁻¹, lower (10-30%) in >0.4D-Gly-slow and <0.4D-Gly-rapid embryos, and lowest (10%) in Suc CP and non-CP+D embryos. Mean time to germinate within these eight treatments was most often higher than partially dried embryos and in at least six cases, higher than fresh embryos (Table 2.1B). Also, GI in these treatments was often lower than fresh and partially dried embryos due to the combined decline of peak value and total germination (Table 2.1B).

Root and shoot meristems did not appear to be differentially sensitive to cooling in *A*. *belladonna* (Table 2.1A) but in *H. montanus*, shoot meristems were generally more sensitive to cooling than root meristems (Table 2.1B). Seedlings recovered from cryopreserved embryos of both species appeared to be less vigorous than those from fresh embryos, even after 2 months of *in vitro* growth. These observations were, however, purely qualitative (Fig. 2.1).

Α			YEAR 2								
Treatment	$WC^1(g g^{-1})$	Roots (%)	Shoots (%)	Viability ² (%)	*MTG ³	*GI ⁴	WC ($g g^{-1}$)	Roots (%)	Shoots (%)	Viability (%)	Treatment categories
Fresh	4.67±0.57	100	100	100	9.3	69.4	5.71±1.25	100	100	100	Control + dehydrated to < or > 0.4 g g-1, with or without cryoprotection.
>0.4D	0.42±0.09	100	100	100	5.3	204.1	0.53±0.09	90	90	90	
<0.4D	0.32±0.10	80	93	80	10.0	14.2	0.29±0.05	80	90	80	
Gly	3.16±0.46	100	100	100	7.0	82.6	3.28±0.64	100	100	100	
Suc	1.86±0.15	100	100	100	8.7	51.0	1.81±0.10	100	100	100	
>0.4D-Gly	0.44±0.02	100	100	100	6.6	61.5	0.46±0.03	90	90	90	
>0.4D-Suc	0.41±0.09	73	93	73	10.5	16.0	0.43±0.13	70	70	70	
<0.4D-Gly	0.31±0.07	87	93	87	6.8	37.6	0.31±0.04	80	80	80	
<0.4D-Suc	0.29±0.05	73	100	73	12.4	13.0	0.31±0.02	60	60	60	
Fresh-rapid	4.67±0.57	0	0	0	0	0	5.71±1.25	0	0	0	Cooled rapidly or slowly with or without cryoprotection; no flash drying.
Fresh-slow	4.67±0.57	0	0	0	0	0	5.71±1.25	0	0	0	
Gly-rapid	3.16±0.46	0	73	0	0	0	3.28±0.64	0	80	0	
Suc-rapid	1.86±0.15	0	0	0	0	0	1.81±0.10	0	0	0	
Gly-slow	3.16±0.46	0	0	0	0	0	3.28±0.64	0	0	0	
Suc-slow	1.86±0.15	0	0	0	0	0	1.81±0.10	0	0	0	
>0.4D-rapid	0.42±0.09	7	0	0	0	0	0.53±0.09	20	0	0	$\begin{array}{c} Dehydrated \ to \\ < \ or > 0.4 \ g \ g^{-l} \\ and \ cooled \\ rapidly \ or \ slowly. \end{array}$
>0.4D-slow	0.42±0.09	7	7	7	9.0	1.2	0.53±0.09	0	0	0	
<0.4D-rapid	0.32±0.10	0	0	0	0	0	0.29±0.05	0	0	0	
<0.4D-slow	0.32±0.10	0	0	0	0	0	0.29±0.05	0	0	0	
>0.4D-Gly-rapid	0.44 ± 0.02	27	20	20	13.0	0.6	0.46±0.03	10	10	10	Dehydrated to $< or > 0.4 \text{ g g}^{-1}$, with cryoprotection, and cooled rapidly or slowly.
>0.4D-Suc-rapid	0.41±0.09	0	0	0	0	0	0.43±0.13	10	0	0	
>0.4D-Gly-slow	0.44 ± 0.02	27	20	20	11.0	0.8	0.46±0.03	20	10	10	
>0.4D-Suc-slow	0.41±0.09	0	0	0	0	0	0.43±0.13	0	0	0	
<0.4D-Gly-rapid	0.31±0.07	73	80	73	19.4	4.9	0.31±0.04	80	90	80	
<0.4D-Suc-rapid	0.29±0.05	0	0	0	0	0	0.31±0.02	0	0	0	
<0.4D-Gly-slow	0.31±0.07	67	47	47	16	2.5	0.31±0.04	50	40	40	
<0.4D-Suc-slow	0.29±0.05	7	7	7	9	1.2	0.31±0.02	0	0	0	

Table 2.1 Water content, vigour and viability for fresh, cryoprotected, partially dried and cooled [A] A. belladonna and [B] H. montanus zygotic embryos.

¹ water content; ² viability = root and shoot production; ³ mean time to germinate; ⁴ germination index. >0.4D = dried to >0.4 g g⁻¹; <0.4D = dried to <0.4 g g⁻¹; -Gly = cryoprotected with glycerol; -Suc = cryoprotected with sucrose; -slow = cooled slowly; -rapid = cooled rapidly. MTG and GI were based on viability. Water content, viability and root and shoot data were tested for significant inter-treatment differences within years: p < 0.05 for WC (Mann-Whitney-U test, n = 10); p < 0.001 for viability and % root and shoot production (null-model chi-squared analysis, n = 10 except for *A. belladonna* year 1 where n = 15). * Replicated once; not tested for significant differences.

В			YEAR 2								
Treatment	$WC^1(g g^{-1})$	Roots (%)	Shoots (%)	Viability ² (%)	*MTG ³	[*] GI ⁴	WC (g g ⁻¹)	Roots (%)	Shoots (%)	Viability (%)	Treatment categories
Fresh	5.05±0.92	100	100	100	12.6	44.4	4.66±0.76	100	100	100	$Control + dehydrated to < or > 0.4 g g^{-1}, with or without cryoprotection.$
>0.4D	0.55±0.09	100	90	90	15.9	6.2	0.43±0.13	80	70	70	
<0.4D	0.34±0.09	80	70	70	13.7	4.8	0.33±0.13	70	70	70	
Gly	4.77±1.59	90	90	90	14.0	3.9	4.26±0.35	100	100	100	
Suc	1.79±0.28	100	90	90	13.3	32.0	1.49±0.13	90	90	90	
>0.4D-Gly	0.53±0.09	90	90	90	17.3	8.9	0.55±0.08	80	80	80	
>0.4D-Suc	0.50±0.11	80	70	70	12.0	8.5	0.44±0.07	60	60	60	
<0.4D-Gly	0.33±0.05	90	90	70	18.9	4.1	0.32±0.04	70	70	70	
<0.4D-Suc	0.30±0.02	80	60	60	16.3	4.1	0.31±0.02	60	50	50	
Fresh-rapid	5.05±0.92	0	0	0	0.0	0.0	4.66±0.76	0	0	0	Cooled rapidly or slowly with or without cryoprotection; no flash drying.
Fresh-slow	5.05±0.92	0	0	0	0.0	0.0	4.66±0.76	0	0	0	
Gly-rapid	4.77±1.59	0	0	0	0.0	0.0	4.26±0.35	0	0	0	
Suc-rapid	1.79±0.28	40	10	10	16.5	0.6	1.49±0.13	30	0	0	
Gly-slow	4.77±1.59	0	0	0	0.0	0.0	4.26±0.35	0	0	0	
Suc-slow	1.79±0.28	60	0	0	0.0	0.0	1.49±0.13	0	0	0	
>0.4D-rapid	0.52±0.15	20	10	10	13.0	0.6	0.52±0.15	30	0	0	$\begin{array}{c} Dehydrated \ to \\ < \ or \ > \ 0.4 \ g \ g^{-1} \\ and \ cooled \\ rapidly \ or \ slowly. \end{array}$
>0.4D-slow	0.52±0.15	0	0	0	23.0	0.2	0.52±0.15	20	10	10	
<0.4D-rapid	0.34±0.09	50	0	0	0.0	0.0	0.33±0.13	0	0	0	
<0.4D-slow	0.34±0.09	10	0	0	0.0	0.0	0.33±0.13	10	0	0	
>0.4D-Gly-rapid	0.53±0.09	60	50	50	28.2	1.5	0.55±0.08	70	60	60	Dehydrated to < or > 0.4 g g ⁻¹ , with cryoprotection, and cooled rapidly or slowly.
>0.4D-Suc-rapid	0.50±0.11	20	0	0	0.0	0.0	$0.44{\pm}0.07$	20	0	0	
>0.4D-Gly-slow	0.53±0.09	30	10	10	7.0	2.0	0.55±0.08	70	20	20	
>0.4D-Suc-slow	0.50±0.11	0	40	0	0.0	0.0	0.44±0.07	0	0	0	
<0.4D-Gly-rapid	0.33±0.05	50	20	20	27.0	0.2	0.32±0.04	50	30	30	
<0.4D-Suc-rapid	0.30±0.02	10	10	10	23.0	0.2	0.34±0.14	40	20	20	
<0.4D-Gly-slow	0.33±0.05	0	0	0	0.0	0.0	0.32±0.04	0	0	0	
<0.4D-Suc-slow	0.30±0.02	30	20	10	12.0	0.7	0.34±0.14	10	0	0	

Table 2.1 Continued... Water content, vigour and viability for fresh, cryoprotected, partially dried and cooled [A] A. belladonna and [B] H. montanus zygotic embryos.

¹ water content; ² viability = root and shoot production; ³ mean time to germinate; ⁴ germination index. >0.4D = dried to >0.4 g g⁻¹; <0.4D = dried to <0.4 g g⁻¹; -Gly = cryoprotected with glycerol; -Suc = cryoprotected with sucrose; -slow = cooled slowly; -rapid = cooled rapidly. MTG and GI were based on viability. Water content, viability and root and shoot data were tested for significant inter-treatment differences within years: p < 0.05 for WC (Mann-Whitney-U test, n = 10); p < 0.001 for viability and % root and shoot production (null-model chi-squared analysis, n = 10 except for *A. belladonna* year 1 where n = 15). * Replicated once; not tested for significant differences.

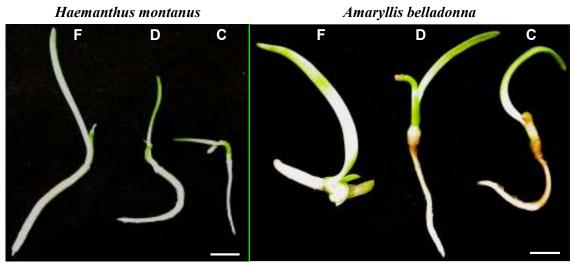


Figure 2.1 60 day old seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) zygotic embryos. Bar = 1 cm.

Electrolyte leakage

In *A. belladonna*, leakage in non-dehydrated CP embryos was significantly higher than fresh embryos (Fig. 2.2A) but this was not true for *H. montanus* (Fig. 2.2B). Partial dehydration, with and without prior CP, increased electrolyte leakage relative to fresh embryos and except for >0.4D, these differences were always significant (Fig. 2.2A, B). These rises in electrolyte leakage with partial dehydration were often, but not always (e.g. >0.4D-Gly in *A. belladonna*), accompanied by a decline in viability. Within the non-cooled treatments, higher leakage was often associated with lower viability but the degree of increase relative to fresh embryos was not proportional to the decline in viability.

In *A. belladonna*, leakage in cooled treatments involving Gly CP (except for <0.4D-Glyslow) were often similar to fresh embryos but post-thaw viability in these treatments was always significantly lower (Fig. 2.2A). Leakage across all other cooled treatments in *A. belladonna* was significantly higher than fresh embryos, comparable to the relatively high leakage associated with most partially dehydrated and cryoprotected non-cooled treatments, and except for three treatments, accompanied by complete viability loss.

In *H. montanus*, leakage across cooled treatments was very rarely as high as that associated with partially dried embryos (Fig. 2.2B). In fact, leakage after cooling was often similar to fresh embryos and in a few treatments significantly lower. However, even when leakage was lower than fresh embryos, post-thaw viability was either completely lost or significantly lower than fresh embryos.

Within the cooled treatments in which post-thaw viability was observed in both species, leakage values were not proportional to the declines in viability (Fig. 2.2A, B) and when data for cooled and non-cooled treatments were pooled for analysis, correlation analyses showed no significant relationships between electrolyte leakage and viability, GI or MTG (*A. belladonna: r* < 0.15, p > 0.60 across all three combinations; *H. montanus: r* < 0.50, p > 0.10 across all three combinations).

Finally, it must be noted that the partially dried and cooled embryos may have leaked extensively into the rehydration/thawing solution and, thus, the leakage of those samples could have been greater than that recorded.

Respiratory activity

Compared to fresh embryos, partial dehydration and cooling led to a significant decline in respiratory activity, being significantly more severe in cooled treatments (Fig. 2.3A-D). The decline in respiratory activity with partial dehydration was often correlated with a decline in viability but this was not without exception; example >0.4D and >0.4D-Gly in *A. belladonna* (Fig. 2.3A). Within the non-cooled treatments the degree of viability was not proportional to the decline in respiratory activity (Fig. 2.3A, C).

While Suc and Gly CP (no drying and no cooling) significantly depressed respiratory activity in *A. belladonna* (Fig. 2.3A), respiratory activities in CP *H. montanus* embryos were comparable to fresh embryos (Fig. 2.3C).

Within the partially dried treatments respiratory activities in CP+D embryos, except for > 0.4D-Gly in *H. montanus*, were significantly lower than non-CP+D embryos (Fig. 2.3A, C). Within the CP+D treatments in *H. montanus*, respiratory activities in embryos dried to $<0.4 \text{ g g}^{-1}$ were often relatively lower than those dehydrated to WC >0.4 g g⁻¹, while respiratory activity within the partially dehydrated treatments was significantly lowest in Suc CP+D embryos.

In *A. belladonna* post-thaw respiratory activities in Gly CP+D embryos were relatively (but not always significantly) higher than Suc+D and non-CP+D treatments (Fig. 2.3B). Cooled treatments involving Gly CP+D *A. belladonna* embryos were associated with the highest post-thaw viabilities, but within these, respiratory activity was not an accurate predicator of post-thaw viability. Respiratory activity in the two treatments in which 10% post-thaw viability was recorded in *A. belladonna* (>0.4D-slow and <0.4D-Suc-slow) was also not significantly higher than many of the treatments in which no post-thaw viability retention was observed.

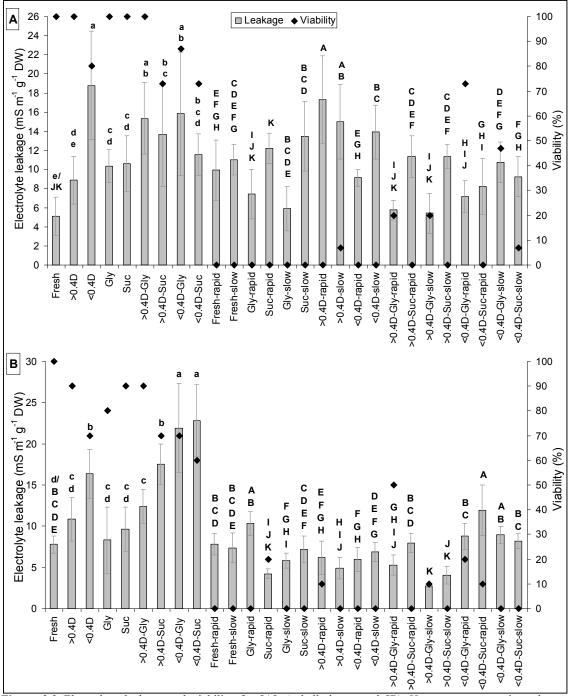


Figure 2.2 Electrolyte leakage and viability for [A] *A. belladonna* and [B) *H. montanus* zygotic embryos. Viability = root and shoot production; >0.4D = dried to >0.4 g g⁻¹; <0.4D = dried to <0.4 g g⁻¹; -Gly = cryoprotected with glycerol; -Suc = cryoprotected with sucrose; -slow = cooled slowly; -rapid = cooled rapidly. When testing for significant inter-treatment differences in electrolyte leakage, cooled and non-cooled treatments were analysed separately, with 'fresh' as the common control group. Leakage values represent mean±SD and are significantly different when followed by different letters (lower-case for non-cooled and upper-case for cooled treatments; *ANOVA*, *n* = 7, *p* < 0.05). *p* < 0.05 when viability data were tested for significant inter-treatment differences (null model chi-squared analysis, *n* = 10).

Within the cooled treatments in *H. montanus*, respiratory activity was often relatively higher in Gly CP+D and >0.4D embryos (Fig. 2.3D). However, except for >0.4D-Gly-rapid, which resulted in 50% post-thaw viability in *H. montanus*, these relatively high post-thaw respiratory activities were accompanied by either very low (i.e. 10-20%), or no, post-thaw viability retention in this species.

When data for cooled and non-cooled treatments were pooled for analysis there were significant positive correlations between respiratory activity and both viability (*A. belladonna: r* = 0.76, p < 0.001; *H. montanus: r* = 0.83, p < 0.001) and GI (*A. belladonna: r* = 0.71, p < 0.001; *H. montanus: r* = 0.85, p < 0.001), but not between respiratory activity and MTG (*A. belladonna: r* = 0.15, p > 0.05; *H. montanus: r* = 0.38, p > 0.05). Thorough qualitative assessments of TTC-staining were not carried out here but it was interesting to note that while the end of the cotyledon devoid of meristematic tissue (see Fig. A1, Appendix A) generally stained negatively, all positively stained tissues were almost always confined to the embryonic axis end.

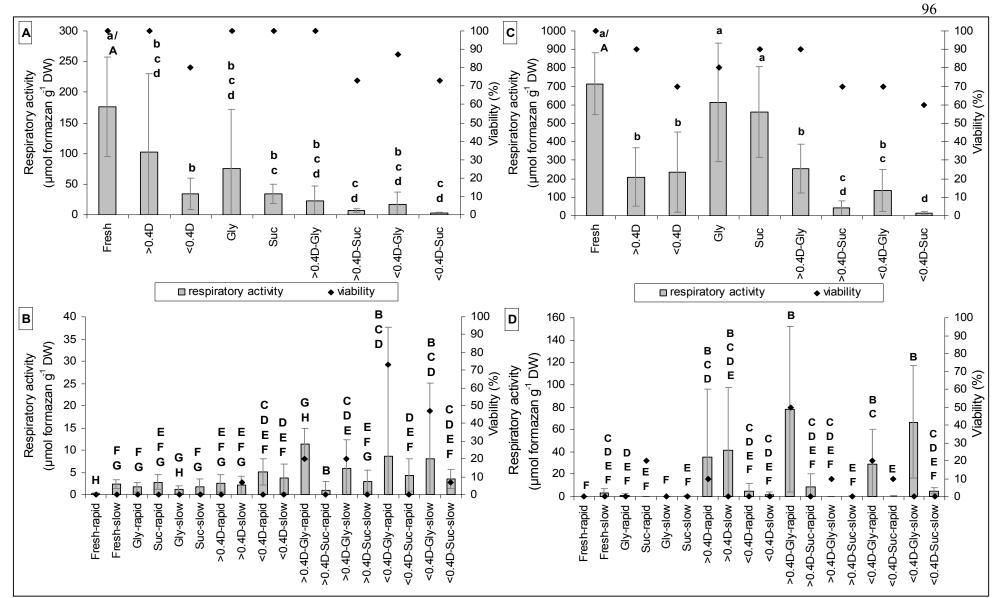


Figure 2.3 Respiratory activity and viability for [A-B] *A. belladonna* and [C-D] *H. montanus* zygotic embryos. [A] and [C] feature non-cooled treatments while [B] and [D] show cooled treatments. Viability = root and shoot production; >0.4D = dried to >0.4 g g⁻¹; <0.4D = dried to <0.4 g g⁻¹; -Gly = cryoprotected with glycerol; -Suc = cryoprotected with sucrose; -slow = cooled slowly; -rapid = cooled rapidly. When testing for significant inter-treatment differences in respiratory activity, cooled and non-cooled treatments were analysed separately, with 'fresh' as the common control group. Respiratory activity values represent mean±SD and are significantly different when followed by different letters (lower-case for non-cooled and upper-case for cooled treatments; *ANOVA*, *n* = 5, *p* < 0.05). *p* < 0.05 when viability data were tested for significant differences (null model chi-squared analysis, *n* = 10).

Rate of protein synthesis

Except for <0.4D-Suc, partial dehydration (with and without CP) and CP generally enhanced protein synthesis relative to fresh embryos (Fig. 2.4A, B). These differences were sometimes marginal and not always significant but the following trends were observed across the non-cooled treatments: (a) dehydration stimulated protein synthesis rates relative to fresh embryos; however, in CP+D treatments this trend was less pronounced in *H. montanus*; (b) within cryoprotectants, post-drying protein synthesis rates were comparable across WC ranges (i.e. < and > 0.4 g g⁻¹); (c) except for <0.4D-Suc, CP, partial dehydration and CP+D never depressed protein synthesis rates below that of fresh embryos; and (d) <0.4D-Suc resulted in the lowest protein synthesis rates and the lowest post-drying viabilities (Fig. 2.4A, B).

With cooling, protein synthesis rates were generally lower than fresh embryos, and by implication, lower than all non-cooled treatments except <0.4D-Suc (Fig. 2.4A, B). Protein synthesis was detectable even in treatments in which post-thaw viability was zero. There was a tendency for cooled treatments involving Gly CP embryos to exhibit relatively higher protein synthesis rates than other cooled treatments; this was often correlated with significantly higher post-thaw viability retention (e.g. *A. belladonna*: >0.4D-Gly-rapid and <0.4D-Gly-rapid; *H. montanus*: >0.4D-Gly-rapid).

Post-thaw viabilities were not proportional to protein synthesis rates (Fig. 2.4A, B). When data for cooled and non-cooled treatments were pooled for analysis there were significant positive correlations between rate of protein synthesis and both viability (*A. belladonna*: r = 0.86, p < 0.001; *H. montanus*: r = 0.82, p < 0.001) and GI (*A. belladonna*: r = 0.83, p < 0.001; *H. montanus*: r = 0.62, p = 0.001), but not between rate of protein synthesis and MTG (*A. belladonna*: r = 0.19, p > 0.10; *H. montanus*: r = 0.07, p > 0.10).

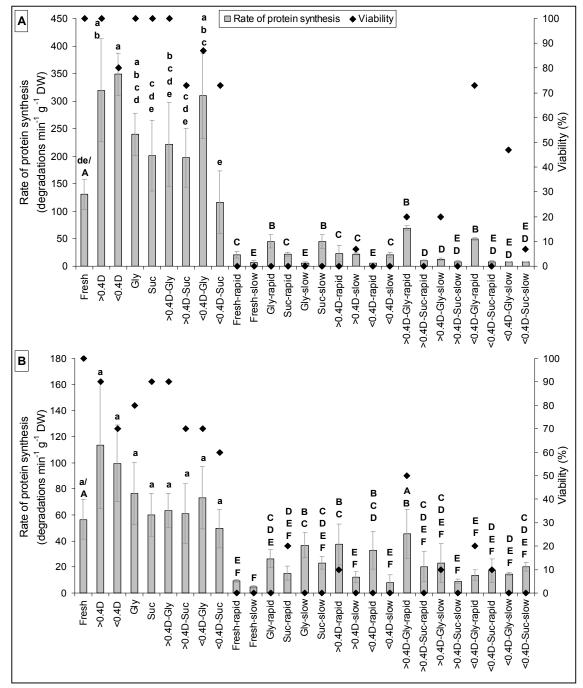


Figure 2.4 Rate of protein synthesis and viability for [A] *A. belladonna* and [B] *H. montanus* zygotic embryos. Viability = root and shoot production; >0.4D = dried to >0.4 g g⁻¹; <0.4D = dried to <0.4 g g⁻¹; -Gly = cryoprotected with glycerol; -Suc = cryoprotected with sucrose; -slow = cooled slowly; -rapid = cooled rapidly. When testing for significant inter-treatment differences in protein synthesis rates, cooled and non-cooled treatments were analysed separately, with 'fresh' as the common control group. Protein synthesis values represent mean±SD and are significantly different when followed by different letters (lower-case for non-cooled and upper-case for cooled treatments; *ANOVA*, *n* = 3, *p* < 0.05). *p* < 0.05 when viability data were tested for significant inter-treatment differences (null model chi-squared analysis, *n* = 10).

2.4 Discussion

The present study aimed to interpret the effects of the various components of cryopreservation on subsequent vigour and viability of recalcitrant *A. belladonna* and *H. montanus* embryos, using three stress markers: electrolyte leakage (an indicator of membrane integrity); spectrophotometric assessment of tetrazolium chloride-reduction (an indicator of respiratory competence); and rate of protein synthesis (an indicator of biochemical competence).

Pre-conditioning for cryopreservation

Like other recalcitrant-seeded species, viz., Landolphia kirkii (Pammenter et al., 1991), Avicennia marina (Farrant et al., 1993), Quercus robur (Finch-Savage and Blake, 1994) and Shorea robusta (Chaitanya et al., 2000), the seeds of A. belladonna and H. montanus possessed highly hydrated embryos at shedding (both >4.0 g g^{-1} ; Table 2.1A, B). Sucrose and Gly CP had a dehydrative effect on the embryos of both species, being significantly more severe with Suc CP (Table 2.1A, B). This was not surprising since non-penetrating cryoprotectants, like Suc, act by dehydrating cells before freezing (Muldrew et al., 2004) but the net effect of all cryoprotectants, be they membrane penetrating (e.g. Gly) or non-penetrating (e.g. Suc), is to effect some measure of dehydration (reviewed by Fuller, 2004). The embryos of both species responded differently to cryoprotection, in that viability was adversely affected by CP in H. montanus, but not in A. belladonna (Table 2.1A, B). However, while the increase in electrolyte leakage relative to fresh embryos induced by cryoprotection was significant in A. belladonna (Fig. 2.2A), this increase was not significant for *H. montanus* (Fig. 2.2B). This suggests that cryoprotectant toxicity rather than osmotic injury may not have been the major cause of the decline in viability observed by cryoprotection in H. montanus. Unlike Gly CP, Suc CP had an adverse effect on vigour (Table 2.1A, B) and respiratory activity in both species (significant for A. belladonna only; Fig. 2.3A, C). Most, if not all, cryoprotectants exhibit some degree of cytotoxicity (reviewed by Fuller, 2004; Mycock et al., 1995) but non-penetrating cryoprotectants such as Suc can result in osmotic injury at high concentrations, since they act by dehydrating cells before freezing (Finkle et al., 1985; Muldrew et al., 2004).

Recalcitrant seeds characteristically lose viability during drying at WCs ranging from 0.6 to 0.2 g g⁻¹, with this wide range in sensitivity being variably attributed to differences in the rate of drying, stage of development and cellular differentiation, metabolic status, climatic conditions during development, and genetic back-ground (Vertucci and Farrant, 1995; Berjak and Pammenter, 1997; Daws *et al.*, 2006). In this study, partial dehydration to embryo WCs >0.4 and <0.4 g g⁻¹ was more detrimental than CP alone (for both species), leading to a significant decline in viability (Table 2.1A, B), respiratory activity (Fig. 2.3A, C) and membrane integrity (as assessed by electrolyte leakage; significant for <0.4D; Fig. 2.2A, B), relative to fresh embryos. In recalcitrant

embryos/axes membrane damage precedes viability loss during desiccation to WCs above and below the level of freezable water (generally taken to be >0.25-0.28 g g⁻¹ [Pammenter *et al.*, 1991; reviewed by Kermode and Finch-Savage, 2002]). As in other recalcitrant-seeded species (e.g. *Ouercus rubra* [Sun, 1999]), the degree of viability loss (Table 2.1A, B) and membrane damage (Fig. 2 A, B) incurred was significantly greater at relatively lower embryo WCs (i.e. <0.4 g g⁻¹). A decline in respiratory activity (as estimated by TTZ-extraction) has been previously correlated with desiccation-induced viability loss in embryos (e.g. Becwar et al., 1982) and the reduction observed here was most certainly a consequence of desiccation-intolerance (and the associated cell death) and not a coordinated down-regulation of metabolism, typical of the desiccation-tolerant state in seeds (Leprince et al., 1994; reviewed by Vertucci and Farrant, 1995). As in other recalcitrantseeded species (e.g. A. marina [Farrant et al., 1985, 1992]) protein synthesis rates in both species were relatively low in fresh embryos but enhanced upon partial dehydration (Fig. 2.4A, B). Studies have shown that slight dehydration can stimulate germination in recalcitrant seeds (Eggers et al., 2007). One of the key steps in seed germination is *de novo* protein synthesis and during the early stages after imbibition it is mediated by preformed mRNA transcribed during embryogenesis (Cheung et al., 1979). The enhancement in protein synthesis in partially dried embryos 48 h into in *vitro* recovery observed here suggests that partial dehydration to embryos WCs >0.25 g g⁻¹ may not have damaged preformed mRNA transcribed during embryogenesis.

Despite their desiccation sensitivity, the embryos of both species could be dried to WCs between 0.34 and 0.29 g g⁻¹ while retaining \geq 70% viability (for both years; Table 2.1A, B). Studies have shown that rapid (as opposed to slow) drying allows for the survival of the embryos/axes of several recalcitrant-seeded species to similarly low WCs (c. 0.20-0.44 g g⁻¹), and as in this study, close to the point where only non-freezable water remains (e.g. Normah et al., 1986; Pritchard and Prendergast, 1986; Berjak et al., 1993; Pammenter et al., 1993; reviewed by Normah and Makeen, 2008). The influence of drying rate is important in studies of this nature, since desiccation damage in recalcitrant seeds appears to be a function of two interrelated parameters: the degree and the duration of dehydration (Pammenter et al., 1998, 2002, 2003; Walters et al., 2001; Liang and Sun, 2002). In the recalcitrant embryos/axes of some species, the faster the drying rate, the lower the WC that can be tolerated without effective loss of viability (Berjak et al., 1984; Farrant et al., 1985; Pritchard, 1991; Pammenter et al., 1998; Wesley-Smith et al., 2001). In the present study the embryos of both species were dehydrated via flash drying (associated with rapid dehydration rates), however, there were marked inter-species differences in drying kinetics in that A. *belladonna* embryos could be dried to WCs around 0.40 g g⁻¹, more than ten times faster than those of H. montanus (also see Fig. A2 and Table A1, Appendix A). Studies on the effects of dehydration rate on desiccation-sensitivity in recalcitrant seeds suggest that when seed tissues spend a longer period of time at intermediate WCs, the time for aqueous-based deleterious

processes to occur is extended, promoting viability loss (Vertucci and Farrant, 1995; Pammenter *et al.*, 1998). So, when the time spent at intermediate WCs is shortened the progress of aqueousbased deleterious reactions and hence, viability loss, is presumably limited (Berjak *et al.*, 1990, 1993; Pammenter *et al.*, 1998; Walters *et al.*, 2001). This may explain why post-drying viabilities at comparable WCs were slightly lower (by 10-20%) in slower-drying *H. montanus* embryos (Table 2.1A, B). Respiratory activity data were not congruent with this hypothesis (Fig. 2.3 A and C) but *H. montanus* embryos appeared to have incurred greater membrane damage (as inferred from the proportion by which electrolyte leakage was increased relative to fresh embryos) than *A. belladonna* upon dehydration to WCs <0.4 g g⁻¹ (Fig. 2.2A, B). Electrolyte leakage is usually a good indicator of membrane damage in seeds, embryonic axes and zygotic embryos (Pammenter *et al.*, 1997; Chaitanya and Naithani, 1998; Sacandé *et al.*, 2001; Varghese and Naithani, 2002).

Compared with Gly CP, Suc CP had a significantly greater dehydrative effect in the absence of physical drying and this may have pre-disposed Suc CP embryos to a greater degree of damage during partial dehydration, since the former exhibited greater post-drying viability losses than Glyand non-CP embryos (at comparable WCs; Table 2.1A, B). This relatively higher post-drying viability loss with Suc CP was correlated with relatively lower respiratory activity (Fig. 2.3A, C) and protein synthesis rates (Fig. 4A, B). As mentioned earlier, impermeable sugar cryoprotectants can inflict severe osmotic injury at high (\geq 1 M) concentrations (Finkle *et al.*, 1985; Muldrew *et al.*, 2004). However, exposure of plant tissue to almost any cryoprotectant causes some degree of stress, the results of which (i.e. damage) can be reflected by an increase in electrolyte leakage, a decrease in germination rate (Verleysen *et al.*, 2004), and/or biochemical abnormalities such as a lag in protein synthesis rates (Fig. 2.4A, B) and (except for >0.4D-Gly in *H. montanus*) respiratory activities that were relatively lower than those of non-CP+D embryos (at comparable WCs) (Fig. 2.3A, C).

The existence of inter-species differences in drying characteristics in this study was further supported by the fact that irrespective of whether embryos were CP or not, root meristems appeared to be more sensitive to dehydration than those of shoots in *A. belladonna*, while the opposite was true for *H. montanus* (Table 2.1A, B). Inter-tissue differences in tolerance of dehydration is well documented for embryos/axes (Fu *et al.*, 1993) and has been variably attributed to differential WC distribution during non-equilibrium drying (Pritchard *et al.*, 1995a), variability in sub-cellular organisation and differences in the matrix-bound water fraction between tissues (Finch-Savage, 1992).

Except for Suc-rapid in *H. montanus* (which resulted in 10% post-thaw viability in year 1), fully hydrated and non-dehydrated CP embryos of both species failed to survive cooling (Table 2.1A, B). This was not surprising since plant tissues seldom survive exposure to cryogenic temperatures at such high (in this case 1.49-4.77 g g⁻¹) WCs (Pence, 1990). However, even with pre-conditioning (i.e. partial dehydration and cryoprotection) cooling most often lead to a significant decline in vigour and viability (Table 2.1 A, B), respiratory activity (Fig. 2.3) and rate of protein synthesis (except for >0.4D-Gly-rapid in H. montanus; Fig. 2.4), relative to fresh and partially dried embryos (with comparable WC \times cryoprotectant combinations). An increase in the lag before the first germination or a decrease in the rate of germination may indicate the ongoing repair of damage (Pammenter et al., 2002) and post-thaw re-growth of plant material (Verleysen et al., 2004) such as recalcitrant embryos/axes is often 'slow' or 'poor' (Abdelnour-Esquivel and Engelmann, 2002; González-Benito et al., 2002; Steinmacher et al., 2007; Sershen et al., 2007). Overzealous decontamination, excessive drying and even poor growth conditions may not produce such symptoms in control (uncooled) embryos/axes, but may do so in cryo-exposed ones by exacerbating freezing damage (Walters et al., 2008). Ironically, cryoprotectants are sometimes also the cause of post-thaw viability loss and can prolong re-growth procedures (Verleysen et al., 2004). A dramatic decline in TTC-reduction post-thawing, as observed here is generally but not always indicative of a decline in viability, since cells that exhibit signs of respiration after thawing can eventually die or regenerate abnormally (e.g. Verleysen et al., 2004). The post-thaw depression in protein synthesis rates observed here, suggests that unlike partial dehydration, cooling may have damaged preformed mRNA transcribed during embryogenesis and/or the cellular machinery involved in protein synthesis.

In *A. belladonna*, post-thaw viability was generally confined to treatments involving Gly CP embryos and when viability was observed in non-Gly CP embryos (e.g. <0.4D-Suc-slow and >0.4D-slow), this was generally very low (\leq 10%) (Table 2.1A). The ability of Gly cryoprotection to limit cryo-induced metabolic and physical damage to non-lethal levels in *A. belladonna*, was evidenced by the fact that >0.4D-Gly-rapid, >0.4D-Gly-slow, <0.4D-Gly-rapid and <0.4D-Gly-slow embryos exhibited the highest over-all post-thaw viabilities (20-73%) and respiratory activities (Fig. 2.3B). One contributory factor could have been that in *A. belladonna* Gly CP (except for <0.4D-Gly-slow) generally decreased freeze-thaw-induced membrane damage, relative to Suc CP+D and non-CP+D embryos (Fig. 2.2). Cell lysis in frozen non-cold acclimated cells has also been shown to occur during thawing and subsequent rehydration (Steponkus and Lynch, 1989) and increased post-thaw survival has been attributed to a reduction in electrolyte leakage (e.g. Verleysen *et al.*, 2004). Additionally, in rapidly cooled Gly CP+D *A. belladonna* embryos this greater retention of post-thaw structural integrity was accompanied by relatively superior post-

thaw metabolic competence, since protein synthesis rates in >0.4D-Gly-rapid and <0.4D-Gly-rapid embryos were significantly higher than all cooled Suc CP+D treatments (Fig. 2.4A).

Of the eight treatments in which post-thaw viability was observed in *H. montanus*, two involved no CP (>0.4D-rapid and >0.4D-slow) and resulted in 10% viability, but for one year only, three involved Suc CP (Suc-rapid [in year 1 only], <0.4D-Suc-rapid and <0.4D-Suc-slow [in year 2 only]) and resulted in 10-20% viability, while viabilities of 10-60% were observed in three treatments involving Gly CP (namely: >0.4D-Gly-rapid; >0.4D-Gly-slow; and <0.4D-Gly-rapid) (Table 2.1B). Except for the treatments involving Suc CP, all those treatments were generally associated with significantly higher post-thaw respiratory activity, with the highest over-all postthaw respiratory activity and viability (50%) occurring in >0.4D-Gly-rapid embryos (Fig. 2.3D). However, high respiratory activity was not necessarily indicative of high post-thaw viability retention in *H. montanus* embryos, since some treatments that exhibited exceptionally low respiratory activities (e.g. Suc-rapid, >0.4D-Gly-slow and <0.4D-Suc-rapid) still resulted in some, though low (10-20%), post-thaw viability. This suggests that viable embryos/axes immediately after recovery from LN and early during *in vitro* recovery can still die during recovery while, on the other hand, post-thaw viability may be facilitated in some embryos by the survival of only a few critical (most likely meristematic) cells. In contrast to A. belladonna, the ability of Gly to promoted post-thaw viability retention in *H. montanus* was not correlated with a reduction in freeze-induced membrane damage, since leakage values across cooled treatments were generally significantly lower than fresh embryos, even when post-thaw viability was completely lost (Fig. 2.2B). This suggests that even though the mechanisms via which Gly conferred cryoprotection were effective enough to promote post-thaw viability retention in both species, these mechanisms may not necessarily have been the same and/or equally effective in both species.

A wide range of cryoprotectants have been applied to desiccation-sensitive zygotic germplasm (alone and in combination with dehydration) with variable success (e.g. de Boucaud *et al.*, 1991, 1996; Pence, 1991; Assy-Bah and Engelmann, 1992a & b; Kioko *et al.*, 1998; Thammasiri, 1999; Walters *et al.*, 2002; Sershen *et al.* 2007). The results obtained here lend support to other suggestions that cryoprotection can improve post-thaw recovery in zygotic germplasm of non-orthodox-seeded species (Engelmann, 1997; Walters *et al.*, 2002; Normah and Makeen, 2008), most especially those of tropical provenance (Walters *et al.* 2008). Penetrating cryoprotectants like Gly are able to diffuse through the plasma membrane and equilibrate in the cytoplasm while non-penetrating CPs like Suc do not enter the cytoplasm but may accumulate apoplastically (Grout, 1995; Muldrew *et al.*, 2004). Differences in the degree/type of protection conferred by Suc and Gly in this study are best understood in terms of the theory of 'colligative cryoprotection' (see Lovelock, 1953; Meryman and Williams, 1980, 1985). Most colligative additives have the potential to enhance viscosity of the cell contents (Benson, 2008) and so inhibit intracellular ice-

crystal formation (Meryman and Williams, 1980, 1985; reviewed by Fuller, 2004); however, one of the essential attributes of the colligative theory of cryoprotection is that cryoprotectants must be able to penetrate the cell, otherwise, they will cause osmotic dehydration, resulting in the very injury they are employed to guard against (Benson, 2008). The superiority of Gly over Suc cryoprotection in this study may therefore have been based, apart from other factors, on glycerol's penetrative ability, which is usually accompanied by an increase in cytoplasmic viscosity but a decrease in the efflux of water from the cytoplasm during cooling (Polge, 1949; Benson, 2008). Additionally, penetrating cryoprotectants may also reduce freezing injury by acting as a solvent for electrolytes that accumulate in the cytoplasm during ice-crystal formation (Finkle *et al.*, 1985; Muldrew et al., 2004) and stabilising proteins at high salt concentration (Popova and Busheva, 2001). The ability of penetrating cryoprotectants to reduce the magnitude of freezing injury by lowering the kinetic freezing point (often referred to as the 'supercooling point' in biological solutions [Wilson et al., 2003]) of cell solution by increasing intracellular osmolality prior to freezing and thus the temperature at which ice nucleation occurs is also well documented (Finkle et al., 1985; Gusta et al., 2004). In fact, they may prevent ice-crystal formation altogether when applied at very high concentrations since the temperature at which nucleation occurs may be substantially decreased while the temperature at which water is transformed into glass increases (Bronshteyn and Steponkus, 1995; Muldrew et al., 2004). Reference to recent and past literature on the use/action of cryoprotectants suggests that the superiority of Gly over Suc cryoprotection in this study was more likely due to a combination of factors, rather than any single one. Some of these factors, such as (1) the superiority of penetrating, compared to non-penetrating, cryoprotectants in extending protection to internal organelles (Fuller, 2004), and (2) the ability of radioprotectants¹ like Gly to confer biochemical protection during cryopreservation, mainly by scavenging harmful free-radicals (Polge et al., 1949; Smirnoff and Cumbes, 1989; Benson and Bremner, 2004), are addressed further in studies featured in Chapters 3 and 4, respectively.

Post-thaw vigour and viability as influenced by embryo water content and cooling rate

While the lower limit of the "optimal hydration window" in recalcitrant embryos/axes is constrained by desiccation sensitivity of the tissue, its upper limit may be constrained by freezing injury due to intracellular ice formation at relatively high WCs (Becwar *et al.*, 1983; Pritchard and Prendergast, 1986; Pence, 1992; Wesley-Smith *et al.*, 1992; Sun, 1999). Additionally, the number, size and location of ice-crystals formed within recalcitrant embryos/axes is influenced by cooling rate (Wesley-Smith *et al.*, 1992, 2004a). As mentioned earlier, water loss enhances cytoplasmic

¹ Penetrating cryoprotectants that can substitute reversibly for water in the hydration sheath of polysaccharides, proteins and nucleic acids, thereby altering their macromolecular structure, which may render them less amenable to radiation-induced injury (Benson and Bremner, 2004).

viscosity in seed tissues (Buitink *et al.*, 1998; Leprince *et al.*, 1999) and rapid cooling of 'sufficiently' dehydrated embryos/axes can slow-down ice nucleation in such tissues (Wesley-Smith *et al.*, 1992, 2004b; Walters *et al.*, 2008). This may explain why post-thaw viabilities across both species in this study were best when partially dehydrated Gly CP embryos were rapidly, as opposed to slowly, cooled (Table 2.1A, B); however, any further discussion of these data demands an appreciation of the relationship between cooling rate and freezing damage.

While slow cooling rates encourage the formation of a few, large extracellular ice-crystals, rapid cooling rates can result in a greater number of ice nuclei, both intra- and extracellularly (Mazur, 1990; Kartha and Engelmann, 1994). During rapid cooling ($\geq 100^{\circ}$ C min⁻¹) exosmosis usually occurs at a rate much slower than the rate of formation of extracellular ice-crystals. As a consequence, the cytoplasm becomes increasingly supercooled, pre-disposing the cells to intracellular ice-crystal formation and hence mechanical shearing of cell membranes (Acker and Croteau, 2004). However, if samples are cooled at rates of $\geq 1000^{\circ}$ C min⁻¹ the ice-crystals that form may be very small and therefore relatively innocuous (Muldrew et al., 2004). The amount of ice formed in cells and solutions at physiological concentration during rapid cooling can be further limited by increasing cooling rates above 100°C s⁻¹ (Luyet et al., 1962). The cooling rates associated with non-equilibrium cooling methods (pioneered by Luyet et al., 1962), such as the rapid cooling method employed here (see section 2.2 for details), are generally in the order of hundreds of °C s⁻¹ (e.g. Wesley-Smith, 2004a & b). Cooling rates in this order are thought to restrict intracellular ice-crystallisation below lethal levels by minimising the time spent by the tissue at temperatures favouring ice formation and growth (generally taken to be 0 to -80°C [Moor. 1973]). Rapid cooling rates have been successfully applied to the recalcitrant embryos/axes of a number of species (e.g. Camellia sinensis [Wesley-Smith et al., 1992]; Aesculus hippocastanum [Wesley-Smith et al. 2001]; Quercus suber and Quercus ilex [González-Benito et al., 2002]; Poncirus trifoliata [Wesley-Smith et al., 2004a]; Ekebergia capensis [Perán et al., 2006]; and a number of amaryllid species [Sershen et al., 2007]).

Step 1 of the 'two-step' cooling method (e.g. Withers and King, 1980; Krøgstrup *et al.*, 1992), i.e. controlled slow cooling (0.5-2.0°C min⁻¹) down to -30 to -40, or even -60°C, is characterised by extracellular ice formation, using components of the cell wall as ice-crystal nucleators (Levitt, 1980). This results in an efflux of water from cells, however, since temperature declines slowly (0.2-10°C min⁻¹) the cells lose water at a rate slow enough to maintain equilibrium with the extracellular solution (Muldrew *et al.*, 2004). This freeze-induced dehydration (during step 1) is often too intense though, giving rise to a variety of deleterious effects such as solute toxicity, osmotic contraction and plasmolysis, and even cell lysis (Mazur, 1990; Pritchard *et al.*, 1995b; Muldrew *et al.*, 2004). On the other hand, some cells may fail to reach the optimum intracellular

concentration during step 1 and upon supercooling can become victim to lethal intracellular icecrystal formation (Mazur, 1990). Freeze-induced dehydration during slow cooling can also affect the transition of the phospholipid bilayer from the liquid crystalline to the gel phase, subsequently limiting the exit of water from cells and increasing the potential for intracellular ice formation (Webb and Steponkus, 1993). The significantly lower post-thaw viabilities achieved with 'twostep' cooling in this study were therefore, not surprising (Table 2.1A, B). In fact, 'two-step' cooling has only been successfully applied to the embryos/axes of a limited number of recalcitrantseeded species (e.g. *Ilex* spp. [Mroginski, 2008]) and there are suggestions that while slow cooling methods may be good at retaining the integrity of individual cells, they may be less efficient at retaining the tissue integrity necessary for the survival of complex tissues (e.g. meristems and embryos [Panis and Lambardi, 2006]).

The optimal WC for cryopreserving desiccation-sensitive embryo/axes varies widely among species, drying rates, cooling rates, storage temperature and embryo maturity (Wesley-Smith et al., 2004a; Makeen et al., 2005; Sershen et al., 2007; reviewed by Normah and Makeen, 2008); being as high as 1.1-1.6 g g⁻¹ for *Camellia sinensis* (Wesley-Smith *et al.*, 1992), to 0.36-0.56 g g⁻¹ in Zizania palustris (Touchell and Walters, 2000), and as low as 0.23 g g⁻¹ for Azadirachta indica (Berjak and Dumet, 1996), and 0.15 g g⁻¹ for Citrus suhuiensis (Makeen et al., 2005). There is now consensus that successful embryo/axis cryopreservation protocols involve optimising cooling rates in conjunction with tissue hydration level, to eliminate or at least minimise, nucleation of lethal intracellular ice-crystals (Wesley-Smith et al., 1992, 2004a; Normah and Makeen, 2008; Walters et al., 2008). Since WC influences cytoplasmic viscosity, it influences the mobility of water within cells (Vertucci and Roos, 1990) and drying seed tissues to c. 0.25 g g⁻¹ ($\Psi \approx$ -1.2 MPa) is said to be sufficient enough to increase cytoplasmic viscosity to such an extent that ice formation during relatively slow cooling is limited, and axis survival becomes independent of the time of exposure to temperatures that promote ice-crystallisation (Wesley-Smith *et al.*, 2004a). However, dehydration to such low WCs is rarely tolerated in desiccation-sensitive embryos/axes and it is now widely accepted that the higher the final embryo/axis WC after drying, the more rapid the rate of cooling should be to restrict ice-crystallisation and associated freezing damage (Vertucci, 1989; Wesley-Smith et al., 1992; Walters et al., 2008). It must be stressed though that even at rapid, non-equilibrium, cooling rates the mobility of water and the cooling rate required to prevent lethal intracellular ice-crystallisation are inter-linked (Luyet et al., 1962).

Additionally, post-thaw viabilities in plant tissue depend to a large extent on the length of the drying period (Niino and Sakai, 1992) and in recalcitrant zygotic germplasm prolonged periods of dehydration, while not lethal in themselves, can act synergistically and lethally with freezing rates (Pritchard *et al.*, 1995b; Berjak *et al.*, 1999; Kioko *et al.*, 1998; Sun, 1999; Wesley-Smith *et al.*, 2001; 2004a). In the present study, while post-thaw viability losses in Gly CP+D embryos were

lowest (27 and 20%) at WCs <0.4 g g⁻¹ in *A. belladonna*, viability losses in *H. montanus* were lowest (50 and 40%) at WCs >0.4 g g⁻¹ (Table 2.1A, B). This species-specific relationship between WC range and post-thaw viability held true for slowly cooled Gly CP+D embryos as well. As discussed earlier, there were marked inter-species differences in drying kinetics, with *A. belladonna* embryos drying so rapidly that the difference in the duration of drying between target WCs (< and >0.4 g g⁻¹) was limited to 10-15 min, as opposed to ≥ 1 h, in *H. montanus*. This may explain why the WC range that was presumably associated with a greater degree of desiccation stress, i.e. <0.4 g g⁻¹, resulted in significantly lower post-thaw viabilities in *H. montanus* (Table 2.1B). However, the opposite was true for *A. belladonna* (Table 2.1A). All that can be proposed at this stage is that, in contrast to *H. montanus*, the difference in the 'duration' of the pre-cooling dehydration stress between target WCs in *A. belladonna* may have been too small to influence post-thaw viability, making water content, or rather the 'intensity' of the dehydration stress the over-riding determinant of post-thaw viability.

2.5 Concluding remarks

In recalcitrant *A. belladonna* and *H. montanus* embryos, stresses and lesions, metabolic and physical, induced at each stage of the cryopreservation protocol appear to be compounded, thus pre-disposing the tissues to further damage and/or viability loss with the progression of each step. Maximum post-thaw viability in both species appeared to be based on the balance between desiccation damage and freezing stress, and the mitigation of both of these via Gly cryoprotection. The results obtained here also suggest that to optimise cryopreservation protocols for recalcitrant zygotic germplasm attention must be paid to pre-cooling dehydration stress, which appears to be the product of both the 'intensity' and 'duration' of the stress. Pammenter *et al.* (2002) suggested that it is the, "concept of 'intensity' vs. 'duration' of a stress that underlies the confusion that has obscured the interpretation of the effects of drying rates on desiccation-sensitive seed material". This concept of 'intensity' vs. 'duration' of a stress appears to be just as relevant to our interpretation of the effects of the various components of cryopreservation on post-thaw viability in recalcitrant embryos/axes.

The present work allowed for an assessment of the value of three markers of cryo-related stress (namely, electrolyte leakage, spectrophotometric assessment of tetrazolium chloride-reduction and rate of protein synthesis) that may be used to better optimise future cryopreservation protocols for recalcitrant zygotic germplasm. Markers such as electrolyte leakage (Jitsuyama *et al.*, 2002) and tetrazolium chloride-staining (reviewed by Verleysen *et al.*, 2004) have previously been shown to be accurate indicators of freezing tolerance in simple homogenous tissue systems. The markers employed here were useful in differentiating among the effects of the various treatments, however, the mixture of living, weakened and dead cells in frozen-thawed embryos appears to

have compromised the accuracy with which some of the markers (e.g. electrolyte leakage and tetrazolium chloride-reduction) forecasted the post-thaw viability associated with these treatments.

Finally, as in other studies on recalcitrant zygotic germplasm (e.g. Steinmacher *et al.*, 2007) pre-conditioning and cooling appeared to compromise vigour in recovered seedlings. However, these observations were purely qualitative (see Fig. 2.1A, B) and this phenomenon was investigated further in studies featured in Chapters 5 and 6.

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CHAPTER THREE:

Ultrastructural responses of recalcitrant *Amaryllis belladonna* and *Haemanthus montanus* zygotic embryos to cryopreservation

Based on

Sershen, Berjak, P., Pammenter, N.W., Wesley-Smith, J. 2011. The effects of various parameters during processing for cryopreservation on the ultrastructure and viability of recalcitrant zygotic embryos of *Amaryllis belladonna*. Protoplasma (In press).

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Sershen, Berjak, P., Pammenter, N.W., Wesley-Smith, J. 2011. Rate of dehydration, state of subcellular organisation and nature of cryoprotection are critical factors contributing to the variable success of cryopreservation: Studies on recalcitrant zygotic embryos of *Haemanthus montanus*. Protoplasma (In press).

Abstract

The ultrastructural responses of recalcitrant Amaryllis belladonna (L.) and Haemanthus montanus (Baker) zygotic embryos to cryoprotection, partial dehydration and thawing after cryogenic exposure were investigated. Cryopreservation studies involved cooling partially dried (to water contents > or <0.4 g g⁻¹) and fully hydrated embryos, with and without prior sucrose (non-penetrative) or glycerol (penetrative) cryoprotection, at rapid or slow cooling rates. Embryos were exposed to these treatments and after a 48 h in vitro recovery period, root meristems were excised and prepared for conventional transmission electron microscopy. Untreated (fresh) embryos (of both species) exhibited 100% viability and ultrastructurally their cells showed a centrally positioned, irregularly shaped nucleus (with dispersed heterochromatin), regular cell walls with contiguous plasmalemmae, a few, small vacuoles, each with intact tonoplast, and signs of ongoing active metabolism, e.g. polysomes, Golgi bodies, mitochondria and rough endoplasmic reticulum. Some viability loss accompanied cryoprotection (CP), partial dehydration, thawing after cryogenic exposure, and combinations of these procedures for both species, being most severe after thawing. For both species, the decline in viability after pre-conditioning and freeze-thawing was generally accompanied by some degree of ultrastructural derangement, increased vacuolation, tonoplast dissolution, and/or total sub-cellular destruction, in some or all cells. While glycerol CP alleviated post-drying ultrastructural derangement and viability loss in both species, sucrose CP exacerbated these adverse effects of drying. For both species, freeze-thaw-induced ultrastructural irregularities were least conspicuous in rapidly cooled, glycerol CP embryos, which also survived cryopreservation best. Results of this study suggest that damage to the sub-cellular matrix and ultrastructural irregularities induced at each stage of the cryopreservation protocol may be compounded with each progressive step, thus pre-disposing tissues to increasing damage and/or viability loss. Cryoprotection and dehydration increased the chances of post-thaw survival but their practical benefits appear to have been realised only when damage to the sub-cellular matrix was minimised. For both species this was best achieved when: (a) pre-conditioning involved the combination of cryoprotection and partial dehydration; (b) the cryoprotectant was penetrating (glycerol) as opposed to non-penetrating (sucrose); and (c) embryos were rapidly cooled at WCs that minimised both dehydration and freezing damage.

3.1 Introduction

Studies described in Chapter 2 suggested that in *Amaryllis belladonna* (L.) and *Haemanthus montanus* (Baker) zygotic embryos, the metabolic and physical stresses and lesions induced at each stage of the cryopreservation protocol appeared to be compounded, thus pre-disposing the tissues to further damage and/or viability loss with the progression of each step. Using transmission electron microscopy (TEM), the present aspect of the study investigated whether the ultrastructural responses of these embryos to the various procedural steps involved in their cryopreservation were consistent with this suggestion.

Ultrastructural and other microscopical studies have been immensely valuable, in providing evidence for the explanation of recalcitrant seed behaviour and responses under a variety of conditions (reviewed by Beriak and Pammenter, 2000). Apart from revealing sub-cellular damage, ultrastructural studies can also reflect the physiological status of particular cell-types in time, and in response to particular stimuli. Mycock (1999) suggested that biochemical imbalances and perturbation of the sub-cellular matrix (including the cytoskeleton) could be prevalent during pre-freezing manipulations. The present study on the ultrastructural responses of A. belladonna and H. montanus zygotic embryos to cryoprotection, partial dehydration and thawing after cryogenic exposure was motivated by reports that sub-cellular changes induced in recalcitrant embryo tissues following dehydration (Berjak et al., 1984, 1999; Farrant et al., 1985; Salmen Espindola et al., 1994; Mycock et al., 2000; Wesley-Smith et al., 2001a; Kioko et al., 2006) and cooling (Wesley-Smith et al., 1992, Chandel et al., 1995; Berjak et al., 1999; Berjak and Pammenter, 2000; Mycock et al., 2000; Wesley-Smith, 2003; Perán et al., 2006), may have severe metabolic and physiological consequences on subsequent growth. This may explain the abnormal growth often observed after cryopreservation of zygotic germplasm from recalcitrant seeds (e.g. Pence, 1992; Dumet et al., 1997; Wesley-Smith et al., 2001b; Sershen et al., 2007; Steinmacher et al., 2007). In relating ultrastructural responses to the most unambiguous indicator of post-cryo survival, germinability and seedling establishment, the present study also aimed to identify ultrastructural markers of cryo-related stresses.

In the present study ultrastructure was assessed after cryoprotection, dehydration, recovery from the cryogen, and after all possible combinations of these procedures. Studies described in Chapter 2 (section 2.3) showed that zygotic embryo WCs <0.4 g g⁻¹ were superior to those >0.4

g g⁻¹, in facilitating post-thaw survival for *A. belladonna*, while the reverse was true for *H. montanus*. Based on those findings, *A. belladonna* and *H. montanus* zygotic embryos in this study were cryopreserved at WCs > and <0.40 g g⁻¹, i.e. at WCs that were presumed to be sufficiently low and at WCs that were potentially too high for successful cryopreservation. Embryos dried to WCs in both these ranges, with and without prior sucrose (Suc; non-penetrative) or glycerol (Gly; penetrative) cryoprotection (CP), were, in turn, cooled rapidly or slowly to observe the interactive effects of WC, cooling rate and cryoprotection on subsequent cellular sub-structure, intracellular organisation and viability.

In desiccation-sensitive tissues, the effect of a stress, particularly a mild stress, is unlikely to be instantaneous. In fact, if a stress induces a metabolic disorder, it takes time for the damage consequent upon that disorder to accumulate (Walters et al, 2001). In most cases the damage incurred may be evident only after the system has rehydrated fully, and metabolism has been reinitiated. Further to this, recovery from cryostorage involves a series of complex events and, while severely damaged tissues undergo degradative processes that lead to cell death, less damaged tissues can stabilise, recover and return to apparently normal metabolic status (Benson and Noronha-Dutra, 1988). Therefore in a study aiming optimise a plant germplasm cryopreservation protocol, knowledge of the tissues ability to recover from the stresses imposed and damage incurred during cryopreservation may be more useful in identifying the treatment combinations that are optimum for post-thaw survival than 'real state' effects (i.e. those evident immediately after the treatment), since real state effects may disappear or worsen in frozenthawed tissues during the *in vitro* recovery period (Kaczmarczyk et al., 2008). Based on the above, freshly-excised embryos and those subjected to pre-conditioning or cooling were processed for TEM after rehydration and a subsequent 48 h in vitro recovery period. Results of these studies are therefore presumed to reflect the damage caused by the treatment as well as that accumulated or ameliorated during early recovery.

3.2 Materials and Methods

Plant material

Mature fruits of *A. belladonna* and *H. montanus* were harvested directly from parent plants and transported in plastic bags to the laboratory with minimum delay (1-2 d). Upon arrival, the seeds were decontaminated and stored in the hydrated condition, as described in Chapter 2 (section 2.2).

Embryo pre-treatment

After 14 d of hydrated storage, individual zygotic embryos were excised with the entire cotyledonary body attached (see Fig. A1, Appendix A) and collected within closed Petri dishes on filter paper moistened with sterile calcium-magnesium solution (CaMg solution: of 0.5 μ M CaCl₂.2H₂O and 0.5 mM MgCl₂.6H₂O [Mycock, 1999]). In order to minimise the potential variation in drying and/or cooling rate as a function of embryo size, only embryos of between 4-6 mm in length were used for all the experiments described below. Excised embryos were flash-dried (rapid dehydration; originally devised by Berjak *et al.*, 1990) to: (a) WCs between 0.53 and 0.41 g g⁻¹ (dry mass basis [dmb]) (referred to as >0.4 g g⁻¹ from here on); (b) WCs between 0.33 and 0.29 g g⁻¹ (referred to as <0.4 g g⁻¹ from here on); and (c) WCs > and <0.40 g g⁻¹ after CP with either aqueous Gly or Suc. The WC ranges used were obtained by constructing WC and viability vs. drying time curves for each species (see Fig. A2, Appendix A). For cryoprotection, freshly excised embryos were immersed in a 5% solution of Gly (v/v) or a 0.5 M solution of Suc for 1 h, and thereafter transferred to a 10% Gly (v/v) or 1.0 M Suc solution for a further hour.

Partially dried embryos (with and without CP) were subsequently cooled at: (a) rapid, nonequilibrium (*c*. 200°C s⁻¹) cooling rates by direct immersion of the naked embryos in nitrogen slush (liquid nitrogen sub-cooled to -210°C [Echlin, 1992]); or (b) slow, equilibrium cooling rates (1°C min⁻¹ in an isopropanol bath [Mr Frosty®] within a -70°C freezer) down to -40°C followed by direct immersion in nitrogen slush. Freshly excised embryos not subjected to dehydration or CP (referred to as 'fresh' embryos from here on), as well as those exposed to CP but no dehydration were also subjected to both cooling rates. After cooling in nitrogen slush, the embryos were transferred under liquid nitrogen (LN) into LN-containing cryovials (GreinerTM), mounted on aluminium cryo-canes (10 embryos per vial) and immersed in LN for up to a week before use. Upon retrieval from LN, embryos were rapidly thawed by direct immersion in sterile CaMg solution at 40°C for 2 min, rehydrated in fresh CaMg solution at ambient temperature for 30 min in the dark, and recovered *in vitro*. Freshly excised embryos not exposed to any of the treatments described above were also recovered *in vitro* to serve as a control.

Water content determination

Immediately after each of excision (referred to as 'fresh' from here on), partial dehydration (D), CP and CP+D, 10 embryos from each of the non-cooled treatment combinations were weighed individually using a 6-place balance (Mettler, MT5; Germany) and dried in an oven at 80°C for 48 h before being re-weighed to determine the dry mass. Water content was expressed on a dry mass basis (dmb; g H₂O per g dry matter [g g⁻¹]).

In vitro recovery and viability assessment

Immediately after rehydration, 15 embryos from each of the 27 treatment combinations (see Table 2.1) were decontaminated and recovered *in vitro* for 60 d, as described in Chapter 2 (section 2.2). Viability was assessed on 10 embryos (as five were used for ultrastructural studies) after 60 d *in vitro* growth and was defined by root and shoot production.

Ultrastructural studies

Fixation

Of the 15 embryos that were recovered *in vitro* for each treatment, the radicle tip (see Fig. A1c, Appendix A) of each of five randomly selected specimens from all treatments and the untreated control was excised after 48 h *in vitro* recovery and fixed for TEM. Samples were fixed in 2.5% phosphate-buffered glutaraldehyde (0.1 M, pH 7.2) for 24 h at 4°C. Following several rinses with phosphate buffer, specimens were post-fixed in 0.5% aqueous osmium tetroxide for 1 h at room temperature and rinsed three times with phosphate buffer. Specimens were then dehydrated in a graded acetone series (30%, 50%, 75%, each for 5 min and 100% for 10 min) followed by infiltration and embedding in low-viscosity resin (Spurr, 1969) and polymerisation for 8 h at 70°C. Specimens were sectioned using an Ultracut E ultramicrotome (Leica, Austria). Ultra-thin sections of root meristem showing copper/gold interference colours were collected on 600 mesh copper grids and contrasted for electron microscopy using a standard double-staining procedure: saturated (2.5%) uranyl acetate followed by lead citrate (Reynolds, 1963).

Microscopy, image processing and analysis

Ultra-thin sections of root meristem cells were viewed using a Jeol JEM 1010 transmission electron microscope (JEOL, Japan) at 100 kV. The ultrastructure of root meristem cells from each of five embryos for all treatments, including the untreated control, was assessed and images that represented the general appearance of cells from each treatment were captured digitally for subsequent analysis. Image-Pro[®] Plus (Version 6.1; MediaCybernetics, USA) was used for measuring vacuolar area and abundance across 25 root meristem cells for each of five embryos, for all treatments and the untreated control. The area within the wall of each cell was also measured, and the corresponding percentage area occupied by vacuoles was calculated. As in other ultrastructural studies on recalcitrant seeds (e.g. Farrant *et al.*, 1997; Wesley-Smith, 2003) degree of vacuolation was expressed here as area, and not volume, in line with the

thinness of the section. This measure was based on the assumption that area equates to volume within the meristematic cells investigated here.

Statistical analysis

As data for vacuolar area and quantity were not normally distributed (p > 0.05; Komolgirov-Smirnov test), even with transformation, inter-treatment differences in vacuolar area and number were tested for using a Mann-Whitney-U test (SPSS, Version 15). Inter-treatment differences in viability were tested for using null-model chi-squared analyses (specifically designed to assess non-parametric data) (EcoSim Version 7.72 [developed by Gotelli and Entsminger, 2009]). All statistical tests were performed at the 0.05 level of significance.

3.3 Results and Discussion

Untreated embryos (Fresh)

For both species, axes excised from fresh seeds were at WC >4.0 g g⁻¹ (*c*. 4.67 g g⁻¹ for *A*. *belladonna* and 5.05 g g⁻¹ for *H. montanus*; data not shown) and viability was 100% (Fig. 3.1A, B). Ultrastructurally, root meristem cells of fresh embryos of both species showed a centrally positioned, irregularly shaped nucleus (N) with dispersed heterochromatin, regular cell walls (CW) with contiguous plasmalemmae, and normally-distributed organelles (Fig. 3.2a & e), an indication of their underlying cytoskeletal organisation (Berjak *et al.*, 1999). These cells also showed signs of ongoing active metabolism: abundant polysomes (Fig. 3.2d & f) and Golgi bodies (Gb; Fig. 3.2a & f-insert); mitochondria (M; Fig. 3.2b & f); and peripherally located profiles of rough endoplasmic reticulum (rER; Fig. 3.2c & e, f).

While the irregular profiles of the nuclei were similar to those reported for the recalcitrant seeds of *Trichilia emetica* (Kioko *et al.*, 2006), the presence of cytomatrical polysomes is indicative of *de novo* protein synthesis (e.g. Farrant *et al.*, 1985). Membrane proteins and also proteins contained within membranous vesicles are processed to glycoproteins within Golgi bodies (Zhang and Staehelin, 1992), making these organelles important indicators of physiological status. Their presence within the cells of fresh embryos here was therefore suggestive of membrane flow and carbohydrate metabolism (reviewed by Berjak and Pammenter, 2000). Short cristae were visible within the mitochondria of both species (Fig. 3.2b & f); such mitochondria are generally taken as sign of active respiration (e.g. as described for root meristems of the recalcitrant seeds of *Avicennia marina* [Farrant *et al.*, 1992]). Mitochondria within *A. belladonna* cells also showed a central membranous formation (black arrow; Fig. 3.2b), the functional significance of which is unknown. The rough endoplasmic reticulum (rER) is the starting point of the protein secretory pathway. Proteins destined for other

compartments of the endomembrane system or the apoplast are initially inserted into the rER and then transported to the Golgi complex *en route* to their final destinations (Vitale *et al.*, 1993; Galili et al., 1998). Apart from being the site of aggregation and accumulation of some classes of storage proteins in seeds of some plants, the rER is the site of synthesis of all the endomembranes within cells (Vitale et al., 1993; Berjak and Pammenter, 2000). The strong development of rER, as in the cells of fresh embryos of both species presently studied (Fig. 3.2c & e), is held to be indicative of active membrane synthesis (Novikoff, 1976; Berjak and Pammenter, 2000). Profiles of rER within the cells of fresh embryos of both species were also generally peripherally located (Fig. 3.2c & e). In plant cells this situation is thought to be indicative of the functional role that the endoplasmic reticulum (ER) plays in vesicle-mediated secretion and/or membrane recycling (Craig and Staehelin, 1988). The ultrastructural signs of active metabolism observed within cells of fresh embryos for both species investigated here are typical of mature recalcitrant seeds (e.g. those of *Podocarpus henkelii* [Dodd et al., 1989]; A. marina [Farrant et al., 1992]; Artocarpus heterophyllus [Wesley-Smith et al., 2001a]; and Trichilia emetica [Kioko et al., 2006]), which grade into germinative metabolism after shedding.

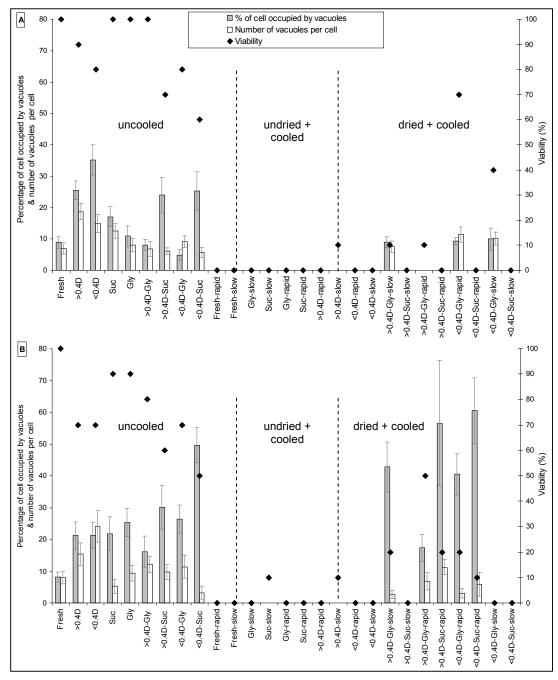


Figure 3.1 Viability, percentage of cell occupied by vacuoles and number of vacuoles per cell, for fresh, cryoprotected, partially dried and cooled [A] *A. belladonna* and [B] *H. montanus* zygotic embryos. Viability = root and shoot production; >0.4D = dried to >0.4 g g⁻¹; <0.4D = dried to <0.4 g g⁻¹; -Gly = cryoprotected with glycerol; -Suc = cryoprotected with sucrose; -slow = cooled slowly; -rapid = cooled rapidly. p < 0.001 when viability data were tested for significant differences across treatments (null-model chi-squared analysis, n = 10). Values for percentage of cell occupied by vacuoles and number of vacuoles per cell represent mean±SD and when tested for significant differences across treatments p was < 0.05 (Mann-Whitney-U test, n = 25).

The cells within fresh embryos of both species showed many plastids (P), containing starch grains (SG; Fig. 3.2a & e) in keeping with the observation that starch is the only complex form of carbohydrate reserve in fresh *A. marina* embryos (Farrant *et al.*, 1992, 1997) and the predominant insoluble reserve in recalcitrant embryos of other species (e.g. those of *P. henkelii* [Dodd *et al.*, 1989] and *Camellia sinensis* [Berjak *et al.*, 1993]). Internal membranes were observed within the matrices of a few plastids in *A. belladonna* cells (Fig. 3.2b) but such detail could not be visualised within the starch-occluded matrices of the plastids in *H. montanus* cells (Fig. 3.2e; f-insert). However, there were substantial areas of cytomatrix between starch-containing plastids within the cells of both species (Fig. 3.2a & e). This suggests that dehydration would probably bring about considerable intracellular collapse as one factor in their desiccation-sensitivity, since such cytomatrical 'space' is minimised in cells of orthodox embryos by accumulation of insoluble reserves (Vertucci and Farrant, 1995; Farrant *et al.*, 1997).

Vacuoles, which are the major lytic compartments of plant cells, may be formed from large ER-derived vesiculations, or by cytolysome formation, i.e. the sequestration of volumes of the cytomatrix, which may or may not contain organelles (Matile and Moor, 1968; Lamb and Berjak, 1981; Staehelin, 1997). Root meristem cells of fresh embryos of both species investigated here showed a few, small clearly defined (i.e. no obvious signs of tonoplast dissolution) vacuoles (V; Fig. 3.2d & g), which is typical of meristems of axes of newly-harvested recalcitrant seeds (e.g. those of *Landolphia kirkii* [Farrant *et al.*, 1989] and *T. emetica* [Kioko *et al.*, 2006]). For both species investigated here, vacuoles within cells of fresh embryos collectively occupied less than 10% of the cell area (Fig. 3.1A, B) and showed intra-vacuolar inclusions (Fig. 3.2d & g). These inclusions, which were in some cases membranous in appearance (encircled; Fig. 3.2b), may be indicative of intracellular turnover (Berjak and Pammenter, 2000), or what is more likely in embryo cells, of the ontogeny of the vacuoles themselves (Lamb and Berjak, 1981).

Lamb and Berjak (1981) described two possible modes of vacuolation, including cytolysome formation with or without regional differentiation, both of which involve sequestration of a considerable volume of cytomatrix, initially by a barrier consisting of double membranes, originating from the endoplasmic reticulum. In the first mode, the sequestered cytoplasm contains a variety of organelles, while regional differentiation involves clearing the cytoplasm isolated within the nascent cytoplysome (vacuole) of organellar components. In both cases ultimately only the outermost membrane of the sequestering ER sheets persists so that the provacuole and ultimately the vacuole, is typically single-membrane bounded (Lamb and Berjak, 1981). In the present study, some vacuoles within cells of fresh embryos of both species

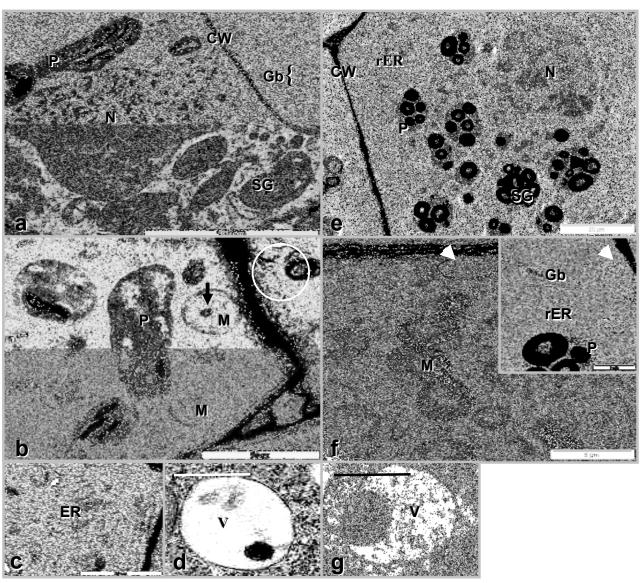
resembled the single-membrane-bound provacuoles that were shown to develop via cytoplasmic regional differentiation during early imbibition in *Zea mays* root cap cells (Lamb and Berjak, 1981). According to those authors, vacuolation by regional differentiation of the cytoplasm has the advantage of the rapid removal of large volumes of ground cytoplasm, while still conserving organelles.

A few cells within *H. montanus* fresh embryos showed vesiculation of the plasma membrane (white arrowhead; Fig. 3.2f & f-insert). From their occasional observation it was not possible to tell whether these were indicative of endo- or exocytosis; however, these vesiculations are suggestedly more likely to be endocytotic, in view of importation of substances from the endosperm via the cotyledon, which would be expected in these developing/germinating axes.

Root meristem cells of fresh (untreated) *A. belladonna* [a-d] and *H. montanus* [e-g] zygotic embryos are illustrated. a) whole cell showing centrally positioned, irregularly shaped, nucleus with nucleolus and heterochromatin, starch grains within plastids, Golgi bodies, and regular cell wall with contiguous plasmalemma; b) plastids, mitochondria (one showing central membranous formation and both showing short cristae) and polysomes; c) profiles of rough endoplasmic reticulum located peripherally; d) small vacuole with inclusion; note the abundant polysomes; e) whole cell showing segment of centrally positioned, irregularly shaped, nucleus with heterochromatin, plastids with starch grains, rough endoplasmic reticulum and regular cell wall with contiguous plasmalemma; f) mitochondria showing cristae, apparent vesiculation of the plasmalemma and insert showing plastid, Golgi body, profiles of rough endoplasmic reticulum, polysomes and apparent vesiculation of the plasmalemma; g) small vacuole with inclusion. N=nucleus; P=plastids; SG=starch grains; M=mitochondria; rER=rough endoplasmic reticulum; V=vacuole; Gb=Golgi body; CW=cell wall; white arrowheads=apparent vesiculation of the plasmalemma; black arrow=central membranous formation within mitochondrion. Bar=100 µm for (a), 20 µm for (b, c, e), 5 µm for (f, f-insert) and 0.5µm for (d, g).

A. belladonna

H. montanus



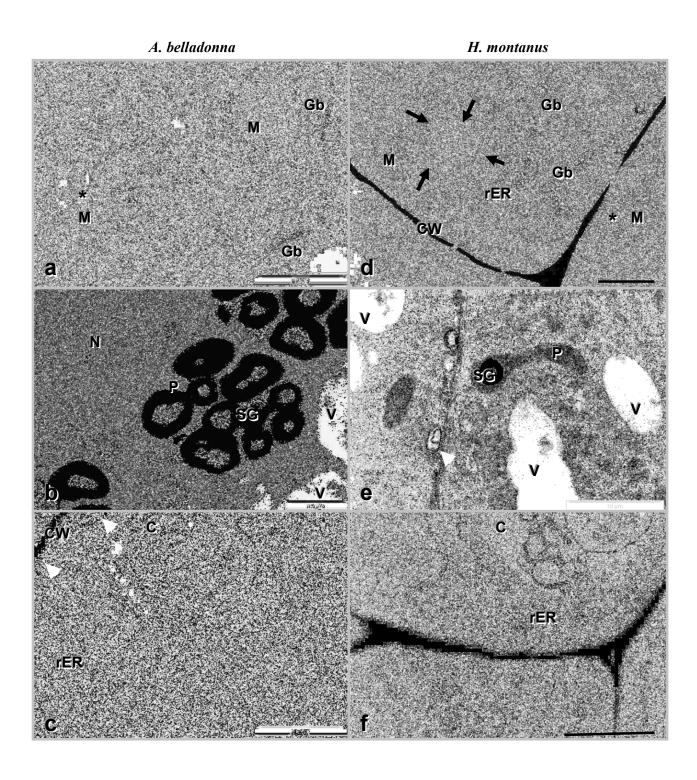
Glycerol (penetrative) cryoprotection in the absence of drying and cooling (Gly)

Glycerol CP had no adverse effect on viability in A. belladonna embryos and in view of the uncompromised ultrastructure and small degree of dehydration (see below) the apparent 10% decline in viability in Gly CP H. montanus embryos may well indicate nothing more than one poor quality embryo among the 10 assessed (Fig. 3.1A, B, respectively). Cells of Gly cryoprotected embryos of both species were similar to those of fresh embryos, showing highly organised ultrastructure: centrally positioned, irregularly shaped, nucleus (not shown) with dispersed heterochromatin (N; Fig. 3.3b); regular cell walls (CW; Fig. 3.3c & d) with contiguous plasmalemmae; and normally-distributed organelles. Intracellular constituents included: cytomatrical polysomes (Fig. 3.3a, c & d, f), Golgi bodies (Gb), mitochondria (M) (Fig. 3.3a & d); and long profiles of rough endoplasmic reticulum (rER) (Fig. 3.3c & d, f). The presence of these various organelles and the frequency of polysomes were indicative of ongoing active metabolism. Cristae were not clearly visible within the mitochondria of Gly CP cells for either species, although central membranous formations could be discerned (*; Fig. 3.3a & d). While plastids (P) in A. belladonna Gly CP cells contained many prominent starch grains (SG; Fig. 3.3b), such prominent starch-filled plastids were not observed in *H. montanus* cells, in contrast to the situation in control material (Figs 3.3e & 3.2e, respectively). There was an increased incidence of plasmalemma-associated vesiculations (white arrowheads; Fig. 3.3c & e) perhaps indicating an endocytotic route of Gly uptake.

Glycerol CP led to a reduction in embryo WC relative to fresh embryos (A. belladonna: by ~32% to c. 3.16 g g⁻¹ but only by ~5.5% in *H. montanus*: to c. 4.77 g g⁻¹ [data not shown]), as well as an increase in the proportion of the area occupied by vacuoles (which showed no obvious signs of tonoplast dissolution) (Fig. 3.3b & e) particularly in the cells of H. montanus, but little increase in the number of vacuoles per cell in either species (Fig. 3.1A, B). Ultrastructural studies have shown many similarities in plant cell responses during exposure to cryoprotection and desiccation (Wilkinson et al., 2003; Kaczmarczyk et al., 2008). Vacuolation often occurs in response to stresses to which cells are subjected (Berjak and Pammenter, 2000) and an increase in the degree of vacuolation in response to physical drying has been reported for tissues of axes from recalcitrant seeds of a variety of species (Berjak et al., 1989, 1990; 1999; Farrant et al., 1989; Mycock et al., 2000; Wesley-Smith et al., 2001a). Since the number of vacuoles in cells of Gly CP embryos did not increase relative to cells of fresh embryos in this study (Fig. 3.1A, B), the accumulation of Gly (or products of CP) and/or the osmotic dehydration accompanying Gly CP, may have increased solute concentrations within vacuoles, rendering these compartments more sensitive to osmotic expansion upon rehydration during in vitro recovery. Digestion by hydrolysis of intravacuolar content too, could have contributed to the osmotic potential of these compartments. In view of the disappearance of plastidial starch in Gly CP *H. montanus* embryos, a further factor possibly contributing both to vacuolar osmotic properties and the very small drop in water content could have been starch hydrolysis.

The observation of relatively amorphous areas of cytoplasm that appeared to have been sequestered by ER (black arrows; Fig. 3.3d) in cells of Gly CP *H. montanus* embryos was suggestive of cytoplasmic regional differentiation which precedes cytolysome formation (e.g. Lamb and Berjak, 1981). Those authors showed that in certain instances, cytolysome formation may involve many ER membrane shells which are invariably concentric about one or more provacuoles or small vacuoles typified by their dense content. The interior of the resulting structure then becomes packed with degenerating membrane, which disappears with time. Noting that this phenomenon is also a form of autophagy, such advanced stages of cytolysome formation, forming vacuoles, were observed within the cells of Gly CP embryos of both species (Fig. 3.3c & f). It is not possible to state unequivocally that the vacuolar structures described above were of ER origin but long profiles of ER were often found in relatively close proximity to these structures (Fig. 3.3c & d, f). This is a common response to stress in plant cells and is regarded as indirect evidence for the conversion of ER membranes to vacuolar membranes – i.e. for the specialisation of membrane being differentiated at the rER, to form cytolysomes and hence vacuoles (reviewed by Staehelin, 1997).

Root meristem cells of glycerol cryoprotected *A. belladonna* [a-c] and *H. montanus* [d-f] zygotic embryos are illustrated. a) numerous polysomes, Golgi bodies and mitochondria, one showing central membranous formations; b) plastids crammed with starch grains, segment of nucleus with heterochromatin, and vacuoles; c) regular cell wall and apparent vesiculation of the plasmalemma, long profiles of rough endoplasmic reticulum and evidence of cytolysome formation; d) regular cell wall with contiguous plasmalemma, polysomes, Golgi bodies, long profiles of rough endoplasmic reticulum, signs of vacuolation by cytoplasmic regional differentiation (arrowed region) which precedes cytolysome formation, and mitochondria, one showing inner non-cristate membranous formations; e) small plastid with starch grain, vacuoles, and apparent plasmalemma vesiculations; f) long profiles of rough endoplasmic reticulum, signs of advanced cytolysome formation forming a vacuole, and abundant polysomes. N=nucleus; P=plastids; SG=starch grains; M=mitochondria; *=central membranous formation; rER=rough endoplasmic reticulum; C=cytolysome formation; V=vacuole; Gb=Golgi bodies; CW=cell wall; white arrowheads=apparent vesiculation of the plasmalemma; black arrows=signs of vacuolation by cytoplasmic regional differentiation. Bar=0.5 µm for (a-c) and 10 µm for (d-f).

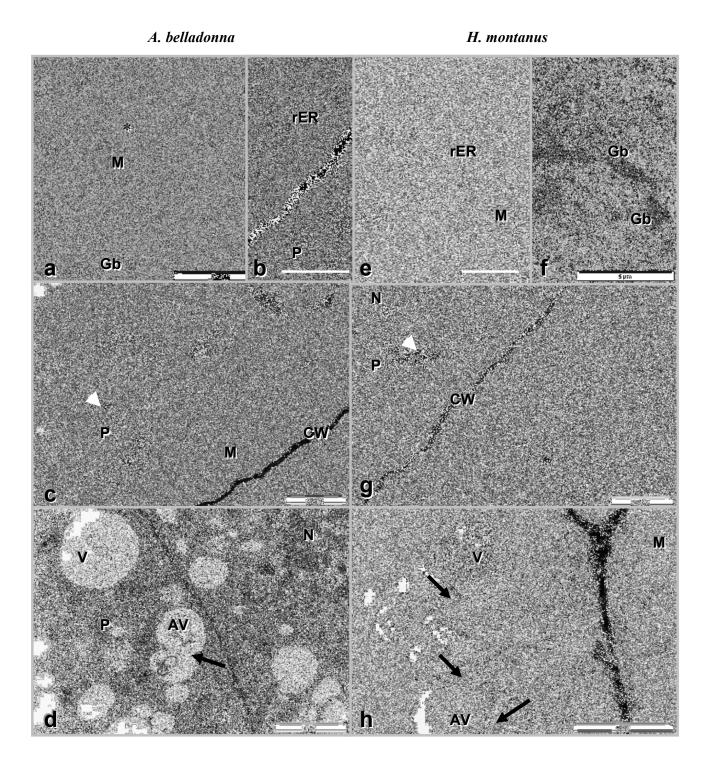


As observed for the Gly CP treatment, Suc CP had no adverse effect on embryo viability in A. belladonna but again a 10% decline in viability in those of H. montanus was recorded (Fig. 3.1A, B, respectively). Cells of Suc CP embryos of both species were similar to those of fresh and Gly CP specimens in that the nucleus was centrally positioned and irregularly shaped (not shown) with the heterochromatin dispersed (N; Fig. 3.4d), and organelles were normallydistributed. The metabolic potential of these cells was evidenced by the presence of: cytomatrical polysomes and Golgi bodies (Gb) (Fig. 3.4a & f), and mitochondria, some with long cristae (M; Fig. 3.4c & h). The mitochondrial development observed could indicate that Suc CP was associated with respiratory enhancement. If this conjecture is correct, then these embryos would have had an enhanced metabolic rate as a consequence of Suc CP, and therefore may have become *more* desiccation sensitive (with reference to the work of Berjak *et al.*, 1989) and Farrant et al., 1992). This could have been one major factor in the greater viability loss upon flash drying and cryopreservation, than shown by Gly CP embryos (discussed later). Long parallel profiles of rough endoplasmic reticulum (rER), which were generally peripherally located, were apparent in cells of both species (Fig. 3.4b & e). Impermeable sugar cryoprotectants can inflict severe osmotic injury at high (≥ 1 M) concentrations (Finkle *et al.*, 1985; Muldrew et al., 2004). In the present study, a concentration of 1 M was used in the second phase of Suc CP, which may explain why, despite the signs of active ongoing metabolism and the intact cell walls with contiguous plasmalemmae (Fig. 3.4c & g), cells of Suc CP embryos showed a number of ultrastructural irregularities, not seen in fresh and Gly CP specimens. These included cell walls with slightly undulating profiles as well as dense plastids (P) containing what are presumed to be small plastoglobuli (white arrowheads) (Fig. 3.4c & g). The electron dense appearance of plastidial stroma is generally indicative of the presence of intraplastidial phenols (e.g. Vaughn and Wilson, 1981; Saranpää, 1988). If this was true for the plastids in cells of Suc CP A. belladonna embryos here, then this could have been a stress-induced response, since plants generally synthesize and accumulate phenols, often within plastids (Wise and Hoober, 2006), in response to physiological stress (Dixon and Paiva, 1995). Additionally, in some instances in A. belladonna cells, plastids were occasionally abnormally attenuated (Fig. 3.4d).

There were no visible signs of plasmalemma-associated vesiculation within Suc CP cells of either species and irrespective of the species or WC range, the proportion of the area occupied by vacuoles (which showed no obvious signs of tonoplast dissolution) was increased relative to fresh embryos (Fig. 3.4d & h; Fig. 3.1A, B). There were also signs of vacuolar fusion (black arrows; Fig. 3.4d & h) and what may have been autophagically-derived content within the

vacuoles in these cells (Fig. 3.4d & h). As discussed for cells of Gly CP embryos, the responses of plant cells to cryoprotection have been likened to those observed after dehydration, e.g. increased vacuolation and autophagy (Wilkinson *et al.*, 2003; Kaczmarczyk *et al.*, 2008). Ultrastructural studies on the responses of vegetative tissues to the procedures involved in cryopreservation report damage inflicted during cryoprotection to be associated with a loss of ultrastructural integrity (Wilkinson *et al.*, 2003; Ding *et al.*, 2008; Kaczmarczyk *et al.*, 2008), the forerunner of which could be the vacuolar fusion presently observed (black arrows; Fig. 3.4d & h). The increased vacuolation and autophagy observed in Suc CP embryos could be a consequence of the fact that Suc CP led to a significantly greater reduction in embryo WC than Gly CP (*A. belladonna: c.* 1.86 g g⁻¹; *H. montanus:* 1.79 g g⁻¹ [data not shown]). With reference to the work of Berjak *et al.* (1989), increased autophagy in Suc CP cells may have provided a mechanism for the removal of structures that were damaged during (osmotic) dehydration.

Root meristem cells of sucrose cryoprotected *A. belladonna* [a-d] and *H. montanus* [e-h] zygotic embryos are illustrated. a) polysomes, Golgi bodies and a mitochondrion showing formation of cristae; b) parallel profiles of rough endoplasmic reticulum and a plastid showing a dense matrix; c) dense plastids with small starch grains and cell wall having a slightly undulating profile; d) part of an irregularly shaped nucleus with dispersed heterochromatin, vacuoles, signs of autophagy and vacuolar fusion, and an attenuated plastid; e) long profiles of endoplasmic reticulum and a mitochondrion showing central membranous formations; f) Golgi bodies and cytomatrical polysomes; g) segment of nucleus showing heterochromatin, plastids containing what are presumed to be small plastoglobuli, and cell wall having a slightly undulating profile; h) vacuoles, signs of autophagy and vacuolar fusion. N=nucleus; P=plastids; M=mitochondria; rER=rough endoplasmic reticulum; V=vacuole; Gb=Golgi bodies; CW=cell wall; white arrowheads=plastoglobuli; black arrows=signs of vacuolar fusion; AV=autophagic vacuole. Bar=0.5 µm for (a, b c, g); 5 µm for (e, f) and 1 µm for (d, h).



For both species, cells within four of the five specimens sampled for each WC range showed regular cell walls with contiguous plasmalemmae (Figs 3.5d & f; 3.6b & d). The metabolic potential of these cells was evidenced by the presence of: Golgi bodies (Gb; Figs 3.5b & f; 3.6b & e), plastids (P) with starch grains (SG) (Figs 3.5c & f; 3.6a-insert & d); mitochondria (M; Figs 3.5b & f; 3.6b-insert & d); and cytomatrical polysomes (Fig. 3.5b; 3.6binsert). Other studies have similarly shown recalcitrant embryonic axes to retain intracellular organisation and viability after dehydration to WC ≥ 0.3 g g⁻¹ and subsequent rehydration, provided they were dried rapidly, and not slowly (e.g. those of T. emetica [Kioko et al., 2006] and A. heterophyllus [Wesley-Smith et al., 2001a]). This is presumed to be the outcome of the limited time allowed during rapid drying, in which damaging metabolism-linked reactions can occur (reviewed by Pammenter and Berjak, 1999; Walters et al., 2001; Berjak and Pammenter, 2008). However, even when recalcitrant embryos/axes of some species are dried rapidly to WC much higher than 0.3 g g^{-1} , a measure of ultrastructural disruption may be observed (e.g. A. heterophyllus flash-dried to c. 0.7 g g⁻¹ [Wesley-Smith et al., 2001a]). In the present study, partial dehydration by ~91% to WC >0.4 g g⁻¹ (c. 0.42 g g⁻¹) and by ~93% to WC <0.4 g g⁻¹ (c. 0.32 g s^{-1}) was accompanied by a decline in viability in A. belladonna embryos, and this was exacerbated in the latter case (Fig. 3.1A). Partial dehydration was also detrimental in H. *montanus* embryos, with viability losses of 30% being recorded both at WC > and <0.4 g g⁻¹ (~89% to c. 0.55 g g⁻¹ and ~93% to c. 0.34 g g⁻¹, respectively; Fig. 3.1B). Partially dried embryos of both species also showed a number ultrastructural irregularities, the frequency and/or severity of which was greater at WC $< 0.4 \text{ g g}^{-1}$.

Ultrastructural studies on the responses of plant tissues to dehydration have shown many similarities in cell response across a number of species and a variety of tissue types. While slight rapid dehydration can result in ultrastructure that is consistent with normal functioning in recalcitrant seed tissues (reviewed by Berjak and Pammenter, 2000; Kioko *et al.*, 2006) dehydration to lethal WCs is often immediately accompanied by an increase in the vacuole:cytoplasm ratio, or at very low WC, total sub-cellular destruction (e.g. Pritchard and Prendergast, 1986; González Arnao *et al.*, 1993; Isaacs and Mycock, 1999; Mycock *et al.*, 2000; Wilkinson *et al.*, 2003; Kioko *et al.*, 2006). Nuclear architecture and spatial arrangement of the organelles that is normally observed at high WC is often lost after dehydration to relatively low WC (Wesley-Smith *et al.*, 1995, 2001a; Berjak *et al.*, 1999; Kioko *et al.*, 2006), possibly as a result of damage to the nucleo- and cytoskeleton (Kioko *et al.*, 1998; Mycock, 1999; Berjak and Pammenter, 2000). However, this post-drying loss of ultrastructural integrity, and associated viability, is far more severe when tissues are dried slowly (Wesley-Smith 2001a; Berjak and

Pammenter, 2000; Kioko *et al.*, 2006); presumably due to the extended time for deleterious metabolism-linked reactions to occur (reviewed by Pammenter and Berjak, 1999; Walters *et al.*, 2001). Membranes are a primary site of desiccation damage (Senaratna and Mckersie, 1986) and damage to sub-cellular membranes, which is curtailed during rapid drying, is prominent among the damaging consequences of slow drying (Kioko *et al.*, 1998; Pammenter *et al.*, 1998).

The WCs attained after flash drying here are greater than those reported to induce structural damage in membranes (~0.25 g g⁻¹ or -11 MPa; Vertucci and Farrant, 1995) and, although dehydration was relatively rapid, some metabolic lesions caused by de-regulated metabolism may nevertheless have occurred (reviewed by Vertucci and Farrant, 1995; Pammenter and Berjak, 1999; Leprince et al., 2000). Partial dehydration was seen to be associated with a number of ultrastructural irregularities in both species: fragmented profiles of endoplasmic reticulum (ER) (Figs 3.5d & f; 3.6b & e); essentially spherical nuclei (N; Figs 3.5a & e-insert; 3.6a), some showing chromatin condensation (Fig 3.5a, e-insert & 3.6a, c); and cell walls (CW) having an undulating profile (Figs 3.5a & 3.6a, f). Condensation of the nuclear chromatin and aberrant nuclear profiles (e.g. Fig. 3.6c) have been cited as signs of desiccation injury in recalcitrant embryonic axes (e.g. Berjak et al., 1999; Wesley-Smith et al., 2001a; Kioko et al., 2006) and germinating axes of Zea mays (Crévecoeur et al., 1976). The change in nuclear shape to spherical in some cells of both species presently investigated is taken to indicate derangement of the nucleoskeleton with concomitant loss of nuclear morphology. As organisation of the nucleoskeleton is intrinsic to spatial localisation of the chromatin (Shumaker et al., 2003), the abnormalities seen in some of the cells in the present study are considered to be incipiently lethal. As shown for Araucaria angustifolia axes (Salmen Espindola et al., 1994), in recalcitrant embryos/axes such damage is unlikely to be repaired upon rehydration.

The undulating profiles of cell walls observed in some dehydrated *A. belladonna* and *H. montanus* specimens here, could have been induced by a reduction in cell volume (Wesley-Smith *et al.*, 2001a) and persisted after rehydration, possibly because cells of desiccation-sensitive, unlike those of desiccation-tolerant seeds (Webb and Arnott, 1982), are not preprogrammed to contract. Alteration of ER disposition following partial dehydration has been reported for recalcitrant embryonic axes (e.g. those of *A. heterophyllus* [Wesley-Smith *et al.*, 2001a]) and during maturation of orthodox seeds (e.g. mustard [Bergfeld and Schopfer, 1984]). The functional significance of the short profiles of ER observed within the cells of partially dehydrated embryos in this study is not yet known; however, in light of reports that the derangement of ER is associated with a cessation of growth (Bergfeld and Schopfer, 1984) and protein synthesis (Kandasamy and Kristen, 1989) in plant tissues, ER fragmentation during dehydration of *A. belladonna* and *H. montanus* embryos may well have had the potential to precondition them adversely, when exposed to cryogenic conditions.

Another striking feature of partially dried A. belladonna and H. montanus embryos was the significant increase in the number of vacuoles (V; which were clearly defined) and the proportion of the area occupied by these vacuoles, relative to fresh embryos (Figs 3.5a & e; 3.6b & f; 3.1A, B). The degree of this increase was greater at WC <0.4 g g⁻¹ in A. belladonna (Fig. 3.1A) and vacuoles within many of the cells belonging to this treatment were exceptionally large, dominating the intracellular space to such an extent that organelles were confined to a narrow strip of cytoplasm along the periphery of the cell (Fig. 3.6c). An increase in the degree of vacuolation in response to physical drying has been reported for tissues of axes from recalcitrant seeds of a variety of species (Berjak et al., 1989, 1990; 1999; Farrant et al., 1989; reviewed by Berjak and Pammenter, 2000; Mycock et al., 2000; Wesley-Smith et al., 2001a). Working on the recalcitrant embryonic axes of A. heterophyllus, Wesley-Smith et al. (2001a) suggested that drying increased solute concentration within vacuoles, rendering these compartments increasingly susceptible to osmotic expansion upon rehydration. Similar results have been reported for non-acclimated tubers of Jerusalem artichoke that had been subjected to freeze-dehydration (Murai and Yoshida, 1998). Irrespective of the species or WC range, there was definite evidence of autophagy (AV; Figs 3.5a & g; 3.6b & d) and vacuolar fusion (black arrows; Figs 3.5a & e; 3.6b & d) within cells of non-CP+D embryos of both the species presently investigated. Vacuoles are critical to the maintenance of homeostasis within cells (Marty, 1999) and when partially dried, desiccation-sensitive cells are rehydrated, autophagy may provide a mechanism for the elimination of intracellular components that have been damaged during drying. Autophagy may thus represent a mechanism to re-establish cellular homeostasis in such cells (Berjak et al., 1989, 1990; Wesley-Smith et al., 2001a).

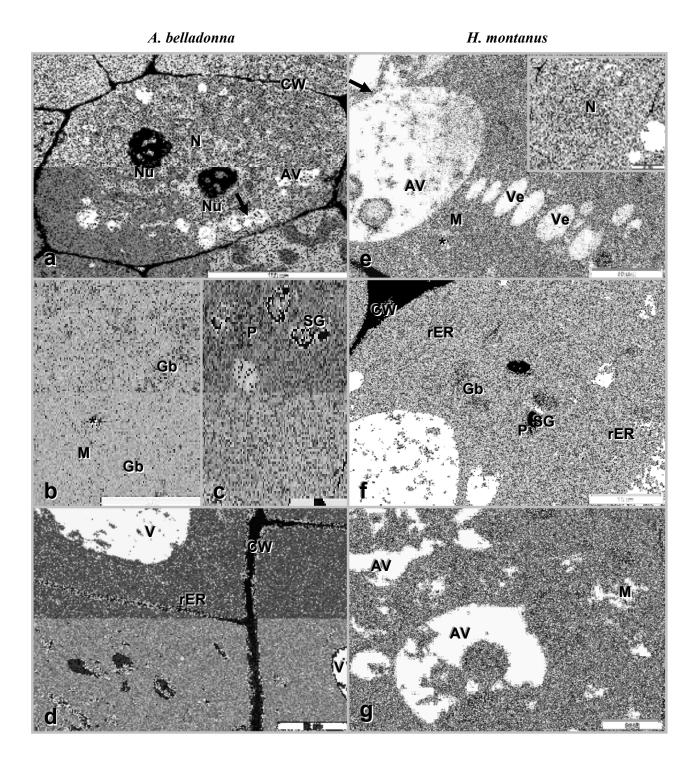
Responses of recalcitrant axis/embryo cells to dehydration stress are not manifested on an all-or-none basis (e.g. Salmen Espindola *et al.*, 1994; Kioko *et al.*, 2006). It has become apparent that cells - which may be near neighbours – show disparate responses, and apparently functional, highly organised cells may be contiguous with others which are extensively deteriorated (e.g. Berjak *et al.*, 1999; Mycock *et al.*, 2000). In the present study, ultrastructural integrity appeared to have been retained after dehydration (irrespective of the WC range) in some embryo cells of both species, as evidenced by: mitochondria (M; Figs 3.5b & e; 3.6a & d); cytomatrical polysomes (Figs 3.5b & f; 3.6b-insert & d); and Golgi bodies (Figs 3.5b & f; 3.6b & e).

However, *H. montanus* embryos were more sensitive to partial dehydration than were those of *A. belladonna* (Fig. 3.1A, B). Consistent with this, intracellular responses to partial

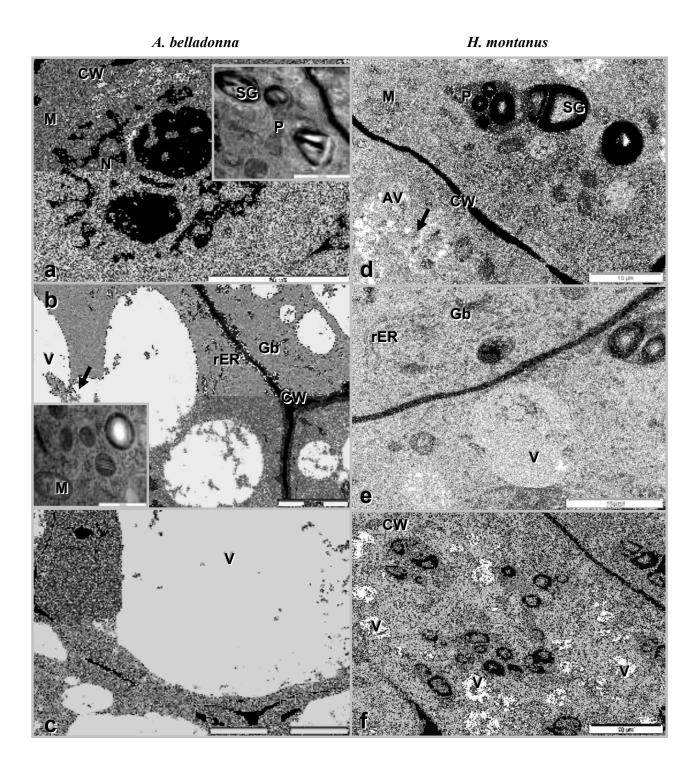
dehydration such as autophagy were more pronounced in *H. montanus* embryos (see Fig. 3.5g) and in contrast to the situation in *A. belladonna* cells (Fig. 3.5b), matrices within some mitochondria in *H. montanus* cells were translucent with the organelles, generally lacking internal structure (Fig. 3.5g). Poorly differentiated or deranged mitochondria are a sign of reduced respiratory competence (Farrant *et al.*, 1992). These inter-species differences are suggested to be the consequence of *H. montanus* embryos requiring significantly longer drying times to reach comparable WCs and hence being exposed to a greater degree of dehydration stress. While embryos of *A. belladonna* could be dried to WCs of *c.* 0.42 and 0.32 g g⁻¹ in 5 and 15 min, respectively, *H. montanus* embryos took 240 and 300 min to reach *c.* 0.55 and

 0.34 g g^{-1} , respectively (data not shown). In recalcitrant seed tissues, the effects of dehydration are based on two inter-related parameters, *viz*. intensity and duration, and when such tissues spend a longer period at intermediate WCs, the time for aqueous-based deleterious processes to occur is extended (Pammenter *et al.*, 1998; reviewed by Pammenter and Berjak, 1999; Walters *et al.*, 2001).

Root meristem cells of non-cryoprotected *A. belladonna* [a-d] and *H. montanus* [e-g] zygotic embryos dried to >0.4 g g⁻¹ are illustrated. a) whole cell showing essentially spherical nucleus with condensed heterochromatin, two nucleolar sites, signs of autophagy and vacuolar fusion, and cell wall having a slightly undulating profile; b) Golgi bodies, mitochondria (one showing the central membranous formation) and abundant polysomes; c) plastids with starch grains; d) profiles of fragmented rough endoplasmic reticulum, vacuoles and regular cell wall; e) mitochondria showing central membranous formations, vacuole showing signs of autophagy and vacuolar fusion and large vesicles, and insert showing essentially spherical nucleus with condensed heterochromatin; f) regular cell wall, short (fragmented) profiles of rough endoplasmic reticulum, Golgi bodies, and plastid with starch grains; g) signs of intensive autophagy and mitochondria with translucent matrices, generally lacking internal structure. N=nucleus; Nu=nucleolus; P=plastids; SG=starch grains; M=mitochondria; *=central membranous formations; rER=rough endoplasmic reticulum; V=vacuole; Ve=vesicles; Gb=Golgi bodies; CW=cell wall; black arrows=signs of vacuolar fusion; AV=signs of autophagy. Bar=100 µm for (a); 10 µm for (b, e & f); 20 µm for (c & d) and 5µm for (g).



Root meristem cells of non-cryoprotected *A. belladonna* [a-c] and *H. montanus* [d-f] zygotic embryos dried to <0.4 g g⁻¹ are illustrated. a) whole cell showing spherical nucleus with condensed heterochromatin, mitochondria, cell wall having markedly undulating profile, and insert showing plastids with starch grains; b) Golgi body, signs of vacuolar fusion, and short profiles of rough endoplasmic reticulum, and insert showing polysomes and mitochondria; c) highly vacuolated cell; d) signs of intensive autophagy and vacuolar fusion, plastids with large starch grains, and regular cell wall; e) short profiles of rough endoplasmic reticulum, Golgi body and vacuole; f) cell showing portion of irregular cell wall (top, left), and many relatively small vacuoles with considerable content suggested to be autophagically-derived. N=nucleus; P=plastids; SG=starch grains; M=mitochondria; *=central membranous formations; rER=rough endoplasmic reticulum; V=vacuole; Gb=Golgi body; CW=cell wall; black arrows=signs of vacuolar fusion; AV=signs of autophagy. Bar=50 µm for illustration (a); 20 µm for (a, b, f); 100 µm for (c) and 10 µm for (b-insert, d, e).



Partial dehydration with prior cryoprotection

Sucrose cryoprotection + partial dehydration (>0.4D-Suc and <0.4D-Suc)

There has been much conjecture about the role of sucrose, particularly in the desiccated state of seed tissues (Koster and Bryant, 2005; Halperin and Koster, 2006; reviewed by Berjak et al., 2007). Orthodox seeds accumulate sucrose and certain raffinose series oligosaccharides during maturation drying (Koster and Leopold, 1988; Leprince et al., 1993; Obendorf, 1997) and upon dehydration these constituents together with others, contribute towards a highly viscous, supersaturated solution known as a glass (Leopold *et al.*, 1994). Intracellular glasses have been suggested to curtail molecular diffusion and so minimise the potential for, and extent of, unregulated metabolism in the dehydrated state of desiccation-tolerant material (reviewed by Pammenter and Berjak, 1999; Berjak and Pammenter, 2001, 2004; Hoekstra et al., 2001; Kermode and Finch-Savage, 2002; Alpert, 2006; Berjak, 2006; Lehner et al., 2006). However, when applied exogenously to hydrated/partially hydrated specimens, non-penetrating CPs like Suc are held not to enter the cytoplasm and may accumulate apoplastically, whereas penetrating cryoprotectants, like Gly, diffuse through the plasma membrane or perhaps enter cells via endocytotic vesicles (e.g. Fig. 3.3c) and equilibrate in the cytoplasm (Grout, 1995; Muldrew et al., 2004). Non-penetrating cryoprotectants such as Suc can also lose their cryoprotective effect at high concentrations, since they act by dehydrating cells before freezing and can inflict severe osmotic injury (Finkle et al., 1985; Muldrew et al., 2004).

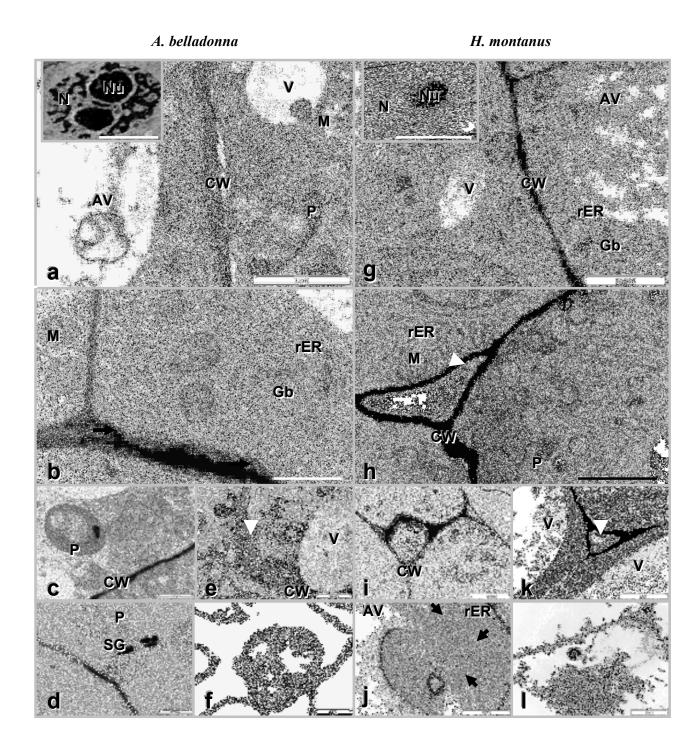
In light of the above, the injurious effects of non-penetrative CP (osmotic) and physical (flash drying) dehydration may have been additive in this study since viabilities for Suc CP+D embryos were significantly lower than those associated with non-CP embryos dehydrated to comparable WCs (Fig. 3.1A, B). In fact, when partial dehydration was preceded by CP post-drying viabilities for both species were significantly lower in Suc CP embryos (Fig. 3.1A, B). This was true for WCs greater (0.41 and 0.50 g g⁻¹ for *A. belladonna* and *H. montanus*, respectively) and less than (0.29 and 0.30 g g⁻¹ for *A. belladonna* and H. *montanus*, respectively) 0.4 g g⁻¹. Within each WC range, for both species, root meristem cells of some of the Suc CP+D embryos appeared well organised with normally-distributed organelles (Fig. 3.7a, b, c & g, i) and indications of ongoing active metabolism in terms of polysomes and Golgi bodies (Gb) (Fig. 3.7b & g); plastids (P) (Fig. 3.7a & h); rough endoplasmic reticulum (rER; Fig. 3.7b & g, h); and mitochondria (M; Fig. 3.7a, b, c & h). Nevertheless, a few cells in some of these embryos showed wall abnormality (Figs 3.7a, b), or incipient and complete autolysis (Fig. 3.7h, k). Additionally, all cells in the remaining specimens (one to two of five) were autolysed in both species (Fig. 3.7f & l).

However, even when cells were not autolysed, Suc CP+D was associated with a number of ultrastructural irregularities: short profiles of rER (Fig. 3.5b & g); cell walls having an irregular profile (Fig. 3.7e & h) and some showing middle lamella separation (Figs 3.7a, b); spherical nuclei (N) with highly condensed heterochromatin (Fig. 3.5a-insert & g-insert); plastids, largely devoid of starch grains (Fig. 3.7a & h), and some having abnormal attentuated or lobed profiles (Fig. 3.7a & d). Autolysing and autolysed cells, referred to above (white arrows), were scattered amongst others which were ultrastructurally intact (Fig. 3.7e, h & k). Aberrant nuclear profiles, condensation of the chromatin (e.g. Berjak et al., 1999; Wesley-Smith et al., 2001a; Kioko et al., 2006), and abnormal ER disposition (Wesley-Smith et al., 2001a) have all been cited as signs of desiccation injury in seed tissues, while sparse starch deposition within plastids attests to a reduced metabolic state (Berjak and Pammenter, 2000). Enhanced autophagy (AV; Fig 3.7a & g) was observed within cells of Suc CP+D embryos of both species and, as discussed for non-CP+D embryos, this could represent a mechanism for the re-establishment of cellular homeostasis (Berjak et al., 1989, 1990; Wesley-Smith et al., 2001a). Additionally, as in the cells of non-CP+D embryos, cells of Suc CP+D embryos of both species showed a significant increase in the proportion of area occupied by vacuoles (which were clearly defined), but this was not associated with an increase in the number of vacuoles, relative to cells of fresh embryos (Figs 3.1A, B; 3.7a, e & g, k). Dehydration generally leads to an increase in the degree of vacuolation in tissues of axes from recalcitrant seeds (e.g. Berjak et al., 1989, 1990; 1999; Farrant et al., 1989; Mycock et al., 2000; Wesley-Smith et al., 2001a). Also, non-penetrating cryoprotectants, like Suc, act by dehydrating cells before freezing (Muldrew et al., 2004), and in the present study autolytic breakdown products and osmotic dehydration accompanying this CP, could have acted synergistically with physical dehydration to increase solute concentration within vacuoles, rendering them more sensitive to osmotic expansion upon rehydration during in vitro recovery.

In terms of survival between the species, *H. montanus* embryos were more adversely affected than were those of *A. belladonna* after Suc CP+D (Fig. 3.1A, B). Ultrastructural observations were consistent with this; for instance, while autolysed cells occurred amongst others which were ultrastructurally intact only at <0.4 g g⁻¹, in *A. belladonna* (Fig. 3.7e), they were a common feature at both WC ranges in *H. montanus* (Fig. 3.7h & k). Also, while some vacuoles within the cells of Suc CP+D embryos of both species showed signs of autophagically-derived content, only cells of *H. montanus* Suc CP+D embryos showed relatively amorphous areas of cytomatrix that appeared to be being sequestered by rER (black arrows; Fig. 3.7j). This is suggestive of *de novo* vacuolation via the cytolysome route (Lamb and Berjak, 1981), which is a common response to stress in plant cells (reviewed by Staehelin, 1997). These inter-species

differences are suggested to be the consequence of the longer drying times to which *H. montanus* embryos were exposed and reinforce the suggestion that the deleterious effects of Suc-CP and physical dehydration may have been additive in the embryos presently studied.

Root meristem cells of sucrose cryoprotected A. belladonna [a-f] and H. montanus [g-l] zygotic embryos dried to >0.4 g g⁻¹ [a, b & g, h] and <0.4 g g⁻¹ [c-f & i-l] are illustrated. a) vacuole, attenuated plastid, mitochondria, cell wall with middle lamella separation, signs of autophagy; insert showing spherical nucleus with highly condensed chromatin and nucleoli; b) polysomes, short rough endoplasmic reticulum profile, Golgi bodies, and mitochondria; g) vacuole, polysomes, short fragments of rough endoplasmic reticulum, Golgi body, signs of autophagy; insert showing spherical nucleus with nucleolus and condensed chromatin; h) irregular cell wall, lysed cell (white arrowhead), mitochondria and plastid. c) cylindrical plastid, surrounding cytomatrical material, and regular cell wall; d) abnormally, lobed plastid with small, dark starch grains; e) vacuole within ultrastructurally intact cell showing irregular cell wall, adjacent to deteriorating (autolysed) cell (lower left); f) autolysed cells; i) regular cell walls; j) early signs of cytolysome formation (long circularly-disposed profiles of rough endoplasmic reticulum to right of autophagic vacuole); k) Vacuole within ultrastructurally intact cell adjacent to deteriorating cell (middle); 1) autolysed cells. N=nucleus; Nu=nucleolus; P=plastids; SG=starch grains; M=mitochondria; rER=rough endoplasmic reticulum; V=vacuole; Gb=Golgi body; CW=cell wall; AV=signs of autophagy; black arrows=signs of cytolysome formation; white arrowheads=lysed cell. Bar=1 μ m for (a, f, k); 0.5 μ m for (b, c, d, e); 10 μ m for (g, h, j); 20 μ m for (i, l); and 50 µm for (a-insert, g-insert).



<u>Glycerol cryoprotection + partial dehydration (>0.4D-Gly and <0.4D-Gly)</u>

Though, not as severe, Gly (penetrating) like Suc (non-penetrating) CP, led to dehydration of the embryos of both species (discussed earlier). When this was followed by flash drying viabilities were either marginally higher than (as in >0.4D-Gly: 0.44 and 0.53 g g⁻¹ for *A. belladonna* and *H. montanus*, respectively) or similar to (as in <0.4D-Gly: 0.32 and 0.33 g g⁻¹ for *A. belladonna* and *H. montanus*, respectively) those associated with non-CP+D embryos, at comparable WCs. Further to this, within species, viabilities after Gly CP+D were always higher than those following Suc CP+D, at comparable WCs (Fig. 3.1A, B).

One of the essential attributes of the colligative theory of cryoprotection is that the relevant cryoprotectants must be able to penetrate the cell, otherwise they will cause osmotic dehydration, resulting in the very injury against which they are employed to prevent (Benson, 2008). The superiority of Gly over Suc cryoprotection in limiting post-drying ultrastructural damage and viability loss of the species presently studied, may have been the outcome of a combination of factors: glycerol penetrates cells (Polge *et al.*, 1949) and may confer protection on internal organelles (reviewed by Fuller, 2004) as well as on the plasmalemma during dehydration; glycerol acts as a solvent for electrolytes that accumulate in the cytoplasm when water is removed (Finkle *et al.*, 1985; Muldrew *et al.*, 2004), limiting injury otherwise resulting from high solute concentrations (Popova and Busheva, 2001); and glycerol may confer biochemical protection, mainly by scavenging harmful free-radicals (Polge *et al.*, 1949; Smirnoff and Cumbes, 1989; reviewed by Benson and Bremner, 2004).

Ultrastructural observations provided evidence for the basis of the superior post-drying viability retention in Gly CP embryos. Gly CP+D cells across all the specimens sampled for both species (five at each WC range) showed retention of regular cell walls with contiguous plasmalemmae; normally-distributed organelles (Fig. 3.8a, d & e, f); and an absence of lysed cells. The features of cells were indicative of ongoing active metabolism (e.g. cytomatrical polysomes and Golgi bodies [Fig. 3.8b & f]) and, in contrast to the situation in Suc CP+D embryos, nuclei showed well dispersed (but somewhat condensed) heterochromatin (Fig. 3.8a & h), small plastids having regular morphology and containing some starch grains (Fig. 3.8b, c, d-insert & f), and an absence of fragmented profiles of rER (Fig. 3.8b, d & e, f). Some Gly CP+D *H. montanus* cells also showed very long profiles of rER arranged in parallel ranks (Fig. 3.8e, f) which is generally taken to be indicative of active membrane synthesis (Novikoff, 1976; Berjak and Pammenter, 2000).

Irrespective of the WC range or species, vacuoles within cells of Gly CP+D embryos were clearly defined (Fig. 3.8a, d & e, h) and often showed signs of what probably was autophagically-derived content (Fig. 3.8d & e), which may indicate that some structures were

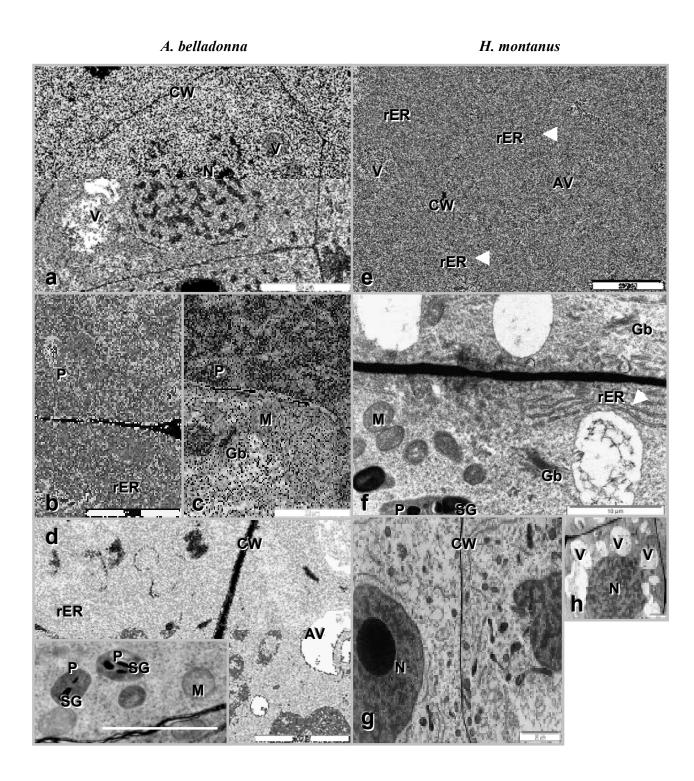
damaged during dehydration and subsequently removed autophagically (with reference to the work of Berjak *et al.*, 1989, 1990 and Wesley-Smith *et al.*, 2001a).

As in the Suc CP+D treatments, viability loss was more severe (Fig. 3.1A, B) after Gly CP+D in *H. montanus* specimens with occasional cells/small groups of cells appearing ultrastructurally compromised. As an example, Fig. 3.7g shows neighbouring cells in which the nuclear matrices are dense, while the cytomatrix shows a 'cleared' appearance suggestive of a diminution of the occurrence of polysomes (indicative of reduced protein synthesis [e.g. Farrant *et al.*, 1985]), and perhaps incipient loss of cytomatrical organisation.

Some cells of Gly CP+D *H. montanus* embryos also showed long profiles of rER arranged peripherally in parallel ranks, adjacent to vacuoles (white arrowheads; Fig. 3.8e, f). This is a common response to stress in plant cells and is regarded as indirect evidence for the synthesis of vacuolar membrane (and lytic enzymes) from specialised ER membranes (reviewed by Staehelin, 1997). Additionally, the peripheral location of profiles of rER could be a consequence of the functional role this system plays in vesicle-mediated secretion and/or membrane recycling (Craig and Staehelin, 1988).

In *A. belladonna*, the proportion of the cell occupied by vacuoles in > and <0.4D-Gly embryos was essentially similar to cells of fresh specimens; however, in cells of > and <0.4D-Gly *H. montanus* embryos, this parameter was higher than that in cells of fresh specimens (Fig. 3.1A, B; also see Fig. 3.8h). These data suggest that penetrative cryoprotectants like Gly may limit the degree of post-drying vacuolation in recalcitrant seed tissues, but that this may be negated by protracted drying times.

Root meristematic cells of glycerol cryoprotected A. belladonna [a-d] and H. montanus [e-h] zygotic embryos dried to >0.4 g g⁻¹ [a, b, c & e, f] and <0.4 g g⁻¹ [d & g, h] are illustrated. a) whole cell showing centrally positioned, spherical nucleus with dispersed, somewhat condensed chromatin, vacuoles with inclusions, and regular cell walls; b) profiles of rough endoplasmic reticulum and plastid; c) mitochondrion, plastid, Golgi body and polysomes (noting the darker aspect of the cell above); e) evidence of autophagy, regular cell walls, parallel ranks of rough endoplasmic reticulum, peripherally located and adjacent to vacuole; f) mitochondria, plastids with starch grains, Golgi bodies, polysomes and parallel, peripheral ranks of rough endoplasmic reticulum adjacent to vacuole. d) rough endoplasmic reticulum, regular cell wall, signs of autophagy; insert showing mitochondrion and plastids with dense starch grains; g) nuclei with denser matrices than in control material (see Fig. 3.2e) and organelles scattered in relatively amorphous cytoplasm which had a 'cleared' appearance; h) whole cell showing centrally positioned, spherical nucleus with dispersed chromatin and vacuoles with inclusions. N=nucleus; P=plastids; SG=starch grains; M=mitochondria; rER=rough endoplasmic reticulum; V=vacuole; Gb=Golgi body; CW=cell wall; AV=signs of autophagy. Bar=50 µm for (a); 20 µm for (b, c, d, d-insert, e, g, h); and 10 µm for (f).



Exposure to cryogenic temperatures and subsequent thawing

The extent of ultrastructural deformation exhibited by cryopreserved zygotic embryo cells often makes it difficult to account for the survival obtained but germination is believed to be more likely when cells in the primary meristem retain their capacity to divide following recovery from cryostorage (Wesley-Smith, 2003). In this regard, survival of even a small number of suitably located meristematic cells can subsequently result in organised growth (e.g. Sussex, 1952; Pritchard and Prendergast, 1986; Wilkinson *et al.*, 2003; Kaczmarczyk *et al.*, 2008). In cryopreserved plant cells, e.g. those of potato shoot tips, ultrastructural changes became apparent within 1 h after re-warming but the totality of cell death was evident only days later (Kaczmarczyk *et al.*, 2008). Wesley-Smith (2003) proposed that recovery of cryopreserved recalcitrant embryos/axes *in vitro* can be assumed to progress at three levels, *viz.* the ability of individual cells to repair damage, the recovery of a critical number of cells to allow function within each tissue, and the ability of tissues to resume interactions that facilitate growth and development.

<u>Cooling of fully hydrated (Fresh-rapid and Fresh-slow), cryoprotected (Suc-rapid and Suc-slow;</u> <u>Gly-rapid and Gly-slow) and flash-dried (>0.4D-rapid and >0.4D-slow; <0.4D-rapid and <0.4D-slow) material</u>

In the present study, almost without exception, zygotic embryos (of both species) that were cooled after cryoprotection but not flash-dried, or flash-dried without cryoprotection, generally did not survive cryogenic cooling, irrespective of whether this was at slow or rapid rates (Fig. 3.1A, B). Similarly, no survival was obtained when fresh embryos were exposed to cryogenic temperatures (Fig. 3.1A, B). Embryos from these treatments displayed total sub-cellular destruction: the cytoplasm showed typical signs of extraction, indicating rupture of the plasmalemma (Fig. 3.9 a-d & e-g). Nucleoli (Nu) from disintegrated nuclei generally persisted among the débris (Fig. 3.9d & g), besides which nothing could be discerned other than starch grains (SG; 3.9c & e). Of the numerous explanations of freezing injury, damage to sub-cellular compartments and to the plasmalemma appear to be most relevant in the current observations (e.g. Mazur, 1966; Sherman and Kim, 1967; Fujikawa, 1980; Mazur, 1984; Chandel *et al.*, 1999; Acker and McGann, 2001; Perán *et al.*, 2006; Kaczmarczyk *et al.*, 2008).

Physical damage to cellular compartments during freezing and upon recovery from cryostorage can destroy the ordered partitioning of ions, macromolecules and enzymes across selectively permeable membranes, but while all cellular compartments are presumably equally important to survival, some of these may survive freezing to a better extent than others (Wesley-

Gb

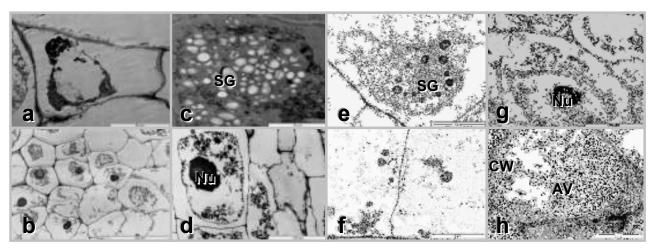
Smith, 2003). Further to this, all individuals of a particular organelle type, or cells within the same tissue, may not be similarly affected (Grout and Henshaw, 1980; Fukai, 1995; Wesley-Smith, 2003; Wilkinson *et al.*, 2003). For instance, in one of five Suc-slow *H. montanus* embryos sampled here, some cells (c. <5%) displayed intact, but irregular, cell walls (CW) with contiguous plasmalemmae and large autophagic vacuoles (Fig. 3.9h), while the remaining cells were lysed. This may explain the 10% post-thaw viability observed in this treatment for *H. montanus* (Fig. 3.1B). The limited number of apparently intact cells did not allow for a statistically robust estimation of the degree of vacuolation but Suc-slow *H. montanus* cells with intact plasmalemmae generally showed 1-2 very large vacuoles that dominated the intracellular space. Despite the 10% post-thaw viability observed in >0.4D-slow for both species, all cells, within all (five) of the other specimens sampled for this treatment showed total sub-cellular destruction (Fig. 3.9c & g).

The results described above are in agreement with other studies (e.g. Pence, 1990; Sershen et al., 2007) which have shown that zygotic germplasm from recalcitrant seeds rarely survive freezing at WCs as high as those associated with fully hydrated and undried+CP embryos in this study $(1.49-4.77 \text{ g s}^{-1})$. It is now well established that removal of most freezable water is necessary to reduce the damage caused to the cells by ice formation during freezing and thawing (Grout and Henshaw, 1980; Wesley-Smith et al., 1992; Benson, 1999). However, while the upper limit of the 'optimal hydration window' in recalcitrant zygotic germplasm is constrained by freezing injury due to intracellular ice formation at relatively high WCs, its lower limit may be constrained by desiccation sensitivity of the tissues (Becwar et al., 1983; Pritchard and Prendergast, 1986; Pence, 1992; Wesley-Smith et al., 1992; Sun, 1999). In this regard, results obtained here and elsewhere suggest that excessive partial dehydration can disrupt intracellular and intranuclear spatial organisation and induce an abnormally high degree of vacuolation (e.g. Figs 3.5 & 3.6) (Berjak et al., 1989, 1990, 1999; Mycock et al., 2000; Wesley-Smith et al., 2001a; Kioko et al., 2006). Such damage may be exacerbated by freezing (Berjak et al., 1999; Mycock, 1999) and probably precluded the successful cryopreservation of non-CP+D embryos in this study.

Root meristem cells of non-cryoprotected and undried treatments that resulted in little, or no, post-thaw viability for *A. belladonna* [a-d] and *H. montanus* [e-h] zygotic embryos are illustrated. a) <0.4D-slow: lysed cell; b) Suc-rapid: lysed cells; c) <0.4D-slow: starch grains amongst cellular débris; d) Gly-rapid: disintegrated nucleolus within plasmolysed cell; e) <0.4D-rapid: starch grains amongst cellular débris; f) >0.4D-rapid: lysed cells; g) Gly-rapid: nucleolus from disintegrated nucleus within completely deteriorated cell; h) Suc-slow: one of the isolated cells that showed some cytomatrical organisation, irregular cell walls and indications of possible autophagy. SG=starch grains; Nu=nucleolus; CW=cell wall; AV=signs of autophagy. Bar=50 µm for (a, c); 200µm for (b); 100µm for (d, g); 5 µm for (e, f); 2 µm for (h).

A. belladonna

H. montanus



<u>Cooling after cryoprotection + flash drying (>0.4D-Suc-rapid and >0.4D-Suc-slow; <0.4D-Suc-rapid and <0.4D-Suc-slow; >0.4D-Gly-rapid and >0.4D-Gly-slow; <0.4D-Gly-rapid and <0.4D-Gly-slow; <0.4D-Gl</u>

a) Treatments that resulted in low post-thaw viability retention.

Of the five CP+D treatments that enabled post-thaw viability retention for some *H. montanus* embryos, two involved Suc CP+D embryos (namely, < and >0.4D-Suc-rapid) and resulted in 10-20% viability retention while three involving Gly CP+D embryos (namely, >0.4D-Gly-slow, <0.4D-Gly-rapid and >0.4D-Gly-rapid) yielded post-thaw viabilities of 20-50% (Fig. 3.1B). Several cryoprotectant treatments have been applied to recalcitrant zygotic germplasm (alone and in combination with dehydration) with variable success (e.g. de Boucaud *et al.*, 1991, 1996; Pence, 1991; Assy-Bah and Engelmann, 1992a & b; Kioko *et al.*, 1998; Thammasiri, 1999; Walters *et al.*, 2002a; Sershen *et al.* 2007). The results obtained here lend support to other suggestions that cryoprotection can improve post-thaw survival in zygotic germplasm of non-orthodox-seeded species (Engelmann, 1997; Walters *et al.*, 2002a; Normah and Makeen, 2008; Walters *et al.* 2008).

However, cryoprotection and prolonged periods of dehydration, though not lethal in themselves, can act synergistically and lethally with freezing stress (Berjak *et al.*, 1999; Mycock, 1999; Wilkinson *et al.*, 2003). In the present study, Suc CP+D was accompanied by a considerable degree of vacuolation and ultrastructural perturbation (see Fig. 3.7), and when followed by freezing and subsequent thawing, just one of the five Suc CP+D-rapid *H. montanus* embryos sampled (at each WC range) showed a few non-lysed cells having some evidence of cytomatrical organisation (Fig. 3.10a, b). Organelles could be discerned within the cytomatrix of non-lysed Suc CP+D-rapid *H. montanus* cells but nuclei (N) (presumably out of the plane of the

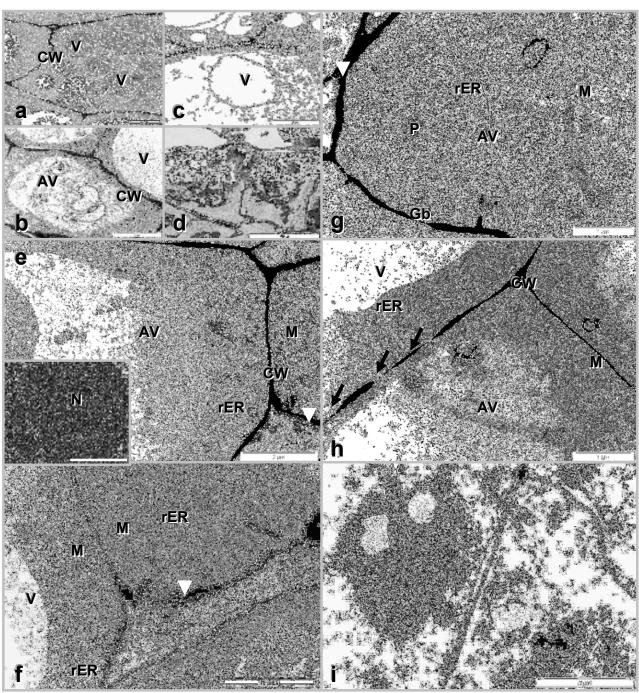
section), rough endoplasmic reticulum (rER) and Golgi bodies (Gb) were not visualised (Fig. 3.10a, b). These cells were also highly vacuolated (V) (Fig. 3.10a, b), with the proportion of the area occupied by vacuoles being the highest across all treatments (Fig. 3.1B). All cells in the remaining four Suc CP+D-rapid *H. montanus* specimens (Fig. 3.10d), and all Suc CP+D+slowly cooled *H. montanus* and Suc CP+D+cooled (slow and rapid) *A. belladonna* specimens, were lysed. Loss of sub-cellular organisation and organellar integrity is often observed during *in vitro* recovery of recalcitrant zygotic germplasm retrieved from cryostorage (e.g. Berjak *et al.*, 1999; Perán *et al.*, 2006) while increased vacuolar sensitivity to osmotic expansion is a common post-drying (e.g. Wesley-Smith *et al.*, 2001a) and post-freezing (e.g. Murai and Yoshida, 1998) response in plant tissues (probably due to increased vacuolar solute concentrations). Suc CP+D-rapid *H. montanus* embryos also showed considerable evidence of autophagy (AV; Fig. 3.10b), which if operationally effective, may have provided a mechanism for the removal of damaged structures and the re-establishment of cellular homeostasis (e.g. Berjak *et al.*, 1989, 1990; Wesley-Smith *et al.*, 2001a).

Consistent with the 20% post-thaw viability recorded for >0.4D-Gly-slow and <0.4D-Glyrapid *H. montanus* embryos, a few of the specimens sampled from these treatments (two of five for >0.4D-Gly-slow and one of five for <0.4D-Gly-rapid) showed a similar general ultrastructure to fresh embryos: irregularly shaped nuclei (N; Fig. 3.10e-insert) with well dispersed heterochromatin; regular cell walls (CW; Fig. 3.10e & h) with contiguous plasmalemmae (but portions of some of these cell walls were irregular [black arrows; Fig. 3.10h]); and signs of ongoing active metabolism such as, long profiles of rER (Fig. 3.10e, f & g, h), mitochondria (Fig. 3.10e, f & g, h), Golgi bodies and plastids (Fig. 3.10g), and cytomatrical polysomes (Fig. 3.10f & g). The proportion of the area occupied by vacuoles within these cells was also not as high as in those of Suc CP+D-rapid embryos, while the number of vacuoles was relatively lower than fresh embryos (Fig. 3.1B). All that can be suggested at this stage, is that the vacuoles in cells of Suc CP+D embryos may fuse and contract during cooling and/or upon retrieval from cryostorage, so reducing their number and volume during in vitro recovery. There were also signs of autophagy within these cells (AV; Fig. 3.10e & g, h), possibly contributing to the re-establishment of cellular homeostasis (Berjak et al., 1989, 1990; Wesley-Smith et al., 2001a). The cells within >0.4D-Gly-slow and <0.4D-Gly-rapid embryos that showed intracellular organisation were, however, frequently contiguous with lysed cells (white arrowheads; Fig. 3.10e, f & g). Also, all the cells in the remaining H. montanus >0.4D-Gly-slow and <0.4D-Gly-rapid specimens examined were extensively deteriorated (Fig. 3.10i), which is in keeping with viability loss of 80% of the embryos after retrieval from cryostorage (Fig. 3.1B).

Figure 3.10

Root meristem cells of treatments that resulted in low post-thaw viabilities for *H. montanus* zygotic embryos are illustrated. (a, b) >0.4D-Suc-rapid: a) highly vacuolated cells showing irregular cell walls; and b) large autophagic vacuole and irregular cell wall. (c, d) <0.4D-Suc-rapid: c) highly vacuolated cell; and d) lysed cells. (e, f) >0.4D-Gly-slow: e) signs of autophagy, long scattered profiles of rough endoplasmic reticulum, cell wall with regular profile, mitochondria; note adjacent lysed cells; insert showing irregularly shaped nucleus with dispersed heterochromatin; and f) organised cell with long profiles of rough endoplasmic reticulum, mitochondria, polysomes and adjacent autolysed cell. (g, h, i) <0.4D-Gly-rapid: g) long profiles of rough endoplasmic reticulum, signs of autophagy, Golgi body, mitochondria, polysomes, plastids; note adjacent lysed cell (mid-left); h) signs of autophagy, long profiles of rough endoplasmic reticulum, signs of autophagy, long profiles of rough endoplasmic reticulum, signs of autophagy, Golgi body, mitochondria, polysomes, plastids; note adjacent lysed cell (mid-left); h) signs of autophagy, long profiles of rough endoplasmic reticulum, mitochondria and irregular portion of cell wall (black arrows); and i) a region of extensively deteriorated cells. N=nucleus; M=mitochondria; rER=rough endoplasmic reticulum; V=vacuole; Gb=Golgi body; CW=cell wall; AV=signs of autophagy; white arrow heads=lysed cell. Bar=2 µm for (a, b, c, e, e-insert, f, i); 100 µm for (d); and 1 µm for (g, h).

H. montanus



Of the four treatments that resulted in some post-thaw viability retention in A. belladonna, all involved Gly CP (Fig. 3.1A). Of these treatments viability was lowest (10%) when Gly CP embryos were rapidly cooled at WC >0.4 g g⁻¹ and relatively higher (40%) when they were cooled slowly at WC <0.4 g g⁻¹ (Fig. 3.1A). Cells within the majority of >0.4D-Gly-slow A. belladonna embryos were lysed (Fig. 3.11c) but cells within two of the embryos examined showed spherical nuclei with dispersed heterochromatin and signs of ongoing active metabolism evidenced by plastids with starch grains, long profiles of rER, mitochondria and Golgi bodies (Fig. 3.11a). Also, despite the high viability loss associated with >0.4D-Gly-slow A. belladonna embryos, in intact cells of >0.4D-Gly-slow A. belladonna embryos the proportion of the area occupied by vacuoles, as well as the number of vacuoles was comparable to that measured for cells within fresh embryos (Fig. 3.1A). However, cell walls were irregular (Fig. 3.11a), having a 'washed-out' appearance. Additionally, vacuoles within these cells were poorly defined and showed signs of tonoplast dissolution (white arrowheads; Fig. 3.11a-insert). In one of the five >0.4D-Gly-rapid A. belladonna embryos sampled, some cells showed disintegrated organelles, no visible vacuoles, highly irregular cell walls and an abundance of starch grains within plastids aggregated around the nucleus, which is indicative of cytoskeletal collapse (Berjak *et al.*, 1999) (Fig. 3.11b). All cells in the remaining four specimens for this treatment were lysed (not shown).

Consistent with the 40% post-thaw viability observed for <0.4D-Gly-slow *A. belladonna* embryos, two of the five embryos sampled for this treatment showed non-lysed cells, within which organelles could be discerned (Fig. 3.11d), but all cells in the remaining three specimens were lysed (not shown). Organelles that could be discerned within non-lysed <0.4D-Gly-slow *A. belladonna* cells included: plastids with starch grains; nuclei with condensed chromatin (Fig. 3.11d); and long profiles of rER (Fig. 3.11e, e-insert). Apart from these signs of metabolic potential, the proportion of the area occupied by vacuoles in <0.4D-Gly-slow *A. belladonna* cells, as well as the number of vacuoles per cell, was comparable to fresh embryos (Fig. 3.1A). This was possibly a consequence of dehydration of *A. belladonna* embryos with prior Gly CP, which may have limited the extent of vacuolation, compared with dehydration after no, or after Suc CP (Fig. 31.A). These data also suggest that the degree of vacuolation is not the over-riding determinant of post-thaw survival, at least in *A. belladonna* zygotic embryos.

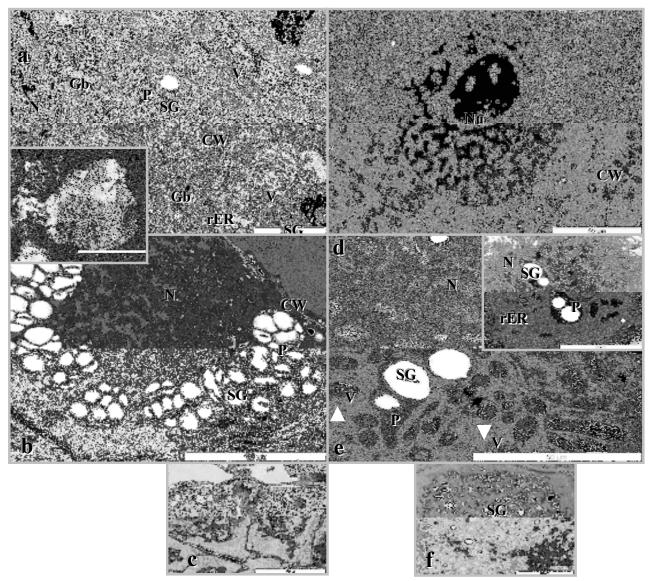
However, vacuoles within <0.4D-Gly-slow *A. belladonna* cells were poorly defined and some showed signs of localised tonoplast dissolution (white arrowheads; Fig. 3.12e). Other ultrastructural irregularities observed in <0.4D-Gly-slow *A. belladonna* cells included: irregular cell walls (Fig. 3.11d); spherical nuclei in some cells, within which chromatin was condensed (Fig. 3.12d); and the presence of lysed cells (Fig. 3.11f) amongst ultrastructurally intact ones.

Tonoplast dissolution, the signs of which were observed in cooled >0.4D-Gly and slowly cooled <0.4D-Gly *A. belladonna* embryos in this study, is a phenomenon that has been previously reported to occur in frozen-thawed recalcitrant embryonic axes (e.g. those of *Acer saccharinum* [Wesley-Smith, 2003]), and can be lethal, as shown in non-acclimated freeze-dried Jerusalem artichoke protoplasts (Murai and Yoshida, 1998). Tonoplast dissolution results in the leakage of vacuolar contents into the cytoplasm, which generally precedes autolysis. As at least a proportion of vacuoles are lytic compartments (Pitt and Stewart, 1981; reviewed by Marty, 1999), leakage of hydrolytic enzymes normally sequestered within vacuoles is rapidly fatal to cells in which this occurs. Tonoplast dissolution is therefore generally regarded as the beginning of the end and the amorphous appearance of the cytomatrix in some <0.4D-Gly-slow (Fig. 3.11e, e-insert) and >0.4D-Gly-rapid (Fig. 3.11b) *A. belladonna* cells, together with the perinuclear arrangement of plastids (Fig. 3.11b), essentially implies intracellular collapse.

Figure 3.11

Root meristem cells of treatments that resulted in low post-thaw viabilities for A. belladonna zygotic embryos are illustrated. >0.4D-Gly-slow: a) irregular 'washed-out' cell wall, Golgi bodies, plastid with starch grain, a few profiles of rough endoplasmic reticulum, nucleus with relatively condensed chromatin, poorly defined vacuoles, and insert showing vacuoles with signs of tonoplast dissolution (white arrowheads). >0.4D-Gly-rapid: b) whole cell showing disintegrated organelles, except for distended plastids containing many starch grains in close proximity to the nucleus, relatively amorphous cytoplasm and irregular cell walls; and c) extensively deteriorated cells. <0.4D-Gly-slow: d) whole cell showing spherical nucleus with condensed chromatin, discernible organelles and irregular, 'washed-out' cell wall; e) irregularly shaped lobe of nucleus, plastids with starch grains, and vacuole showing signs of tonoplast dissolution; e-insert) long profiles of rough endoplasmic reticulum, irregularly shaped nuclear lobe and plastids with starch grains; and f) lysed cell showing cellular débris and starch grains. N=nucleus; Nu=nucleolus; P=plastid; SG=starch grains; M=mitochondria; rER=rough endoplasmic reticulum; V=vacuole; Gb=Golgi body; CW=cell wall; white arrowheads=signs of tonoplast dissolution. Bar=20 μm for (a); 0.5 μm for (a-insert); 100 μm for (b, c); and 50 μm for (d, e, f).

A. belladonna



b) Treatments that resulted in the best post-thaw viability retention.

Post-thaw viability for *H. montanus* embryos was best (50%) when Gly CP embryos were rapidly cooled at WC >0.4 g g⁻¹ (Fig. 3.1B). The cells of three of five *H. montanus* embryos sampled for this treatment showed irregularly shaped nuclei (Fig. 3.12c), intact cell walls (Fig. 3.12a, d) with contiguous plasmalemmae, and signs of intracellular organisation and organellar integrity (Fig. 3.12a, b, d, e). The metabolic potential of these cells was also evidenced by the presence of cytomatrical polysomes (Fig. 3.12b, e), long and shorter profiles of rER (Fig. 3.12a, b, e); plastids (Fig. 3.12a, b, e), some containing starch grains (Fig. 3.12e); mitochondria (Fig. 3.12d); and Golgi bodies (Fig. 3.12a, e). There was also evidence of autophagic activity having occurred in >0.4D-Gly-rapid *H. montanus* cells (Fig. 3.12b, e). However, tonoplast dissolution was also seen in some otherwise highly organised cells (black arrow; Fig. 3.12d, e), which would have presumably led to autolysis as shown in Fig. 3.12f. The proportion of the area occupied by vacuoles (Fig. 3.12a, b) within these cells was significantly greater than in cells of fresh material, but significantly lower than in all the other cooling treatments after which post-thaw viability retention was observed for *H. montanus* (Fig. 3.1B).

There is no doubt that Gly CP promoted post-thaw viability retention in partially dried H. *montanus* embryos, which was best when drying time (i.e. the duration of the dehydration stress) and the proportion of the area occupied by vacuoles were minimised, as in >0.4D-Gly-rapid H. *montanus* embryos (Fig. 3.1B). Dehydration damage can be exacerbated by freezing (Berjak *et al.*, 1999; Mycock, 1999). In the present study Gly CP followed by flash drying may have rendered H. *montanus* cells less susceptible to vacuolation during the recovery phase following cryogen exposure by limiting the extent of post-drying vacuolation preceding freezing. As indicated above, however, some cells showed signs of tonoplast dissolution, probably indicative of freezing damage (Murai and Yoshida, 1998; Wesley-Smith, 2003).

The endoplasmic reticulum plays a pivotal role during dehydration (e.g. Bergfeld and Schopfer, 1984; Wesley-Smith *et al.*, 2001a) and the dynamic and varied response of this membrane system to stress has been highlighted by conformational changes that occur during equilibrium freezing in parenchyma cells of cold-acclimated mulberry (Fujikawa and Takabe, 1996). The endoplasmic reticulum system is also involved in the formation of vacuoles via several routes (Matile and Moor, 1968; Lamb and Berjak, 1981; Staehelin, 1997; Berjak and Pammenter, 2000) and in stressed cells, these ER-derived lytic organelles are often observed to be involved in autophagy of cellular components – an event that has been suggested to be an important survival mechanism (reviewed by Staehelin, 1997; Marty, 1999). There was no direct evidence of vacuolation via ER vesiculation within the cells of >0.4D-Gly-rapid *H. montanus* embryos, but isolated cells within the >0.4D-Gly-rapid embryos showed long profiles of rER

arranged in parallel ranks adjacent to large vacuoles (Fig. 3.12a, b), which is a common response to stress in plant cells and is regarded as indirect evidence for the specialisation of ER membranes as vacuolar membranes (reviewed by Staehelin, 1997). Considering the present evidence for vacuolation via cytolysome formation (e.g. after Gly CP), the ranked ER could well represent a prelude to cytolysome formation, perhaps encompassing pre-existing vacuoles.

As in the cells of fresh and Gly CP embryos, it was not possible to tell whether the plasmalemma-associated vesiculation observed in >0.4D-Gly-rapid *H. montanus* cells (encircled; Fig. 3.12d) were indicative of endo- or exocytosis; however, importation of substances from cotyledons is typical of germinating axes, making endocytosis the more probable phenomenon. As in all the *H. montanus* treatments in which post-thaw viability was recorded, some lysed cells were present amongst ultrastructurally intact ones in >0.4D-Gly-rapid *H. montanus* embryos (white arrowheads; Fig. 3.12f). In contrast, in embryos representative of the 50% of non-survivors, all cells were lysed (not shown).

For *A. belladonna*, post-thaw viability was best (70%) when Gly CP embryos were rapidly cooled at WCs <0.4 g g⁻¹ (Fig. 3.1A). Cells within four of the five specimens sampled for this treatment showed irregularly shaped lobed nuclei (Fig. 3.13a), intact cell walls with contiguous plasmalemmae, and clear signs of intracellular organisation and organellar integrity (Fig. 3.13a). The metabolic potential of these cells was evidenced by the presence of long profiles of rER (Fig. 3.13c, d), plastids containing many lightly-contrasted starch grains (Fig. 3.13c), cytomatrical polysomes (Fig. 3.13e), mitochondria and Golgi bodies (Fig. 3.13b). The proportion of the area occupied by vacuoles in cells of <0.4D-Gly-rapid embryos (Fig. 3.13a) was comparable with fresh embryos and the other Gly CP+D treatments in which relatively lower post-thaw viabilities were recorded, but the number of vacuoles per cell was significantly higher than fresh embryos (Fig. 3.1A). For *A. belladonna* embryos, Gly CP promoted post-thaw viability retention in partially dried embryos, which was maximised when embryo WC (i.e. potential for damaging ice-crystal formation) and the proportion of the area occupied by vacuoles were low, as in <0.4D-Gly-rapid (Fig. 3.1A).

Vacuoles in <0.4D-Gly-rapid *A. belladonna* cells showed signs of what could have been autophagically-derived content (Fig. 3.13b) but unlike the cells of >0.4D-Gly-rapid *H. montanus* embryos, tonoplast dissolution was rarely observed. This could have been the basis of the better post-thaw viability retention for *A. belladonna* than that for *H. montanus* (Fig. 3.1A, B) However, despite the relatively high post-thaw viability retention associated with <0.4D-Gly-rapid *A. belladonna* embryos, cells within these embryos did show a number of ultrastructural irregularities: irregular ('washed-out') cell walls (Fig. 3.13c); amorphous areas of cytoplasm in some cells (black arrows; Fig. 3.13c, d), which suggest initiation of deterioration

in the cells concerned, perhaps presaging their lysis (although regional differentiation of the cytoplasm [Lamb and Berjak, 1981] cannot be precluded); the presence of a few (<5%) lysed cells (white arrowhead; Fig. 3.13f); and highly attenuated plastids in a few cells (Fig. 3.13e). Also, in one of the five <0.4D-Gly-rapid *A. belladonna* embryos sampled, all cells were lysed (not shown), which is in keeping with the 30% of non-surviving embryos (Fig. 3.1A).

Figure 3.12

Root meristem cells following the treatment that resulted in the best post-thaw viability for *H. montanus* zygotic embryos, *viz.* >0.4D-Gly-rapid, are illustrated. a) cell wall with contiguous plasmalemma, cytomatrical polysomes, Golgi body, plastids with little starch apparent, vacuoles, long profiles of rough endoplasmic reticulum ranked adjacent to vacuole; b) signs of autophagy, shortish (slightly distended) rough endoplasmic reticulum ranked near a large vacuole and plastid with plastoglobuli; c) irregularly shaped nuclear lobe with heterochromatin; d) mitochondria, irregular cell wall, vacuoles showing signs of tonoplast dissolution and apparent vesiculation of the plasmalemma (encircled); e) Golgi body, autophagic vacuoles showing signs of tonoplast dissolution, plastid with starch grain and short profiles of rough endoplasmic reticulum; and f) lysed cell flanked by ultrastructurally intact cells. N=nucleus; P=plastid; SG=starch grains; pg=plastoglobuli; M=mitochondria; rER=rough endoplasmic reticulum; V=vacuole; Gb=Golgi body; CW=cell wall; black arrows=signs of tonoplast dissolution; white arrowheads=plasmolysed cell. Bar=1 µm for (a, b, c, e, f); and 0.5 µm for (d).

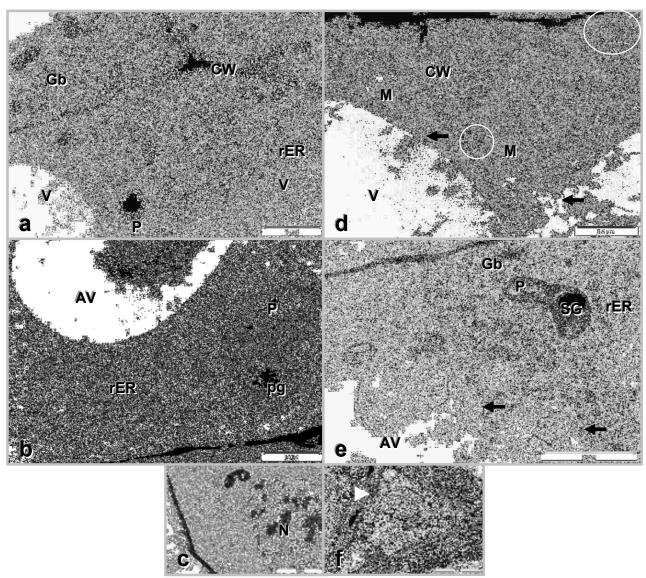
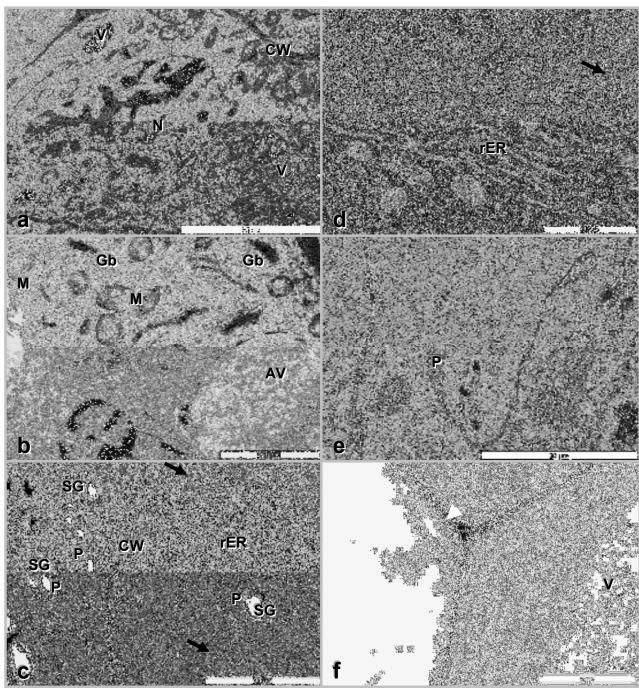


Figure 3.13

Root meristem cells following the treatment that resulted in the best post-thaw viability for *A. belladonna* zygotic embryos, *viz.* <0.4D-Gly-rapid, are illustrated. a) whole cell showing intact cell wall with contiguous plasmalemma, vacuoles, irregularly shaped, lobed nucleus with heterochromatin, and normally-distributed organelles; (b) mitochondria, Golgi bodies and vacuoles showing what could be autophagically-derived, hydrolysed flocculent content; (c) plastids containing many, lightly-contrasted starch grains, irregular cell wall, long profiles of rough endoplasmic reticulum and regions of relatively non-granular cytomatrix (black arrows); note the 'washed-out' appearance of the wall between the two cells to the right; d) long profiles of rough endoplasmic reticulum and a relatively amorphous region of cytomatrix; e) highly attenuated plastid; and f) vacuole within ultrastructurally intact cell, adjacent to lysed cell (left). N=nucleus; P=plastid; SG=starch grains; M=mitochondria; rER=rough endoplasmic reticulum; V=vacuole; Gb=Golgi body; CW=cell wall; black arrows=signs of tonoplast dissolution; white arrowhead=lysed cell. Bar=1 µm for (a, b, c, e, f); and 0.5 µm for (d).



3.4 Concluding remarks

The ultrastructural responses of recalcitrant *A. belladonna* and *H. montanus* zygotic embryos to cryoprotection, partial dehydration and thawing after cryogenic exposure were investigated. Untreated (fresh) embryos (of both species) exhibited 100% viability and ultrastructurally their cells showed a centrally positioned, irregularly shaped nucleus (with dispersed chromatin), regular cell walls with contiguous plasmalemmae, a few, small vacuoles, each with intact tonoplast, and signs of ongoing active metabolism, e.g. polysomes, Golgi bodies, mitochondria and rough endoplasmic reticulum. Some viability loss accompanied cryoprotection (CP), partial dehydration, thawing after cryogenic exposure, and combinations of these procedures for both species, being most severe after thawing. For both species, viability loss after pre-conditioning and upon thawing was generally accompanied by some degree of ultrastructural derangement, increased vacuolation, tonoplast dissolution, and/or total subcellular destruction, in some or all cells. While Gly CP alleviated post-drying ultrastructural derangement and viability loss in both species, sucrose CP exacerbated these adverse effects of partial drying. For both species, freeze-thaw-induced ultrastructural irregularities were least conspicuous in rapidly cooled, Gly CP embryos, which also survived cryopreservation best.

The better retention of viability associated with Gly CP+D, as opposed to Suc CP+D, may be based on the penetrative ability of glycerol, which is held to bring about an increase in cytoplasmic viscosity but a decrease in the efflux of water from the cytoplasm during cooling (Polge *et al.*, 1949; Benson, 2008), i.e. limiting freeze-dehydration. The cooling rate required to prevent the formation of ice-crystals and the mobility of water are closely related (Luyet *et al.*, 1962; Rall, 1987) and increasing intracellular cytoplasmic viscosity limits ice-crystallisation, and hence promotes post-thaw survival in recalcitrant embryonic axes (as shown for *Poncirus trifoliata* [Wesley-Smith *et al.*, 2004a]). The ability of penetrating cryoprotectants to reduce the magnitude of freezing injury by lowering the freezing point of cell solution by increasing intracellular osmolality prior to freezing; decreasing the temperature at which ice nucleation occurs; and increasing the temperature at which water is transformed into glass, is well documented (Finkle *et al.*, 1985; Bronshteyn and Steponkus, 1995; Gusta *et al.*, 2004; Muldrew *et al.*, 2004).

However, the incidence of ultrastructural irregularities in Gly CP+D+cooled embryos, even within treatments in which post-thaw viability retention were relatively good, suggests a further factor: i.e. the protective benefits of Gly may also have been based on the limitation of metabolic lesions. In this regard, damage by dehydration (Pammenter *et al.*, 2000; Roach *et al.*, 2008; Walters *et al.*, 2002b, 2008) and freezing (Touchell and Walters, 2000; Dussert *et al.*, 2003; Normah and Makeen, 2008; Varghese and Naithani, 2008; Pammenter *et al.*, 2010) in

recalcitrant seed tissues is thought to be largely free-radical-mediated. Radioprotectants like Gly

have been suggested to confer biochemical protection during cryopreservation, mainly by scavenging harmful free-radicals (Polge *et al.*, 1949; Smirnoff and Cumbes, 1989; Benson and Bremner, 2004). The possibility that Gly CP promoted post-thaw viability retention in partially dried *A. belladonna* and *H. montanus* embryos, at least partly, by alleviating the oxidative stress associated with cryopreservation, is explored in studies described in Chapter 4.

Even though dehydration and cryoprotection may facilitate successful cryopreservation of plant germplasm, post-thaw survival is critically related to the rate of cooling (Meryman and William, 1985; Wesley-Smith 2001b, 2004a; reviewed by Walters et al., 2008). During cooling at $\geq 100^{\circ}$ C min⁻¹, exosmosis is likely to occur at a rate much slower than the rate of formation of extracellular ice-crystals. As a consequence, the cytoplasm becomes increasingly supercooled, pre-disposing the cells to intracellular ice-crystal formation and hence the potential for mechanical shearing of cell membranes (Acker and Croteau, 2004). However, if samples are cooled at rates of $\geq 1000^{\circ}$ C min⁻¹, the ice-crystals that form may be very small and therefore relatively innocuous (Muldrew et al., 2004). Rapid cooling rates have been reported to hinder ice nucleation in hydrated seed tissues (Wesley-Smith et al., 1992, 2004b; reviewed by Walters et al., 2008) facilitating successful cryopreservation of the zygotic germplasm of a number of recalcitrant-seeded woody dicot species at relatively high WCs (e.g. Camellia sinensis [Wesley-Smith et al., 1992]; Aesculus hippocastanum [Wesley-Smith et al. 2001b]; Ouercus suber and *Quercus ilex* [González-Benito *et al.*, 2002]; *Poncirus trifoliata* [Wesley-Smith *et al.*, 2004a]; Ekebergia capensis [Perán et al., 2006]). The rapid non-equilibrium cooling method employed here facilitated cooling rates that permitted the maintenance of ultrastructural integrity in a sufficient number of cells to account for the high post-thaw viabilities observed in >0.4D-Glyrapid *H. montanus* and <0.4D-Gly-rapid *A. belladonna* embryos.

The relatively poorer retention of post-thaw cytomatrical organisation, organellar integrity and viability in slowly cooled Gly CP+D embryos in this study may be related to the fact that while slow cooling rates encourage the formation of a few, large extracellular ice-crystals (Mazur, 1990; Kartha and Engelmann, 1994), the freeze-induced dehydration associated with slow cooling can be too intense. Such freeze-dehydration could give rise to a variety of deleterious effects, *viz.* solute toxicity, osmotic contraction and plasmolysis, and even cell lysis (Mazur, 1990; Pritchard *et al.*, 1995; Muldrew *et al.*, 2004). In fact, there are suggestions that while slow cooling methods may retain the integrity of individual cells, they may be less efficient at retaining the tissue integrity necessary for the survival of complex tissues (e.g. meristems and embryos [Panis and Lambardi, 2006]).

It is difficult to separate the effects of cooling rate and WC on axis/embryo survival following non-equilibrium cooling, since these variables are co-dependent (Wesley-Smith et al., 2004a). In general though, with increasing hydration the greater mobility of water demands that the axes/embryos be cooled correspondingly faster, if lethal ice-crystallisation is to be avoided (Wesley-Smith et al., 1992, 2001b). This suggests that more rapid cooling rates may have allowed for the improved retention of ultrastructural integrity and associated viability in >0.4D-Gly A. belladonna and H. montanus embryos. Slow cooling can lead to intracellular icecrystallisation at higher WCs (Franks, 1985), and is therefore most successful when explants are dehydrated to (or close to) non-freezable WCs (e.g. González-Benito et al. 2002). However, it is only at WCs ≤ 0.2 g g⁻¹ that all the water within recalcitrant seed tissue is believed to be nonfreezable (Pritchard and Prendergast, 1986; Pammenter et al., 1991; Berjak et al., 1993) and embryos/axes of recalcitrant seeds will seldom survive dehydration below this level, at least in terms of organised growth (Pammenter et al., 1993). Amaryllis belladonna and H. montanus embryos in this study were subjected to slow cooling at WCs considerably higher than 0.2 g g⁻¹, which may explain the greater degree of ultrastructural damage and lower post-thaw viability retention associated with slowly cooled specimens of both species, even after Gly CP and flash drying.

In summary, the results obtained here complement those described in Chapter 2, in showing that intracellular and apoplastic damage, induced at each stage of the cryopreservation protocol may be compounded, thus pre-disposing tissues to further damage and ultimately viability loss with each progressive step. Cryoprotection and dehydration increased the chances of post-thaw survival in these amaryllid embryos but the practical benefits appear to have been realised only when damage to the cells was minimised. For both species this was best achieved when: (a) pre-conditioning involved the combination of cryoprotection and partial dehydration; (b) the cryoprotectant was penetrating (glycerol), as opposed to non-penetrating (sucrose); and (c) embryos were rapidly cooled at WCs that minimised dehydration and freezing damage.

The loss of cytomatrical organisation, organellar integrity and/or simply the induction and retention of certain ultrastructural irregularities within the meristematic cells of recalcitrant embryos/axes during processing for cryopreservation may underlie the abnormal growth often recorded after retrieval of cryopreserved recalcitrant zygotic germplasm from cryogenic conditions (e.g. Pence, 1992; Dumet *et al.*, 1997; Sershen *et al.*, 2007; Steinmacher *et al.*, 2007). Results of studies reported in Chapter 2 (see section 2.3) showed seedlings recovered from cryopreserved embryos of *A. belladonna* and *H. montanus* to be less vigorous than those from fresh embryos. The potential consequences of the ultrastructural damage and irregularities induced within the meristematic cells of recalcitrant *A. belladonna* and *H. montanus* embryos

during cryopreservation, on subsequent *ex vitro* seedling growth, are reported in Chapters 5 and 6.

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CHAPTER FOUR:

Ability of *Amaryllis belladonna* and *Haemanthus montanus* zygotic embryos to tolerate cryopreservation in relation to oxidative stress

Abstract

Oxidative stress is a major component of cryoinjury in plant tissues. The present study investigated the ability of Amaryllis belladonna (L.) and Haemanthus montanus (Baker) zygotic embryos to tolerate the various components of cryopreservation (i.e. short-term hydrated storage; cryoprotection [CP]; partial dehydration [D]; and freeze-thawing) as related to changes in extracellular superoxide (O_2) production and lipid peroxidation. The study also investigated whether glycerol (Gly) CP promoted post-thaw viability retention in H. montanus and A. belladonna embryos by protecting enzymic antioxidant activities. Short-term hydrated storage of whole seeds was accompanied by O_2^- production and lipid peroxidation but O_2 levels were lower than D and cooled embryos and viability was 100%, possibly associated with the high activities of certain antioxidant enzymes. Partial dehydration and CP (in H. montanus only) increased O_2 production (especially in CP+D embryos) and was associated with some viability loss, but this was not correlated with enhanced lipid peroxidation. Partial dehydration was always accompanied by some viability loss but this was not correlated with enhanced lipid peroxidation. Enzymic antioxidant activities often declined relative to fresh embryos after D but this decline was consistently less severe in Gly CP, as opposed to non-CP, embryos. Post-drying activities of certain antioxidant enzymes were even enhanced relative to fresh embryos in some Gly CP+D treatments. Cooling generally led to the greatest increase in O_2 production, and decline in viability. Post-thaw lipid peroxidation levels were generally higher than fresh and pre-conditioned embryos, but in A. belladonna only. Partial dehydration and cooling decreased enzymic antioxidant activities but this decrease was consistently less severe in Gly CP+D, as opposed to non-CP+D, embryos. Oxidative stress was a major component of cryoinjury in the embryos investigated here. Post-thaw viability retention in Gly CP+D embryos was significantly higher than non-CP+D embryos, possibly associated with the relatively lower post-drying lipid peroxidation levels and relatively higher post-drying and postthawing enzymic antioxidant activities in the former. Pre-conditioning treatments, such as Gly CP, may enhance post-thaw viability in recalcitrant zygotic embryos by providing/protecting ROS scavenging agents during pre-conditioning for, and after retrieval from, cryostorage.

4.1 Introduction

While there is some understanding of the physical factors (e.g. ice formation and intracellular dehydration) associated with freezing sensitivity of recalcitrant seeds (see Wesley-Smith *et al.*,

1992, 2001, 2004; Walters *et al.*, 2008), there is a paucity of information on the physiological and biochemical basis of post-thaw survival, and death, in such seeds. In this regard, the role of oxidative stress metabolism in determining post-thaw survival is of particular interest since oxidative stress has been identified as a major component of chilling and cryoinjury in plant tissues (Tapell, 1966; Levitt, 1980; Steponkus, 1985; Benson and Withers, 1987; Benson, 1990; Benson *et al.*, 1992, 1995; Prasad *et al.*, 1994; Doke 1997; Park *et al.*, 1998; Fleck *et al.*, 1999, 2000, 2003; Day *et al.*, 1998; Johnston *et al.*, 2007). Any assessment of oxidative stress in cryopreserved germplasm should consider the two key factors of dehydration (osmotic and evaporative) and ice formation in freezing injury (for reviews see Levitt, 1980; Benson, 2008), as both can have effects on the ability of a system to participate in free-radical chemistry (Benson and Bremner, 2004).

In cryopreservation studies on desiccation-sensitive germplasm such as recalcitrant seeds, embryonic axes and zygotic embryos, for which partial dehydration is now a standard pretreatment (for reviews see Walters et al., 2008; Engelmann, 2009), the effects of dehydration on oxidative stress metabolism are of particular interest. When water is removed from cells damage sensu stricto results from mechanical stresses that perturb organelle structures at high (>-5 MPa) moisture levels (reviewed by Levitt, 1980) or macromolecule structures at slightly lower levels (reviewed by Wolfe and Bryant, 1999). However, when metabolically active cells are dehydrated to intermediate moisture levels these cells may continue to respire but may be incapable of scavenging toxic metabolic by-products that accumulate (Leprince et al., 1990, 2000; reviewed by Hand and Hardewig, 1996; Leprince and Hoekstra, 1998) and cause freeradical-associated damage (McKersie et al., 1988; Hendry et al., 1992; Finch-Savage et al., 1996). Dehydration of recalcitrant seeds to water contents (WCs) above their upper limit of desiccation sensitivity (generally taken to be ~ 0.25 g g⁻¹ [Vertucci and Farrant, 1995; Wesley-Smith et al., 2004]) has been suggested to result in deleterious aqueous-based reactions that lead to what is referred to as 'metabolism-derived damage' (Pammenter et al., 1998, 2000; Walters et al., 2001). This damage is thought to be largely free-radical-associated (Pammenter et al., 2000; Walters et al., 2002, 2008). For successful cryopreservation recalcitrant zygotic germplasm generally need to be dried to WCs between 0.4 and 0.10 g g^{-1} (e.g. Normah *et al.*, 1986; Pritchard and Prendergast, 1986; de Boucaud et al., 1991; Berjak and Dumet, 1996; Touchell and Walters, 2000; Wesley-Smith et al., 2004; Makeen et al., 2005; Sershen et al., 2007) but as alluded to above, dehydration of recalcitrant seeds to WCs in, or slightly above, this range generally results in the generation of damaging free-radical species and/or the failure of antioxidant systems (e.g. Hendry et al., 1992; Chaitanya and Naithani, 1994; Krinsky, 1994; Côme and Corbineau, 1996; Greggains et al., 2001; Varghese and Naithani, 2002; Dussert et al., 2006; Francini *et al.*, 2006; Pukacka and Ratajczak, 2006; Roach *et al.*, 2008). Suggestions that oxidative stress may be a major component of cryoinjury in recalcitrant seed tissues are therefore not surprising (Touchell and Walters, 2000; Dussert *et al.*, 2003; Normah and Makeen, 2008; Varghese and Naithani, 2008; Pammenter *et al.*, 2010; Whitaker *et al.*, 2010).

Since oxidative stress is essentially the consequence of imbalances between prooxidative and antioxidative processes (Kranner et al., 2006; Roach et al., 2008), antioxidant status is an important consideration in studies on cryo-tolerance (Harding and Benson, 1995). Protection from oxidative stress in orthodox and recalcitrant seeds is believed to arise from the production of enzymic (e.g. superoxide dismutase, catalase and peroxidases) and non-enzymic (e.g. vitamin E, ascorbate and glutathione) antioxidants and metabolically inert, highly water soluble, carbohydrates and proteins (Kermode and Finch-Savage, 2002; Bailly, 2004; Walters et al., 2008). However, the induction of certain components of this antioxidant system is hypothesised to occur to a lesser extent, or not at all, in recalcitrant seeds (Pammenter and Berjak, 1999; Walters et al., 2008). Antioxidant protection has been implicated in post-thaw recovery in vegetative (e.g. Green et al., 1986; Fleck et al., 2000; Johnston et al., 2007) and seed (Dussert et al., 2003; Varghese and Naithani, 2008; Walters et al., 2008) tissues and the susceptibility of non-orthodox seed tissue to oxidative damage during freezing and/or thawing (Dussert et al., 2003; Varghese and Naithani, 2008; Whitaker et al., 2010), and regeneration (Touchell and Walters, 2000) may be a consequence of insufficient and/or inappropriate free-radical scavenging capacity. Reports that recovery media that suppress production of free-radicals or provide free-radical scavenging elements, sustain higher post-thaw recovery rates in nonorthodox zygotic embryos/embryonic axes (e.g. Chandel et al., 1996; Touchell and Walters, 2000; Walters et al., 2008) also lend support to this hypothesis. Cryopreservation protocols for recalcitrant seed germplasm may therefore be improved by a more fundamental understanding of how reactive oxygen species (ROS), free-radical mediated processes, and antioxidant capacity moderate differential tolerance to the various components of cryopreservation.

The present study investigated the ability of *Amaryllis belladonna* (L.) and *Haemanthus montanus* (Baker) embryos to tolerate the various components of cryopreservation (i.e. short-term hydrated storage, cryoprotection, partial dehydration and freeze-thawing) in relation to changes in extracellular superoxide (O_2^-) production and lipid peroxidation (a popular 'marker' for oxidative stress [e.g. Leprince *et al.*, 2000; Varghese and Naithani, 2000, 2008]). Additionally, studies featured in Chapter 2 (see section 2.3) indicated that post-thaw viability of *H. montanus* and *A. belladonna* embryos could be enhanced when partial dehydration was preceded by glycerol (Gly; penetrative), as opposed to sucrose (Suc; nonpenetrative) or no, cryoprotection (CP). The tendency for reactions to proceed at low temperatures is influenced by the terminal freezing temperatures and hydration status of the tissues (Fennema and Sung, 1980), and since the processes by which secondary oxidative reactions proceed during cooling may be the result of both enzymic and non-enzymic reactions, the colligative behaviour of the solute/solvent system is a significant factor in how pro-oxidative enzyme reactions proceed (Benson and Bremner, 2004). While water activity can change, enhance, and suppress free-radical reactions (Heckly and Quay, 1983), ice formation generally accelerates oxidative reactions, possibly related to the concentration of reactants in the nonfrozen portion of water remaining in the cell (Apgar and Hultin, 1982). Based on their colligative mode of action, cryoprotectants like polyols (e.g. Gly) and sugars (e.g. Suc) can differentially, remove freezable water through osmotic dehydration, increase cell viscosity, enhance the glass-forming tendency of aqueous solutions and inhibit ice-crystallisation (for reviews see Fuller, 2004; Benson, 2008). Since the extent and/or localisation (e.g. aqueous or organic phase distributions) of secondary oxidative reactions may be influenced by both icecrystal formation (Apgar and Hultin, 1982) and the characteristics of glasses in water-organic solute systems (Symons, 1982), cryoprotectant mode of action is practically important to the study of oxidative stress in plant material during cryopreservation. In light of the above, the present study also investigated whether Gly CP promoted post-thaw viability retention in H. montanus and A. belladonna embryos by protecting post-drying and post-thaw enzymic antioxidant activities; the enzymes assayed included: superoxide dismutase, catalase, glutathione reductase, ascorbate peroxidase and guaiacol peroxidase.

Extracellular O_2^- production, lipid peroxidation and enzyme activities (for non-CP and Gly CP embryos only) were assessed immediately after the treatment, i.e. immediately after CP, D, CP+D and CP/D/CP+D + freeze-thawing. The O_2^- , lipid peroxidation and enzyme activity measurements are therefore presumed to reflect the damage caused by the treatment and not the damage that accumulated or declined during *in vitro* recovery.

4.2 Materials and Methods

Plant material

Mature fruits of *A. belladonna* and *H. montanus* were harvested directly from parent plants and transported in plastic bags to the laboratory with minimum delay (1-2 d). Upon arrival, the seeds were decontaminated and stored hydrated, as described in Chapter 2 (section 2.2).

Embryo pre-treatment

After 14 d of hydrated storage, embryos were excised with the entire cotyledonary body attached (see Figure A1, Appendix A) and collected within closed Petri dishes on filter paper

moistened with sterile calcium-magnesium solution (CaMg solution: 0.5 μ M CaCl₂.2H₂O and 0.5 mM MgCl₂.6H₂O [Mycock, 1999]). In order to minimise the potential variation in drying and/or cooling rate as a function of embryo size, only embryos of between 4-6 mm in length were used for all the experiments described below. Embryos were rapidly dehydrated via flash drying (devised by Berjak *et al.*, 1990) to: (a) water contents (WCs) between 0.50 and 0.42 g g⁻¹ (dry mass basis [dmb]) (referred to as '>0.4 g g⁻¹, from here on); (b) WCs between 0.33 and 0.30 g g⁻¹ (referred to as '<0.4 g g⁻¹, from here on); and (c) WCs > and <0.40 g g⁻¹ after CP with either aqueous Gly or Suc. As explained in Chapter 2 (section 2.2), embryos were dried to WCs above and below 0.4 g g⁻¹ based on the results of a previous study on the cryopreservation of the zygotic embryos of 15 amaryllids species (Sershen *et al.*, 2007) which showed embryo WCs <0.4 g g⁻¹ to be superior to those >0.4 g g⁻¹ in promoting post-thaw viability. The WC ranges used in the current contribution were defined by constructing WC and viability vs. drying time curves for each species (e.g. Fig. A2, Appendix A). For cryoprotection, freshly excised embryos were immersed in a 5% solution of Gly (v/v) or a 0.5 M solution of Suc for 1 h, and thereafter transferred to a 10% Gly (v/v) or 1 M Suc solution for a further hour.

Partially dried embryos (with and without CP) were subsequently cooled at: (a) rapid, nonequilibrium (*c*. 200°C s⁻¹), cooling rates by direct immersion of naked embryos in nitrogen slush (liquid nitrogen sub-cooled to -210°C [Echlin, 1992]); or (b) slow, equilibrium cooling rates (1°C min⁻¹ in an isopropanol bath [Mr Frosty® Nalgene, Rochester, New York] within a -70°C freezer) down to -40°C followed by direct immersion in nitrogen slush. Freshly excised embryos subjected to no dehydration or CP (referred to as 'fresh' embryos from here on), as well as embryos exposed to CP but no dehydration were also subjected to both cooling rates. After cooling in nitrogen slush embryos were transferred under liquid nitrogen (LN) into LNcontaining cryovials (GreinerTM), mounted on aluminium cryo-canes (10 embryos per vial) and immersed in LN for no longer than a week before use; LN entered the cryovials. Upon retrieval from LN, embryos were rapidly thawed by direct immersion in CaMg solution at 40°C for 2 min, rehydrated in sterile CaMg solution at ambient temperature for 30 min in the dark, and recovered *in vitro*. Freshly excised embryos exposed to none of the treatments described above were also recovered *in vitro* to serve as a control.

Water content determination

Immediately after each of excision (referred to as 'fresh' from here on), partial dehydration (D), CP and CP+D, 10 embryos from each of the non-cooled treatment combinations (see Table 4.1) were weighed individually using a 6-place balance (Mettler, MT5; Germany) and dried in

an oven at 80°C for 48 h before being re-weighed to determine the dry mass. Water content was expressed on a dry mass basis (dmb; $g H_2O$ per g dry matter [$g g^{-1}$]).

In vitro recovery and viability assessment

After rehydration, 10 embryos from each of the 27 treatment combinations (see Table 4.1) were decontaminated and recovered *in vitro* for 60 d, as described in Chapter 2 (section 2.2). Viability was assessed after 60 d *in vitro* growth and was defined by root and shoot production.

Reagents

Epinephrine, dithiothreitol (DTT), ethylene diamine tetra-acetic acid (EDTA), riboflavin, sodium phosphate polyethylene glycol (PEG) 4000, trichloroacetic acid (TCA), polyvinylpolypyrrolidone (PVP), thiobarbituric acid (TBA), methionine, nitroblue tetrazolium (NBT), ascorbic acid, NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate), glutathione and guaiacol and superoxide dismutase from horseradish lyophilized powder (1000-4000 units per mg protein) were purchased from Sigma Chemical Co. (Germany). All other reagents were analytical grade and locally available.

Superoxide assay

The assay of extracellular O_2^{-} was carried out according to Misra and Fridovich (1972), with slight modifications after Beckett *et al.* (2003). Here, O_2^{-} production was measured spectrophotometrically by NADH-mediated oxidation of epinephrine to adrenochrome. Three batches of five embryos from all 27 treatment combinations were shaken in 2.0 ml of 1 mM epinephrine (pH 7.0) for 15 min in the dark, at ~25°C. Fresh embryos were incubated immediately after excision, CP embryos immediately after 1 h incubation at the highest CP concentration (followed by gentle dabbing on filter paper to remove external traces of cryoprotectant solution), D and CP+D embryos immediately after drying and cooled embryos immediately after thawing. After incubation, the oxidation of epinephrine, measured as the increase in absorbance relative to a reagent blank (epinephrine without tissue) at 490 nm (UV-Vis spectrophotometer; Varian), was used to assess O_2^{-} levels. This was calculated using the molar extinction coefficient for adrenochrome (4.47 mM⁻¹ cm⁻¹), and expressed as µmol of epinephrine oxidised min⁻¹ g⁻¹ DW. Embryo batches were dried in an oven at 80°C for 48 h to determine DW. Each data point represents the mean±SD of six measurements carried out with three different extracts (i.e. two measurements on each extract).

Since epinephrine can be oxidised non-specifically and possibly by some enzymes (e.g. tyrosinases) (Baker and Orlandi, 1995), the validity of the epinephrine assay for the detection of

extracellular O_2^- production (see Misra and Fridovich, 1972) in the embryos investigated here was assessed. Here superoxide dismutase (from horseradish lyophilized powder) was added to 2.0 ml of 1 mM epinephrine (pH 7.0) to give a final concentration of 0.10 µg ml⁻¹ before fresh, D and frozen-thawed embryos were incubated in the assay mixture. In all cases, and for both species, the addition of superoxide dismutase inhibited the oxidation of epinephrine by more than 50% after 15 min incubation (data not shown), validating the use of the assay for the detection of extracellular O_2^- production in this study.

Other studies on ROS production in recalcitrant seeds illustrate the value of carrying out time course of ROS production; while initial rates may be high these may remain high or decline with time (Roach *et al.*, 2008; Whitaker *et al.*, 2010). However, the limited number of seeds available did not permit time course measurements of ROS production in the present study.

Lipid peroxidation

The thiobarbituric acid-reactive substances (TBARS) assay (originally described by Heath and Packer, 1968) has been widely used to measure aldehydic lipid peroxidation products in plants (see Hodges *et al.*, 1999 and references therein), but phenolic and carbohydrate compounds can cause interference problems in such colorimetric assays since many of them also absorb at 532 nm (Du and Bramlage, 1992). This threat of over-estimation has therefore demanded assay modification and careful interpretation to limit interference and improve specificity in a number of studies (e.g. Du and Bramlage, 1992; Hodges *et al.*, 1999). The method described by Heath and Packer (1968) involves assessing the thiobarbituric acidmalondialdehyde (TBA-MDA) complex in terms of Equation 1:

MDA equivalents (nmol ml⁻¹) = $[(A_{532} - A_{600}) / (155\ 000)]10^6$ [1]

where 532 nm represents the maximum absorbance of the TBA-MDA complex, 600 nm the correction for non-specific turbidity, and 155 000 the molar extinction coefficient for MDA.

To correct for interference generated by TBA-sugar complexes Du and Bramlage (1992) modified this method, where MDA equivalents (nmol ml⁻¹) were determined according to Equation 2:

 $[[(A_{532} - A_{600})-[(A_{440} - A_{600}) (MA of Suc at 532 nm/MA of Suc at 440 nm)]]/157 000]10⁶ [2] where MA is the molar absorbance of Suc. Du and Bramlage's (1992) results indicated that the absorbance of Suc at 440 nm was proportional to the concentration of Suc being measured and they calculated MA of 1±10 mM sucrose at 532 nm and 440 nm to be 8.4 and 147, respectively, giving a ratio of 0.0571. The results of these studies yielded a modified procedure for MDA$

estimation which involved measuring absorbance at 532, 600 and 440 nm and rectifying the interference of soluble sugars in samples using a standard curve for sucrose (2.5-10 μ mol ml⁻¹).

In the present study, Suc concentrations (estimated via a colorimetric anthrone assay [Jermyn, 1956]) of fresh, Suc CP and Gly CP embryos across both species were between 1.42 and 1.77 mg g^{-1} DW and always <0.10 µmol ml⁻¹ (detailed data [Table B1] and methodology given in Appendix B). Also, within species, concentrations were not significantly different across the three treatments (see Table B1, Appendix B). These data suggested that sucrose concentrations across all three treatments (for both species) were lower than the interfering range (2.5-10.0 µmol ml⁻¹) as suggested by Du and Bramlage (1992). Additionally, if interference did occur, the degree of interference was likely to have been comparable across CP and non-CP treatments. With this in mind, lipid peroxidation in the present study was assessed via the formation of TBARS as described by Heath and Packer (1968), with slight modifications after Varghese and Naithani (2008), who also estimated TBARS production in recalcitrant embryos using the Heath and Packer (1968) method. Here, three batches of 15 embryos each (c. 100 mg) from all 27 treatment combinations, were homogenised in LN and suspended in 1 ml of 0.1% (w/v) TCA, and 3 ml of 0.5% 2-TBA in 20% (w/v) TCA. Fresh embryos were homogenised immediately after excision, CP embryos immediately after 1 h incubation at the highest CP concentration (followed by rinsing with water to remove residual cryoprotectant), D and CP+D embryos immediately after drying and cooled embryos immediately after thawing. The homogenate was heated at 95°C for 30 min and thereafter incubated on ice for 10 min. After cooling the samples were centrifuged for 15 min at 8,000 g and the absorbance of the supernatant taken at 532 nm (UV-Vis spectrophotometer; Varian); this reading was subtracted for any non-specific absorbance at 600 nm. The concentration of MDA was calculated using the molar extinction coefficient of 155 mM⁻¹ cm⁻¹ in terms of µmol MDA g⁻¹ DW. However, since aldehydes other than MDA can also react with TBA to produce pink chromophore (Du and Bramlage, 1992) with an absorbance of 532 nm, we refer to lipid peroxidation in terms of µmol TBARS g⁻¹ DW from here on. Each data point represents the mean±SD of six measurements carried out with three different extracts.

Enzyme extraction and estimation

Three batches of 15 embryos each (*c*. 100 mg) from all partially dried and partially dried + cooled treatments involving non-CP and Gly CP embryos, as well as fresh embryos (not dried or cooled), were homogenised in LN and suspended in 0.1 M sodium phosphate buffer (pH 7.8) containing 2 mM DTT, 0.1 mM EDTA, 1.25 mM polyethylene glycol (PEG) 4000 and 1% (w/v) PVP (after Farrant *et al.*, 2004). Fresh embryos were homogenised immediately after excision,

D and Gly CP+D embryos immediately after dehydration and D + cooled and Gly CP+D + cooled embryos immediately after thawing. Extracts were incubated on ice for 15 min, with gentle vortexing every 5 min, before centrifugation at 16,000 g for 30 min at 4°C. The supernatants were collected and immediately stored at -70°C before use in all subsequent antioxidant enzyme assays. Absorbances were taken using a UV-Vis spectrophotometer (Varian), at a constant temperature of 25°C. The volume of enzyme extract used in the assay mixture was optimised independently for each enzyme. Assay reagent blanks (sample replaced with same volume of extraction buffer) were used to check for any change in absorbance owing to assay reagents, and corrected for, where necessary. Five different antioxidant enzymes (identified below) were assayed twice on three different extracts.

Superoxide dismutase (SOD) assay

Superoxide dismutase activity was assayed in terms of its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) according to Beauchamp and Fridovich (1971). The assay mixture consisted of 50 mM sodium phosphate buffer, 1.17 μ M riboflavin, 0.01 M methionine, 0.056 mM NBT and 10 (for *A. belladonna*) or 100 (for *H. montanus*) μ l enzyme extract. The assay mixture was illuminated for 5 min by a 55 W fluorescent tube light (Phillips, South Africa), in aluminium foil-lined container, and absorbance was read against unilluminated samples. One unit of SOD corresponded to 50% inhibition of photochemical reduction of NBT (measured as the increase in absorbance at 560 nm), and SOD activity was expressed as units of SOD g⁻¹ DW.

Catalase (CAT) assay

Catalase was assayed according to Clairbone (1985). The final assay mixture comprised 37.5 mM potassium phosphate buffer (pH 7.0), 10 mM H₂O₂ and 100 (for *A. belladonna*) or 50 (for *H. montanus*) μ l enzyme extract. The enzymic break-down of H₂O₂, measured as a decline in absorbance at 240 nm, was used to assess CAT activity. This was calculated using the molar extinction coefficient for H₂O₂ (0.0436 mM⁻¹ cm⁻¹), and expressed as μ mol H₂O₂ decomposed min⁻¹ g⁻¹ DW.

Glutathione reductase (GR) assay

The activity of GR was estimated according to Esterbauer and Grill (1978). The assay mixture comprised 50 mM potassium phosphate buffer (pH 7.8), 0.5 mM NADPH, 10 mM glutathione, 3 mM MgCl₂ and 100 μ l enzyme extract. The oxidation of NADPH to NADP, which measured as the decline in absorbance at 340 nm, was used to assess GR activity. This

was calculated using the molar extinction coefficient for NADPH (6.22 $\text{mM}^{-1} \text{ cm}^{-1}$), and expressed as μ mol NADPH oxidised min⁻¹ g⁻¹ DW.

Ascorbate peroxidase (AsPX) assay

Ascorbate peroxidase activity was estimated according to Nakano and Asada (1981). The rate of H_2O_2 dependent oxidation of ascorbate was determined in an assay mixture that contained 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H_2O_2 and 100 µl enzyme extract. The reaction was started by addition of H_2O_2 and the oxidation rate of ascorbate, estimated by monitoring the decline in absorbance at 290 nm, was used to assess AsPX activity. This was calculated using the molar extinction coefficient for ascorbate (2.8 mM⁻¹ cm⁻¹) and expressed as µmol ascorbate oxidised min⁻¹ g⁻¹ DW.

Guaiacol peroxidase (POX) assay

Guaiacol peroxidase activity was estimated according to Chance and Maehly (1955). The assay mixture comprised 25 mM sodium acetate-HCl buffer (pH 5.0), 8.26 mM of guaiacol and 50 (for *A. belladonna*) or 20 (for *H. montanus*) μ l enzyme extract. The reaction was started by the addition of 8.8 mM H₂O₂ and the oxidation of guaiacol to tetraguaiacol, measured as the increase in absorbance at 470 nm, was used to assess POX activity. This was calculated using the molar extinction coefficient for tetraguaiacol (26.6 mM⁻¹ cm⁻¹), and expressed as μ M guaiacol oxidised min⁻¹ g⁻¹ DW. From here on guaiacol peroxidase will be abbreviated as 'POX' and not 'GPX' since the guaiacol-based assay used here tests for the activity of Class III peroxidases and not glutathione peroxidase.

Statistical analysis

Inter-treatment differences in extracellular O_2^- production, lipid peroxidation and enzyme activity were tested for by Analysis of Variance (*ANOVA*; SPSS, Version 15). Multiple comparisons were then made using Duncan's mean separation test. Inter-treatment differences in viability were tested for using null-model chi-squared analyses (specifically designed to assess non-parametric data) (EcoSim Version 7.72 [developed by Gotelli and Entsminger, 2009]). Correlations between viability and extracellular O_2^- , TBARS levels and antioxidant enzyme activity, as well as between extracellular O_2^- and TBARS levels were tested for using Pearson correlation analysis (SPSS, Version 15). For all correlation analyses viability percentages were transformed (arcsin) to conform data to parametric test assumptions. All statistical tests were performed at the 0.05 level of significance.

4.3 Results

All parameters were assessed for both species and unless otherwise stated, the trends reported for these data below, are applicable to both species. Where it was necessary to discuss these parameters in terms of actual values, those for both species are given.

Viability

The embryos excised from seeds stored for 14 d (referred to as 'fresh' from here on) were highly hydrated (>4.0 g g⁻¹) and incurred some (10-30%) viability loss upon rapid dehydration to WCs > and <0.4 g g⁻¹ (Table 4.1); being slightly greater at WCs <0.4 g g⁻¹. The embryos of both species could be dried to WCs of 0.30-0.48 g g⁻¹ while retaining $\geq 60\%$ viability but while A. belladonna embryos could be dried to WCs of 0.48 ± 0.09 and 0.31 ± 0.07 g g⁻¹ in 5 and 15 min, respectively, H. montanus embryos took 180 and 240 min to reach 0.48±0.08 and 0.33±0.09 g g ¹, respectively (data not shown). Cryoprotection led to a significant reduction in embryo WC relative to fresh embryos, the effect being significantly greater with Suc CP (Table 4.1). After Gly CP+D, viabilities were either marginally lower or similar to non-CP embryos dehydrated to comparable WCs, but with Suc CP+D viabilities were significantly lower than Gly CP and non-CP embryos dehydrated to comparable WCs. Cooling generally led to a dramatic reduction in viability, relative to fresh, CP and D embryos (Table 4.1). Fully hydrated embryos did not survive cooling. Of the six treatments in which post-thaw viability was observed in A. *belladonna*, one involved non-CP embryos (>0.4D-slow) and facilitated in 10% viability, while four (>0.4D-Gly-rapid, >0.4D-Gly-slow, <0.4D-Gly-rapid and <0.4D-Gly-slow) involved Gly CP+D embryos and facilitated between 20-70% viability (Table 4.1). Of the six treatments in which post-thaw viability was observed in *H. montanus*, one (>0.4D-slow) involved non-CP+D embryos and facilitated 10% viability, two (Suc-rapid, <0.4D-Suc-rapid) involved Suc CP embryos and facilitated 10% viability, while three involving Gly CP+D embryos (>0.4D-Glyrapid, >0.4D-Gly-slow and <0.4D-Gly-rapid) facilitating 10-50% viability (Table 4.1).

	A. belladonna		H. montanus		
Treatment	$WC^{1}(g g^{-1})$	Viability ² (%)	$WC(g g^{-1})$	Viability (%)	Treatment categories
Fresh	5.19±0.91	100	4.85±0.84	100	Control + dehydrated to $< or > 0.4 \text{ g g}^{-1}$, with or without cryoprotection.
>0.4D	0.48±0.09	90	0.48±0.08	80	
<0.4D	0.31±0.07	80	0.33±0.09	70	
Gly	3.22±0.55	100	4.52±0.07	90	
Suc	1.84±0.13	100	1.64±0.21	90	
>0.4D-Gly	0.43±0.04	80	0.50±0.08	80	
>0.4D-Suc	$0.42{\pm}0.07$	70	0.47±0.09	70	
<0.4D-Gly	0.33±0.06	80	0.32±0.06	80	
<0.4D-Suc	0.30±0.03	60	0.30±0.08	60	
Fresh-rapid	5.19±0.91	0	4.85±0.84	0	Cooled rapidly or slowly with or without cryoprotection; no flash drying.
Fresh-slow	5.19±0.91	0	4.85±0.84	0	
Gly-rapid	3.22±0.55	0	4.52±0.97	0	
Suc-rapid	1.84±0.13	0	1.64±0.21	10	
Gly-slow	3.22±0.55	0	4.52±0.97	0	
Suc-slow	1.84±0.13	0	1.64±0.21	0	
>0.4D-rapid	0.48±0.09	0	0.52±0.15	0	Dehydrated to $< or > 0.4 g g^{-1}$ and cooled rapidly or slowly.
>0.4D-slow	0.48±0.09	10	0.52±0.15	10	
<0.4D-rapid	0.31±0.07	0	0.33±0.11	0	
<0.4D-slow	0.31±0.07	0	0.33±0.11	0	
>0.4D-Gly-rapid	0.43±0.04	20	0.50±0.08	50	Dehydrated to < or > 0.4 g g ⁻¹ , with cryoprotection, and cooled rapidly or slowly.
>0.4D-Suc-rapid	$0.42{\pm}0.07$	0	0.47±0.09	0	
>0.4D-Gly-slow	0.43 ± 0.04	20	0.50±0.08	10	
>0.4D-Suc-slow	$0.42{\pm}0.07$	0	0.47±0.09	0	
<0.4D-Gly-rapid	0.33±0.06	70	0.32±0.06	20	
<0.4D-Suc-rapid	0.30±0.03	0	0.30±0.08	10	
<0.4D-Gly-slow	0.33±0.06	40	0.32 ± 0.06	0	
<0.4D-Suc-slow	0.30±0.03	0	0.30±0.08	0	

 Table 4.1 Water content and viability for fresh, cryoprotected, partially dried and cooled

 A. belladonna and H. montanus zygotic embryos.

¹ water content; ² viability = root and shoot production. >0.4D = dried to >0.4 g g⁻¹; <0.4D = dried to <0.4 g g⁻¹; -Gly = cryoprotected with glycerol; -Suc = cryoprotected with sucrose; -slow = cooled slowly; -rapid = cooled rapidly. p < 0.05 for water content (Mann-Whitney-U test, n = 10) and p < 0.001 for viability (null-model chi-squared analysis, n = 10) when these data were tested for significant differences across treatments.

Extracellular superoxide

Superoxide levels were low in fresh embryos and even though D (in both species) and CP (in *H. montanus*) generally led to an increase in these levels, inter-treatment differences were seldom significant (Fig. 4.1A, B). The post-drying increase in O_2^- levels relative to fresh embryos was slightly (but not significantly) more pronounced in CP+D embryos but did not differ significantly across CP+D treatments. Within the non-cooled treatments, higher O_2^- levels

were generally accompanied by greater viability loss (Fig. 4.1A, B). Cooling led to the greatest increase in O_2^- production, with levels across all cooling treatments being significantly higher than fresh, D, CP and most often CP+D treatments. These high post-thaw O_2^- levels were generally accompanied by high or, very often, complete viability loss (Fig. 4.1A, B).

Within the cooled treatments in A. belladonna, inter-treatment differences in O_2 production were seldom significant but the following trends were observed (Fig. 4.1A): (a) embryos that were not dried or cryoprotected before cooling yielded higher post-thaw O_2 levels than noncooled CP, D and CP+D treatments; (b) across the CP, D and CP+D treatments, post-thaw O_2 levels were often relatively higher in treatments involving Suc CP (e.g. >0.4D-Suc-rapid and Suc-slow); and (c) where post-thaw viability was observed, O_2 levels were often relatively lower (e.g. >0.4D-Gly-slow and >0.4D-Gly-rapid), but this was not without exception (e.g. <0.4D-Gly-Slow). Differences in O₂ production across the cooled treatments in *H. montanus* were also seldom significant but the following trends were observed (Fig. 4.1B): (a) except for <0.4D-Gly-slow, fully hydrated, non-CP+D and Suc CP+D treatments generally exhibited higher post-thaw O_2 levels than treatments involving Gly CP embryos; and (b) lower O_2 levels were not necessarily indicative of post-thaw viability retention (e.g. Gly-slow and Gly-rapid). Within the treatments in which post-thaw viability retention was observed, viability was not proportional to O_2 levels but when data for non-cooled and cooled treatments were pooled, there was a significant negative correlation between extracellular O_2 production and viability (A. belladonna: r = -0.73, p < 0.001; H. montanus: r = -0.88, p < 0.001).

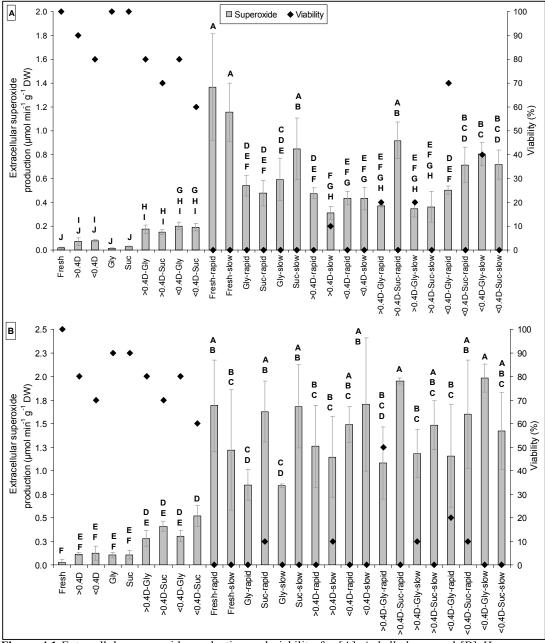


Figure 4.1 Extracellular superoxide production and viability for [A] *A. belladonna* and [B] *H. montanus* zygotic embryos. >0.4D = dried to >0.4 g g⁻¹; <0.4D = dried to <0.4 g g⁻¹; -Gly = cryoprotected with glycerol; -Suc = cryoprotected with sucrose; -slow = cooled slowly; -rapid = cooled rapidly. Superoxide values represent mean±SD and are significantly different when followed by different letters (*ANOVA*, n = 5, p < 0.05). p < 0.05 when viability data were tested for significant differences across treatments (null-model chi-squared analysis, n = 10).

Lipid peroxidation

Cryoprotected and non-CP + partially dried embryos exhibited some (10-40%) viability loss but lipid peroxidation levels (as assessed by TBARS production) in treatments involving such embryos were comparable to fresh embryos (Fig. 4.2A, B). Interestingly, TBARS levels in embryos excised from seeds immediately after harvest (i.e. zero-time control) were marginally, but not significantly (data not shown), lower than those excised from seeds that had been stored for 14 d (i.e. 'fresh' embryos). Lipid peroxidation levels in Gly CP+D embryos were significantly lower than fresh, CP and non-CP+D embryos, while TBARS levels and the degree of viability loss in Suc CP+D treatments were relatively higher than Gly CP+D treatments (Fig. 4.2A, B).

In *A. belladonna*, cooling often facilitated TBARS levels that were higher than fresh, CP, D and Suc CP+D treatments (Fig. 4.2A). Except for Gly-rapid embryos, there was a trend for cooling treatments involving Gly CP embryos to exhibit TBARS levels that were either lower (e.g. <0.4D-Gly-rapid) or similar (e.g. <0.4D-Gly-slow, >0.4D-Gly-slow and Gly-slow) to fresh embryos in *A. belladonna*; this was often accompanied by higher post-thaw viability retention (e.g. <0.4D-Gly-rapid) than treatments involving Suc or no, cryoprotection. When data for cooled and non-cooled *A. belladonna* treatments were pooled, there was a significant negative correlation between viability and TBARS levels (r = -0.68, p < 0.001).

Except for >0.4D-rapid and >0.4D-slow, TBARS levels within the cooled treatments in *H. montanus* were generally similar to or, in a few cases, slightly lower (e.g. <0.4D-Gly-slow and <0.4D-Suc-slow) than fresh embryos (Fig. 4.2B). Low TBARS levels were not necessarily indicative of post-thaw viability retention in *H. montanus* embryos though, and when data for cooled and non-cooled treatments were pooled, there was no significant correlation between viability and TBARS levels (r = -0.20, p = 0.31).

Finally, TBARS levels in either species were not significantly correlated with extracellular O_2^- production (r < 0.04 and p > 0.05 for both).

Antioxidant enzyme activity

As mentioned earlier, post-drying and post-thaw antioxidant enzyme activities were assessed and compared across treatments involving non-CP and Gly CP embryos, only.

Superoxide dismutase

Partial dehydration always led to some (10-30%) viability loss in both species but while SOD activity was high in fresh *A. belladonna* embryos and declined significantly after D (Fig. 4.3A), SOD activity was low in fresh *H. montanus* embryos and increased after D (Fig. 4.3B). This post-drying enhancement of SOD activity in *H. montanus* embryos was marginal for non-CP treatments but significantly higher for Gly CP. The post-drying decline of SOD activity in *A. belladonna* embryos was slightly, but not significantly, more pronounced in Gly CP+D embryos (Fig. 4.3A).

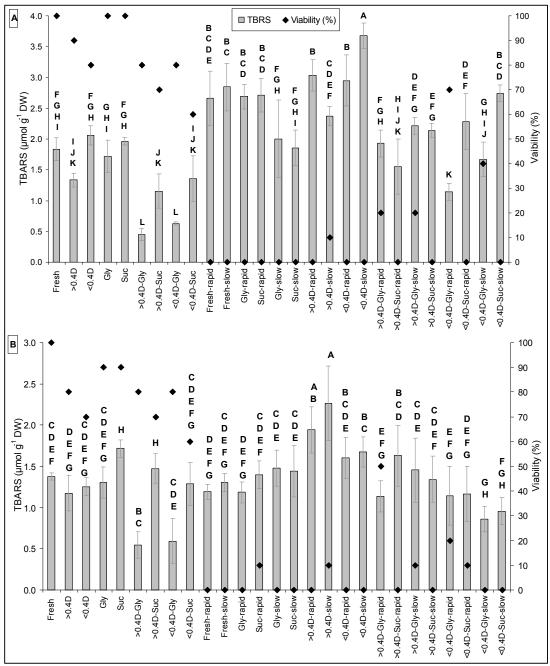


Figure 4.2 Lipid peroxidation (as the concentration of thiobarbituric acid-reactive substances [TBARS]) and viability for [A] *A. belladonna* and [B] *H. montanus* zygotic embryos. >0.4D = dried to >0.4 g g⁻¹; <0.4D = dried to <0.4 g g⁻¹; -Gly = cryoprotected with glycerol; -Suc = cryoprotected with sucrose; -slow = cooled slowly; -rapid = cooled rapidly. TBARS values represent mean±SD and are significantly different across treatments when followed by different letters (*ANOVA*, *n* = 6, *p* < 0.05). *p* < 0.05 when viability data were tested for significant differences across treatments (null-model chi-squared analysis, *n* = 10).

With cooling, SOD activity in *A. belladonna* declined to levels significantly lower than fresh embryos (Fig. 4.3A). In treatments that were associated with no post-thaw viability retention in *A. belladonna* SOD activity was either zero or confined to one replicate while its

activity in treatments that did result in post-thaw viability retention was generally higher and detectable across more replicates (detailed data not shown).

In *H. montanus*, post-thaw SOD activity was detected in just three cooled treatments (Fig. 4.3B), all of which involved Gly-CP+D embryos. These treatments were accompanied by some (10-50%) post-thaw viability and embryos exhibited SOD activities that were significantly higher than fresh and D embryos. However, >0.4D-slow in *H. montanus* exhibited no SOD activity even though it facilitated 10% post-thaw viability.

For both species, post-thaw viability was not proportional to SOD activity (Fig. 4.3A, B). When cooled and non-cooled treatments were pooled, there was a significant positive correlation between SOD activity and viability in *A. belladonna* (r = 0.67, p = 0.01), but not in *H. montanus* (r = -0.02, p = 0.94), despite Gly apparently promoting SOD activity in some embryo treatments of this species.

<u>Catalase</u>

Partial dehydration led to a significant decline in CAT activity in *A. belladonna* relative to fresh embryos and this was always accompanied by some (10-20%) viability loss; however, the post-drying decline in CAT activity was less severe in <0.4D-Gly embryos (Fig. 4.4A). Except for <0.4D-Gly, partial dehydration always brought about a significant decline in CAT activity in *H. montanus* (Fig. 4.4B). As in *A. belladonna*, partial dehydration led to some (20-30%) viability loss across all treatments, even in <0.4D-Gly, which exhibited CAT activities that were statistically comparable to fresh embryos.

Post-thaw CAT activity and viability declined to levels lower than fresh embryos in both species (Fig. 4.4A, B) and, except for >0.4D-Gly-rapid in *H. montanus*, this decline in activity was always significant. This post-thaw decline in CAT activity was less severe across all rapidly cooled Gly-CP+D treatments (although significant for *A. belladonna* only), with post-thaw CAT activities in rapidly cooled Gly-CP+D embryos in *A. belladonna* being just a high as some of the partially dried treatments.

In *A. belladonna*, CAT activity, except for 0.4D-rapid, was detected only in treatments that yielded post-thaw viability (Fig. 4.4A). In *H. montanus*, CAT activity was detected across all cooled treatments and except for >0.4D-Gly-rapid, which exhibited CAT activities that were statistically comparable to fresh embryos, these activities were often statistically comparable to partially dried treatments (Fig. 4.4B).

In both species, the highest post-thaw CAT activity corresponded to the highest post-thaw viability and there was a tendency for cooled treatments that were accompanied by post-thaw viability retention to exhibit CAT activity across relatively more replicates than those that did

not (detailed data not shown). Also, when data for cooled and non-cooled treatments were pooled, there was a significant positive correlation between viability and CAT activity for either species (*A. belladonna*: r = 0.86, p < 0.000; *H. montanus*: r = 0.62, p = 0.02).

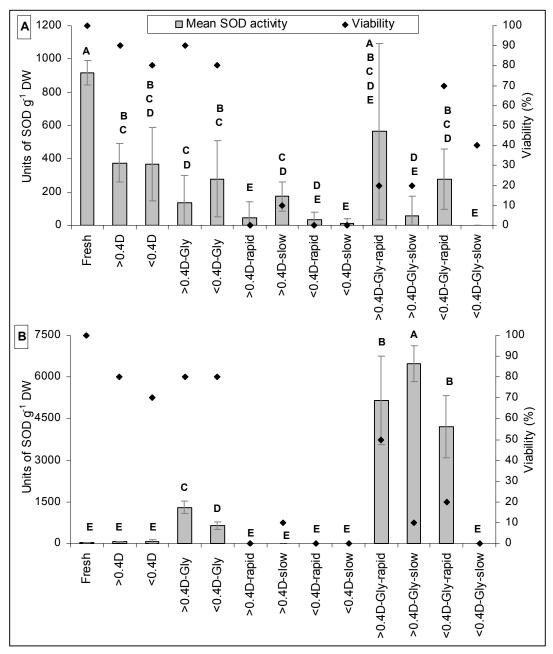


Figure 4.3 Superoxide dismutase (SOD) activity and viability for [A] *A. belladonna* and [B] *H. montanus* zygotic embryos. >0.4D = dried to >0.4 g g⁻¹; <0.4D = dried to <0.4 g g⁻¹; -Gly = cryoprotected with glycerol; -slow = cooled slowly; -rapid = cooled rapidly. Values represent mean±SD and are significantly different across treatments when followed by different letters (p < 0.05, *ANOVA*, n = 6). p < 0.05 when viability data were tested for significant differences across treatments (null-model chi-squared analysis, n = 10).

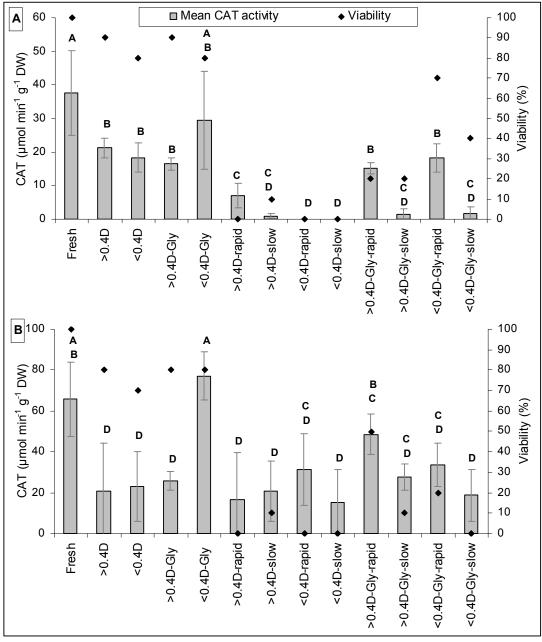


Figure 4.4 Catalase (CAT) activity and viability for [A] *A. belladonna* and [B] *H. montanus* zygotic embryos. >0.4D = dried to >0.4 g g⁻¹; <0.4D = dried to <0.4 g g⁻¹; -Gly = cryoprotected with glycerol; -slow = cooled slowly; -rapid = cooled rapidly. Values represent mean±SD and are significantly different across treatments when followed by different letters (p < 0.05, *ANOVA*, n = 6). p < 0.05 when viability data were tested for significant differences across treatments (null-model chi-squared analysis, n = 10).

Glutathione reductase

Partial dehydration reduced GR activity relative to fresh embryos, in both species (Fig. 4.5A, B). Even though all dried treatments were accompanied by some (10-30%) viability loss,

GR activity in both species was often unaffected (e.g. >0.4D-Gly in *A. belladonna* and <0.4D-Gly in *H. montanus*) or enhanced relative to fresh embryos in treatments involving Gly CP+D embryos (e.g. <0.4D-Gly in *A. belladonna*).

In *A. belladonna*, cooling generally led to a significant decline in GR activity (e.g. >0.4D cooled embryos) relative to fresh embryos, but GR activity in rapidly cooled Gly-CP+D embryos was as high as non-cooled D embryos, and facilitated post-thaw viabilities of 20 and 70% (Fig. 4.5A). In *A. belladonna*, >0.4D-slow exhibited 10% post-thaw viability and very low GR activity; however, there were treatments that exhibited some post-thaw viability (e.g. >0.4D-Gly-slow: 20% and <0.4D-Gly-slow: 40%) but no GR activity, while others showed GR activity but no post-thaw viability (e.g. <0.4D-rapid).

In *H. montanus*, all cooled treatments exhibited GR activity (Fig. 4.5B). Except for <0.4D-Gly-slow there was a trend for Gly CP+D embryos in *H. montanus* to exhibit post-thaw GR activities that were either comparable to, or higher than, fresh and D embryos; this was often accompanied by higher post-thaw viability. In *H. montanus*, the highest post-thaw viability corresponded to the highest post-thaw GR activity but there were treatments that were accompanied by no post-thaw viability, yet still exhibited GR activity (e.g. <0.4D-rapid).

Within the cooled treatments in both species, GR activity was not proportional to viability. Even though Gly protected GR activity in some treatments, when data for non-cooled and cooled treatments were pooled, there was no significant correlation between viability and GR activity for either species (r < 0.3 and p > 0.10 for both).

Guaiacol peroxidase

Partial dehydration was always accompanied by some (10-30%) viability loss (Fig. 4.6A, B). In non-CP+D *A. belladonna* embryos this was accompanied by a significant decline in POX activity, relative to fresh embryos (Fig. 4.6A). This post-drying decline was not apparent in Gly CP+D *A. belladonna* embryos; in fact, POX activity in <0.4D-Gly embryos of this species was significantly higher than fresh embryos. Viability within the non-cooled treatments in *A. belladonna* was not proportional to POX activity. In *A. belladonna* cooling led to a significant decline in POX activity relative to fresh embryos. This decline was significantly less severe in treatments involving rapidly cooled Gly CP+D embryos, which facilitated 20 and 70% post-thaw viability. In *A. belladonna*, POX activity was detected in >0.4D-slow which facilitated a post-thaw viability of 10%, yet it was not detected in slowly cooled Gly CP+D treatments which were accompanied by post-thaw viabilities of 20 and 40%.

In *H. montanus*, except for <0.4D, partial dehydration led to a slight but insignificant decrease in POX activity relative to fresh embryos (Fig. 4.6B). Partial dehydration always led to

some (20-30%) viability loss in *H. montanus*, even when POX activity was not lower than fresh embryos (as in <0.4D). Even though cooling led to a considerable decline in viability, POX activity (except for <0.4D-slow) was detected across all cooled treatments in *H. montanus*. Within the treatments involving Gly CP+D embryos, rapidly cooled embryos exhibited the highest POX activities and post-thaw viabilities. In *H. montanus*, some treatments exhibited post-thaw POX activity but no viability; however, this was usually confined to just one or two replicates (e.g. <0.4D-rapid; detailed data not shown).

Even though Gly CP protected POX activity in some treatments, when data for non-cooled and cooled treatments were pooled, there was no significant correlation between viability and POX activity for either species (r < 0.50 and p > 0.10 for both).

Ascorbate peroxidase

Partial dehydration always led to some (10-40%) viability loss but post-drying AsPX activities were either comparable to or, as in Gly CP+D embryos, slightly higher than fresh embryos (significant for > and <0.4D-Gly in *A. belladonna* and for <0.4D-Gly in *H. montanus*; Fig. 4.7A, B). Within the non-cooled treatments, AsPX activity was not proportional to viability.

In *A. belladonna*, AsPX activity was detected across all cooled treatments (Fig. 4.7A). However, post-thaw AsPX activities in Gly CP+D embryos were significantly higher than fresh, non-cooled and cooled non-CP+D embryos; this was often accompanied by relatively higher post-thaw viabilities.

In *H. montanus*, post-thaw AsPX activity was only detected in treatments involving Gly CP+D embryos and the single non-CP treatment associated with post-thaw viability retention (i.e. >0.4D-slow); activities in these treatments were significantly higher than fresh embryos but not proportional to post-thaw viability (Fig. 4.7B).

Even though Gly protected AsPX activity in some treatments, when data for non-cooled and cooled treatments were pooled, there was no significant correlation between viability and AsPX activity for both species (r < 0.40 and p > 0.20 for both species).

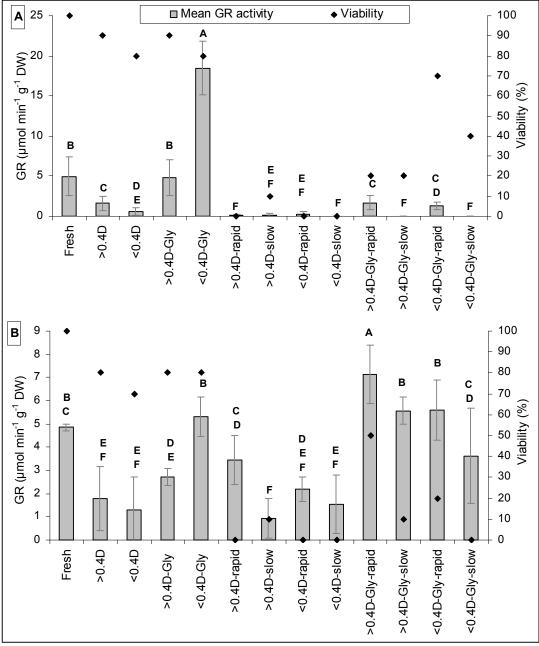


Figure 4.5 Glutathione reductase (GR) activity and viability for [A] *A. belladonna* and [B] *H. montanus* zygotic embryos. >0.4D = dried to >0.4 g g⁻¹; <0.4D = dried to <0.4 g g⁻¹; -Gly = cryoprotected with glycerol; -slow = cooled slowly; -rapid = cooled rapidly. Values represent mean±SD and are significantly different across treatments when followed by different letters (p < 0.05, *ANOVA*, n = 6). p < 0.05 when viability data were tested for significant differences across treatments (null-model chi-squared analysis, n = 10).

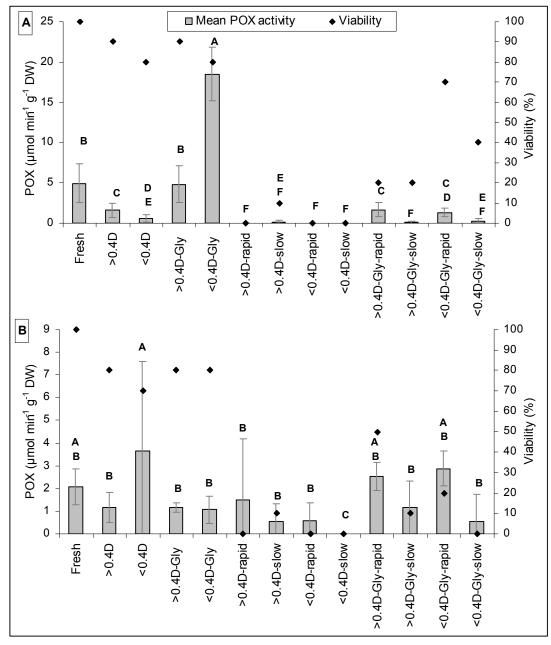


Figure 4.6 Guaiacol peroxidase (POX) activity and viability for [A] *A. belladonna* and [B] *H. montanus* zygotic embryos. >0.4D = dried to >0.4 g g⁻¹; <0.4D = dried to <0.4 g g⁻¹; -Gly = cryoprotected with glycerol; -slow = cooled slowly; -rapid = cooled rapidly. Values represent mean±SD and are significantly different across treatments when followed by different letters (p < 0.05, *ANOVA*, n = 6). p < 0.05 when viability data were tested for significant differences across treatments (null-model chi-squared analysis, n = 10).

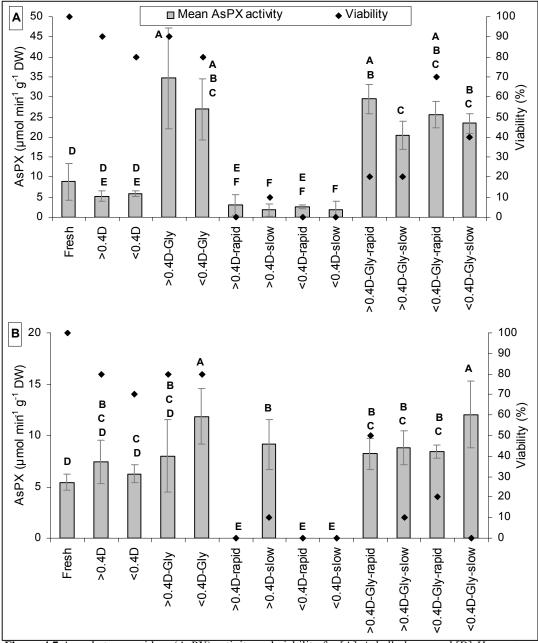


Figure 4.7 Ascorbate peroxidase (AsPX) activity and viability for [A] *A. belladonna* and [B] *H. montanus* zygotic embryos. >0.4D = dried to >0.4 g g⁻¹; <0.4D = dried to <0.4 g g⁻¹; -Gly = cryoprotected with glycerol; -slow = cooled slowly; -rapid = cooled rapidly. Values represent mean±SD and are significantly different across treatments when followed by different letters (p < 0.05, *ANOVA*, n = 6). p < 0.05 when viability data were tested for significant differences across treatments (null-model chi-squared analysis, n = 10).

4.4 Discussion

The present study investigated the ability of *A. belladonna* and *H. montanus* embryos to tolerate the various components of cryopreservation in relation to changes in oxidative

stress. The study also investigated whether Gly CP promoted post-thaw viability retention in *H. montanus* and *A. belladonna* embryos by protecting or promoting post-drying and post-thaw enzymic antioxidant activities.

Zygotic embryo viability in relation to changes in oxidative stress metabolism Hydrated storage

Storage of seed at low (usually -20°C) rather than ultra-low, i.e. cryogenic temperatures (usually -196°C), is still the most popular method of conserving plant germplasm (Rao *et al.*, 2006) but there is considerable evidence that seeds stored at -18 to -20°C are susceptible to oxidative stress (Hendry, 1993 and references therein). The data may be conflicting and mainly correlative due to the physiological complexity of studying seed storage parameters, but what these data do show is that pre-storage status of seeds can significantly influence the oxidative stress profiles obtained with respect to seed hydration, age and biochemical composition (Benson and Bremner, 2004).

Prior to embryo or axis excision for cryopreservation, recalcitrant seeds almost always need to be stored in the short-term (days). As in the current contribution, this is often achieved via hydrated storage which involves the maintenance of their moisture content at, or slightly below, their shedding WC, while at ambient or slightly reduced temperatures (e.g. Berjak *et al.*, 1989). Reactive oxygen species (ROS) metabolism is a characteristic feature of fresh mature nonorthodox (Chaitanya and Naithani, 1994, 1998; Varghese and Naithani, 2002) and orthodox (Garnczarska et al., 2008) seeds and free-radical production in immature and mature nonorthodox seeds appears to be a natural consequence of respiration (Francini *et al.*, 2006). In the present study, extracellular O_2 was detected in embryos excised from seeds that had been stored hydrated for 14 d but these levels were lower than D and cooled embryos (Fig. 4.1A, B), possibly associated with the relatively high activities of certain antioxidant enzymes in fresh, compared with D and cooled embryos (e.g. SOD and POX in A. belladonna; CAT and GR in both species; Figs 4.3-4.6). However, hydrated storage was accompanied by lipid peroxidation with TBARS levels in fresh embryos being just as high as those in CP, D and CP+D embryos (Fig. 4.2A, B). Recalcitrant seeds exhibit ongoing metabolism during storage (Farrant et al., 1989) and as their embryos progress towards germination in storage, an intracellular water stress develops (Farrant et al., 1986; Berjak et al., 1989; Pammenter et al., 1994, 1997). The consequences of this stress appear to be similar to those associated with the oxidative stressinduced deterioration of orthodox seeds in dry storage (Cakmak et al., 1993; Hendry, 1993; Bailly et al., 1998), and include free-radical mediated lipid peroxidation (and hence membrane damage [Côme and Corbineau, 1996; Finch-Savage et al., 1996; Kermode and Finch-Savage, 2002; Dussert *et al.*, 2006; Ratajczak and Pukacka, 2006]). In the present study, TBARS levels in embryos excised from seeds that had been stored for 14 d (i.e. 'fresh embryos') were, however, not significantly higher than those excised from freshly harvested seeds (data not shown). Also, despite the comparable TBARS levels in fresh and D embryos, viability declined in the latter only (i.e. fresh embryos exhibited 100% viability; Table 4.1). The retention of viability in fresh embryos, despite the evident lipid peroxidation, may be related to fact that $O_2^$ production in fresh embryos was limited to lower levels than D embryos (Fig. 4.1A), while the activities of certain antioxidant enzymes in fresh embryos were often higher than D embryos (see Figs 4.3-4.6). Protective mechanisms against ROS during seed storage are predominantly enzymic (Bailly, 2004).

During zygotic embryogenesis SOD, CAT and AsPX activities and/or expression change markedly (Puntarulo *et al.*, 1991; Bailly, 2004). So, the inter-species differences in enzymic antioxidant activities observed in fresh embryos here (e.g. SOD [Fig. 4.3A, B]), may have simply been a consequence of them being at different developmental stages.

Pre-conditioning

One of the common responses during exposure to many different types of abiotic stress is the production of ROS, which if uncontrolled can lead to severe cellular damage and even cell death (Oliver *et al.*, 2001; Pastori and Foyer, 2002). Studies on oxidative stress in seeds, such as an electron paramagnetic resonance study by Leprince *et al.* (1995) which demonstrated that $O_2^$ exacerbated free-radical production in orthodox seeds exposed to desiccation, are of particular interest to studies on the cryopreservation of plant germplasm. The Leprince *et al.* (1995) study did not involve exposing seeds to LN, but informed our thinking around why desiccation tolerance is frequently a prerequisite for cryo-tolerance in plant germplasm (reviewed by Benson, 1990; Dumet *et al.*, 2000; Dumet and Benson, 2000). In the present study, evaporative (i.e. flash drying) and osmotic (i.e. during cryoprotection) dehydration (in *H. montanus* embryos only) increased O_2^- levels relative to fresh embryos and this trend was significantly more pronounced for both species when flash drying and cryoprotection were combined (Fig. 4.1A, B).

Desiccation stress, apart from resulting in the loss of hydrophilic interactions and the associated perturbation of macromolecule structure (reviewed by Crowe and Crowe, 1986; Pammenter and Berjak, 1999; Walters *et al.*, 2002), can significantly enhance free-radical production in non-orthodox seeds (Hendry *et al.*, 1992; Leprince *et al.*, 1993; Varghese and Naithani, 2002; Bailly, 2004; Francini *et al.*, 2006; Pukacka and Ratajczak, 2006; Roach *et al.*, 2008). This increase in free-radical production, believed to be the consequence of uncoordinated

metabolism that occurs at intermediate hydration levels during dehydration (Pammenter *et al.*, 1998, 2000; Côme and Corbineau, 1996; Walters *et al.*, 2002; Bailly, 2004), generally leads to lipid peroxidation and an accompanying decline in viability (Hendry *et al.*, 1992; Chaitanya and Naithani, 1994, 1998; Leprince *et al.*, 1994; Li and Sun, 1999; Greggains *et al.*, 2001; Varghese and Naithani, 2002; Francini *et al.*, 2006). In the present study, elevated post-drying O_2^- levels were accompanied by declines in viability (Fig. 4.1A, B) but in contrast to the studies referenced above, this was not correlated with an increase in TBARS levels (Fig. 4.2A, B). In fact, post-drying TBARS levels in both species were either similar to, or lower than fresh embryos. Xin *et al.* (2010) have similarly shown TBARS accumulation and viability loss to be unrelated in rapidly dried recalcitrant *Antiaris toxicaria* axes. Those authors suggest that viability loss under rapid drying in recalcitrant axes may be associated with mechanical or physical damage, rather than have a metabolic basis.

Cooling

As mentioned earlier, oxidative stress (primary and secondary) appears to be a major component of chilling and cryoinjury in a variety of plant tissues (Tapell, 1966; Benson, 1990; Prasad et al., 1994; Benson et al., 1995; Doke 1997; Day et al., 1998; Park et al., 1998; Harding, 1999; Fleck et al., 2000, 2003; Benson and Bremner, 2004; Blagojević, 2007; Johnston et al., 2007). Cryoinjury in seed tissues has also been attributed to an increase in oxidative stress (Dussert et al., 2003; Walters et al., 2004; Varghese and Naithani, 2008; Whitaker *et al.*, 2010). In the present study, cooling generally led to the greatest increase in O_2 production (Fig. 4.1A, B), and the greatest decline in enzymic antioxidant activity (e.g. SOD in A. belladonna; CAT, GR, POX and AsPX in both species [Figs 4.3, 4.4, 4.6 and 4.7 respectively]). This was accompanied by considerable or, most often complete post-thaw viability loss and when data for cooled and non-cooled treatments were pooled, there was a significant negative correlation between O_2 production and viability for both species (Fig. 4.1A, B). The results of such correlations should always be interpreted with caution since they are not necessarily causative. However, the loss of compartmentalisation and metabolic uncoupling that often accompanies freezing (Singh and Miller, 1985) can have catastrophic effects on both primary metabolism (reviewed by Tapell, 1966) and antioxidant defences (Guy, 1990). This can in turn inflict physical and metabolic 'lesions' on plant tissues, leading to an enhancement in free-radical production and an accompanying decline in viability (Benson and Withers, 1987; Benson and Noronha-Dutra, 1988; Benson et al., 1992, 1995; Okuda et al., 1994; Fleck et al., 1999, 2000, 2003; Varghese and Naithani, 2008).

In the present study, post-thaw TBARS levels in *A. belladonna* were relatively higher than fresh and pre-conditioned embryos, while post-thaw TBARS levels in *H. montanus* (except for

>0.4D-rapid and >0.4D-slow) were generally similar to, or slightly lower than fresh embryos (Fig. 4.2A, B). Potential explanations for these inter-species differences in post-thaw lipid peroxidation patterns, include the possibility that the majority of lipid hydroperoxides (LOOHs) generated as a consequence of lipid free-radical production in *H. montanus*, were reduced to hydroxy acids (e.g. HODEs) *in vivo*, as opposed to be being decomposed and rearranged into secondary reaction products such as MDA (as discussed by Esterbauer *et al.*, 1991), which was the major thiobarbituric acid-reactive substance measured here.

Influence of glycerol cryoprotection on post-drying and post-thaw viability as related to changes in oxidative stress metabolism

The nature of the damage associated with dehydration-induced oxidative stress can vary, as free-radicals can either directly or indirectly cause four types of cellular damage: (1) mitochondrial dysfunction; (2) enzyme inactivation; (3) membrane perturbation; and (4) genetic damage (Hendry, 1993; Riley, 1994; Foyer *et al.*, 2006). In the event of antioxidant enzyme inactivation one must consider that CAT and peroxidase are involved in the metabolism of H_2O_2 , a product of SOD catalyzed dismutation of O_2^- (Fridovich, 1986) and that a decrease in CAT and peroxidase activities could lead to an accumulation of H_2O_2 , which is cytotoxic (in the presence of metal catalysts; i.e. the Fenton reaction). More specifically, H_2O_2 can lead to lethal levels of hydroxyl radicals and related lipid peroxidation (MacRae and Ferguson, 1985).

The actual mechanism by which enzyme inhibition occurs in plants may be enzyme dependent and influenced by physico-chemical factors such as the WC of tissues prior to freezing (Benson and Bremner, 2004). Water content also affects the range of temperatures at which tissue water freezes and the cooling rate required to traverse that temperature range before ice nucleation, but since cryoprotectants actually alter the freezing properties of water, they have the potential to change the relationship between WC and the required cooling rate (Volk and Walters, 2006). Certain cryoprotectant additives may even offer 'biochemical' and 'physical' protection during cryopreservation (Benson and Bremner, 2004; Fuller, 2004). The ability of certain cryoprotectants like Gly (Polge et al., 1949) and dimethyl sulphoxide (DMSO) (Benson and Withers, 1987; Fleck *et al.*, 2000) to scavenge free-radicals for instance, may make a significant contribution to their protective efficacy as well as their colligative and osmotic properties (Benson and Bremner, 2004). Ultrastructural studies described in Chapter 3 (section 3.3) clearly showed Gly cryoprotection to promote the post-drying and post-thawing retention of ultrastructural integrity in the embryos investigated in this study and based on the results obtained here this may have been facilitated by the superior antioxidant protection in Gly CP, as opposed to non-CP, embryos after drying and cooling.

In the present study, except for SOD in *H. montanus* (Fig. 4.3B) and AsPX in both species (Fig. 4.7A, B), enzymic antioxidant activities in both species were often reduced relative to fresh embryos after partial dehydration (Figs 4.4-4.6A, B). In non-orthodox seeds enzymic antioxidants are often inactivated or function sub-optimally under conditions of water stress and, as observed here (Figs 4.4-4.6A, B), this loss of enzymic antioxidant capacity is often accompanied by a decline in viability (e.g. Hendry *et al.*, 1992; Chaitanya *et al.*, 2000; Greggains *et al.*, 2001; Varghese and Naithani, 2002; Francini *et al.*, 2006; Tommasi *et al.*, 2006). The absence or poor expression of mechanisms that allow orthodox seeds to avoid and recover from free-radical-induced injury during dehydration, such as the maintenance and/or stimulation of antioxidant defences (Leprince *et al.*, 2000; De Tullio and Arrigoni, 2003; Bailly, 2004; Boudet *et al.*, 2006), may therefore represent one of the major determinants of desiccation sensitivity in the amaryllid embryos investigated here.

The post-drying decline in enzyme activity observed here was, however, consistently less severe in Gly CP, as opposed to non-CP, embryos (e.g. CAT: <0.4D-Gly for both species [Fig. 4.4A, B]; GR: > and <0.4D-Gly for both species [Fig. 4.5A, B]; POX: > and <0.4D-Gly for A. belladonna [Fig. 4.6A]). In fact, post-drying activities for AsPX (in both species [Fig. 4.7A, B]) and SOD (in *H. montanus* only [Fig. 4.3B]) were even enhanced relative to fresh embryos in some Gly CP+D treatments. A dehydration-induced increase in SOD and/or AsPX activity has been observed in other non-orthodox seeds (e.g. Araucaria bidwilli [Francini et al., 2006]; Azadirachta indica [Varghese and Naithani, 2002, 2008]; Acer saccharinum [Pukacka and Ratajczak, 2006]). As in other seed-based studies (e.g. Varghese and Naithani, 2002; Garnczarska et al., 2008), the post-drying enhancement of SOD activity observed in H. *montanus* here, may have been associated with the desiccation-induced accumulation of O_2 (discussed earlier). Ascorbate peroxidase activity may have been enhanced (in both species) to compensate for the post-drying decline in CAT and GR activity, both of which also scavenge H₂O₂. Compared with orthodox seeds, recalcitrant types are often characterised by enhanced activity of the enzymes of the ascorbate-glutathione pathway, such as AsPX, and in recalcitrant seeds decreased sensitivity to dehydration may be correlated with enhanced AsPX activity (see Tommasi et al., 1999). Such results are often attributed to the presence of AsPX in all cell compartments and its high affinity for H₂O₂ (Pukacka and Ratajczak, 2006).

Both Gly and Suc CP failed to reduce post-drying O_2^- production relative to non-CP embryos (Fig. 4.1A, B). However, the relatively higher post-drying antioxidant enzymic activities in Gly CP compared with non-CP embryos (discussed above) was accompanied by relatively lower lipid peroxidation levels (Fig. 4.2A, B). Post-drying viability retention in non-orthodox seeds has been correlated with reduced lipid peroxidation and the retention of enzymic

antioxidant activity elsewhere (e.g. Chaitanya and Naithani, 1994; Varghese and Naithani, 2001) and this may explain why post-drying viabilities in Gly CP embryos were consistently higher than non-CP embryos (at comparable WCs; Table 4.1). Sucrose and similar sugars are believed to stabilise membranes during the removal of water by interacting with the polar head groups of phospholipids during hypertonic exposure, replacing water that usually forms hydrogen-bonded bridges with the lipids (Rudolph and Crowe, 1985; Strauss and Hauser, 1986). However, membrane impermeable sugars often lose their protective effect at high (≥ 1 M) concentrations, mainly due to the severe osmotic injury they inflict at such concentrations (Finkle *et al.*, 1985; Muldrew *et al.*, 2004). This may have been the case in Suc CP+D embryos in this study. These data suggest that the potential for a cryoprotectant to reduce free-radical mediated damage (e.g. lipid peroxidation) in recalcitrant seed tissues may be realised, only when its osmotic properties are undamaging to the tissue.

Despite the reputation of glycerol as an effective free-radical scavenger (Polge *et al.*, 1949; Benson and Bremner, 2004; Fuller, 2004), Gly CP did not lead to a significant decline in postthaw O_2^- production (Fig. 4.1A, B) or lipid peroxidation (Fig. 4.2A, B), relative to non-CP + cooled embryos. In spite of this, the highest post-thaw viabilities and antioxidant enzyme activities (across all five enzymes), across both species, were associated with Gly CP, as opposed to non-CP, embryos (Figs 4.3-4.7A, B). As in other studies (e.g. Guy, 1990; Thomas *et al.*, 1999; Varghese and Naithani, 2008), SOD (in *A. belladonna* only) and CAT activities often declined after cooling in the embryos investigated here (Figs 4.3A and 4.4A, B respectively). However, SOD activity was detected in a number of Gly CP treatments in which post-thaw viability retention was observed, with its activity in these treatments being relatively higher than the cooled treatments that led to no viability retention, in *A. belladonna*, and relatively higher than fresh and partially dried embryos, in *H. montanus*. Superoxide dismutase-enhanced tolerance to freezing stress in plant tissues has been previously reported (McKersie *et al.*, 1996; Park *et al.*, 1998) and the over-expression of SOD in transgenic plants has been shown to confer some protection against enhanced oxidative stress (Sen Gupta *et al.*, 1993).

A further consideration is that H_2O_2 is produced by the reaction of SOD and if allowed to accumulate in cells, becomes toxic via Fenton chemistry (for reviews see Scandalios, 1997; Benson and Bremner, 2004). Its removal by CAT is therefore a vital component of cellular antioxidant protection (for reviews see Scandalios, 1990, 1997) but AsPX, due to its high affinity for H_2O_2 and presence in all cell compartments, is just as important an enzyme in H_2O_2 detoxification in plant cells (Pukacka and Ratajczak, 2006). Ascorbate peroxidase catalyses the reaction between ascorbic acid and H_2O_2 which forms monodehydroascorbate. Ascorbate is thereafter regenerated from monodehydroascorbate through an enzymic route (monodehydroascorbate reductase) or via spontaneous transformation of monodehydroascorbate into dehydroascorbate (DHA) (see Pukacka and Ratajczak, 2006). The regeneration of ascorbic acid from DHA occurs via a catalytic reaction by dehydroascorbate reductase coupled to a glutathione-oxidised glutathione (GSH-GSSG) cycle in which reduced GSH is oxidised to the disulphide GSSG (for a review see Kranner et al., 2006). The regeneration of GSH is carried out by glutathione reductase (for a review see Sharma and Davis, 1997). So, together with CAT, control of H_2O_2 levels by the ascorbate-glutathione enzymes (mentioned above) is critically important to the amelioration of oxidative stress in vegetative (Asada, 1992) and seed (Pukacka and Ratajczak, 2006) tissues. With reference to other studies (e.g. Aebi, 1983; MacRae and Ferguson, 1985; Fridovich, 1986) the relatively higher post-thaw SOD activities in the Gly CP treatments that facilitated post-thaw viability retention in this study (Fig. 4.3A, B), probably led to increased levels of H_2O_2 , most especially in treatments in which CAT and/or AsPX activities were compromised. This may explain, (1) why there was the tendency for treatments associated with post-thaw viability retention in H. montanus to exhibit CAT activity across relatively more replicates than those associated with complete viability loss (Fig. 4.4B; detailed data not shown); (2) why (except for >0.4D-rapid) post-thaw CAT activity in A. belladonna was detected only in treatments that facilitated post-thaw viability retention (Fig. 4.4A); (3) why across the five enzymes assayed, it was only for CAT that enzyme activity was significantly correlated with viability (for both species; Fig. 4.4A, B); (4) why, for both species, the treatments that were associated with the two highest post-thaw viabilities exhibited SOD and CAT activities that were relatively higher than the other cooled treatments; (5) why the four highest post-thaw AsPX activities in A. belladonna were associated with the four highest postthaw viabilities (Fig. 4.7A); and (6) why (except for <0.4D-Gly-slow) post-thaw AsPX activity in *H. montanus* was detected only in treatments that facilitated post-thaw viability retention (Fig. 4.7B).

The protective roles of SOD, CAT and AsPX in relation to free-radical-mediated damage of cellular membranes are well documented (Touchell and Walters, 2000; Bailly, 2004; Benson and Bremner, 2004; Kibinza *et al.*, 2006), and there are reports on the impairment of cellular metabolism in seeds upon failure of these enzymic antioxidants (Chaitanya and Naithani, 1994, 1998). Hydrogen peroxide levels were not measured here but the post-thaw patterns in SOD, CAT and AsPX antioxidant activities discussed above, suggest that Gly CP embryos may have been more efficient than non-CP embryos at the enzymatic detoxification of H₂O₂. Also, higher post-thaw viabilities in Gly CP embryos were often accompanied by relatively higher POX activities than non-CP embryos (Fig. 4.6A, B). This suggests that protection against substrate level peroxidation by this guaiacol specific peroxidase (for a review see Elstner and Osswald,

1994) may have also favoured post-thaw viability retention in Gly CP embryos. Studies on plant (Fleck *et al.*, 2003) and animal (Zhou *et al.*, 2008) cells have reported post-thaw viability retention in CP cells to be correlated with the maintenance or enhancement of antioxidant activity. The exact mechanism/s upon which this relationship is based have yet to be identified but some possibilities are discussed in Chapter 8.

A further consideration is that viable tissues recovered from cryostorage are influenced by their pre-storage 'metabolic history', such that the status of cell signalling mechanisms and redox states upon freezing, influences the subsequent manifestation of free-radical injury during and after retrieval from storage (Fennema and Sung, 1980; Benson and Bremner, 2004). Incipient, deleterious metabolic reactions may actually be 'fixed' at the point of freezing and 'held in suspension' until their effects are manifested on thawing and devitrification (Fuller *et al.*, 1988; Benson, 1990; Benson and Bremner, 2004). Presently, the post-drying decline in enzymic antioxidant activities, and increase in lipid peroxidation were more severe in the absence of Gly CP. This suggests that the cumulative effects of storage- and dehydration-induced lipid peroxidation (Fig. 4.2A, B), and dehydration-induced decline in enzymic antioxidant activities (Figs 4.3-4.7A, B), may have pre-disposed non-CP+D embryos to greater freeze-thawing damage than Gly-CP+D embryos.

As in other studies (e.g. Fleck *et al.*, 2003), enzymic antioxidant activity was detected in non-viable embryos here. Glutathione reductase in *H. montanus* (Fig. 4.5B) for example, was detected across all cooled treatments, irrespective of whether they facilitated post-thaw viability retention or not. Data about the regulation of enzymes such as GR during periods of abiotic stress are less clear than for CAT and AsPX and do not permit the construction of a clear picture of their possible roles in the tolerance of abiotic stresses (Bailly, 2004). However, Fleck *et al.* (2003) also caution that since abiotic (chemical as opposed to metabolic) free-radical mediated oxidative reactions continue to occur in dead cells, lethally damaged cells can display residual enzyme activity.

4.5 Concluding remarks

Pre-conditioning and freeze-thawing led to an increase in oxidative stress and the accompanying decline in viability suggests that oxidative stress was a major component of cryoinjury in the embryos investigated here. Post-thaw viability retention in Gly CP+D embryos was significantly higher than non-CP+D embryos, possibly related to the relatively lower post-drying lipid peroxidation levels and relatively higher post-drying and post-thawing enzymic antioxidant activities in Gly CP embryos. The results of this study lend support to other suggestions that oxidative stress is a major component of cryoinjury in recalcitrant seeds (e.g. Chandel *et al.*,

1996; Touchell and Walters, 2000; Normah and Makeen, 2008; Walters *et al.*, 2008) and corroborate findings of others who have concluded that enzymic antioxidant protection has a role in low temperature and cryo-tolerance (Green *et al.*, 1986; Prasad, 1996; Fryer *et al.*, 1998; Fleck *et al.*, 2000; Touchell and Walters, 2000; Xin and Browse, 2000; Dussert *et al.*, 2003; Sung *et al.*, 2003; Odani *et al.*, 2003; Johnston *et al.*, 2007; Varghese and Naithani, 2008). However, in the present study the retention of antioxidant activity was not always accompanied by the retention of viability. This reinforces the notion that post-thaw survival in plant germplasm relies on the protection of the suite of integrated systems responsible for gross physiological regulation.

Pre-conditioning treatments that provide pathways for reduction of ROS may play a part in facilitating viability retention in recalcitrant zygotic germplasm thawed after retrieval from cryostorage. Treatments such as Gly cryoprotection, may act either by checking ROS production or by providing/protecting ROS scavenging agents during pre-conditioning for, and after retrieval from, cryostorage. Additionally, adaptations of classical cryo-based 'stress acclimation' methods (e.g. Reed and Yu, 1995; Benson *et al.*, 1996; Dumet *et al.*, 2000) may decrease cryo-sensitivity in recalcitrant zygotic germplasm by enhancing stress tolerance mechanisms such as antioxidant protection; this forms the basis of Chapter 5.

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CHAPTER FIVE: Can osmotic or oxidative stress pre-treatment decrease *Amaryllis belladonna* and *Haemanthus montanus* zygotic embryo cryo-sensitivity?

Abstract

Exposure of certain plant tissues to low levels of oxidative or osmotic stress can improve their tolerance to a wide range of stresses. This study investigated whether exposure of recalcitrant Haemanthus montanus (Baker) zygotic embryos to low levels of oxidative stress provoked by exogenously applied hydrogen peroxide (H₂O₂), or exposure of recalcitrant Amaryllis belladonna (L.) embryos to low levels of osmotic stress provoked by low water potential mannitol and polyethylene glycol solutions, reduces their sensitivity to subsequent dehydration and freeze-thaw stresses associated with cryopreservation. Since increased antioxidant activity has been correlated with enhanced tolerance to abiotic stresses, viability of embryos in this study was related to changes in post-drying and post-thaw total antioxidant activity (TAA). Exposure of A. belladonna zygotic embryos to osmotica decreased embryo water content and very often viability as well. The variability in osmotic stress-induced viability loss across different water potential × temperature × incubation time combinations was considerable but there was a trend for viability loss to be greater at lower water potentials and longer incubation times. With rapid drying and freeze-thawing the decline in viability and TAA relative to fresh embryos, was consistently more severe in osmotica pre-treated, as opposed to untreated, embryos. Exposure of H. montanus zygotic embryos to H_2O_2 concentrations >0.10 mM led to a decline in viability relative to fresh embryos (100% viability). Exposure to 0.10 mM H₂O₂ for 30 min had no adverse effect on viability. However, when this pre-treatment was followed by rapid drying and freeze-thawing, the post-drying and post-thaw decline in viability and TAA relative to fresh embryos was generally more severe when embryos were pre-treated with H_2O_2 . The results suggest that exposure of recalcitrant amaryllid zygotic embryos to osmotic and oxidative stress pre-treatments may pre-dispose such explants to greater post-drying and postfreezing TAA and viability loss than untreated zygotic embryos, rather than inducing some stress tolerance.

5.1 Introduction

Improving the vigour and viability of plant tissues exposed to the dehydration and freezethaw stresses associated with cryopreservation is based on fundamental studies of cold tolerance. A commonly used method of improving cryo-tolerance in plant germplasm is stress acclimation, particularly cold acclimation or cold hardening, during which the tolerance of explants to freezing is enhanced by gradual exposure to low but above zero temperatures in combination with shortened day-length, osmotic changes and/or abscisic acid (ABA) treatment (e.g. Reed and Yu, 1995; Benson *et al.*, 1996; Dumet *et al.*, 2000; reviewed by Panis and Lambardi, 2006). These treatments are based on observations that the tolerance of a number of species (mainly temperate) to freezing can increase during late autumn or early winter, when these plants perceive the seasonal changes in temperature. This triggers a change in gene expression, referred to as 'adaptive metabolism', which leads to enhanced freezing tolerance (Guy, 1990; Xin and Browse, 2000; Kaplan *et al.*, 2004). Adaptive metabolism therefore has important implications for cryopreservation, since it may allow for the manipulation of explants to produce such responses and so improve cryo-tolerance (Benson *et al.*, 1996; Johnston *et al.*, 2007; Benson, 2008).

A number of natural and simulated cold-adaptations have been exploited to improve cryostorage protocols for plant germplasm but these have been successful almost exclusively in temperate species (Reed and Yu, 1995; Benson *et al.*, 1996; Johnston *et al.*, 2007; reviewed by Benson, 2008). Additionally, the low *in vitro* growth temperatures (e.g. Yongjian and Reed, 2000) and/or high-sugar pre-treatments (e.g. Borochov *et al.*, 1989; Johnston *et al.*, 2007) generally associated with simulated cold adaptations preclude their application to recalcitrant seeds, which are desiccation- and very often chilling-sensitive as well (Chin and Roberts, 1980; Ellis *et al.*, 1990; Hong and Ellis, 1996). However, reports of a web of overlapping signals in stress response pathways, where induction of tolerance to one particular stress may result in acquired tolerance to other stresses (a phenomenon referred to as 'cross-tolerance' [Pastori and Foyer, 2002; Kozlowski and Pallardy, 2002; Xiong *et al.*, 2002]), offer some hope for plant tissues that are not amenable to conventional cold acclimation. In this regard, many of the responses observed during cold acclimation are strikingly similar to responses to other stresses, e.g. drought and osmotic stress (Gazzarrini and McCourt, 2001).

As in other plant tissues (Tapell, 1966; Levitt, 1980; Steponkus, 1985; Benson and Withers, 1987; Benson, 1990; Benson *et al.*, 1992, 1995; Prasad *et al.*, 1994a; Doke, 1997; Day *et al.*, 1998; Park *et al.*, 1998; Fleck *et al.*, 1999, 2000, 2003; Johnston *et al.*, 2007) oxidative stress appears to be a major source of chilling and cryoinjury in seed tissues (Touchell and Walters, 2000; Dussert *et al.*, 2003; Varghese and Naithani, 2008; Whitaker *et al.*, 2010). Exposure of certain plant tissues to low levels of oxidative stress (usually in the form of exogenous hydrogen peroxide [e.g. Azevedo Neto *et al.*, 2005; Hung *et al.*, 2005; Wahid *et al.*, 2000) can improve their tolerance to a wide range of abiotic stresses by, among other things, regulating the expression of a number of 'defence' genes. Some of these genes are involved in antioxidant protection (Kovtun *et al.*, 2000; Robert and David, 2004; Pearce, 2004; Hung *et al.*, 2005) and increased

antioxidant activity has been correlated with enhanced tolerance to a number of different stresses in plants (e.g. drought, salinity, chilling and freezing [Dhindsa and Matowe, 1981; Senaratna and McKersie, 1986; Leprince *et al*, 1990; Dhindsa, 1991; Price and Hendry, 1991; Anderson *et al.*, 1992; Seel *et al.*, 1992; Reuzeu and Cavalie, 1995; O'Kane *et al.*, 1996; Scebba *et al.*, 1999; Baek and Skinner, 2003]).

A practical application may be derived from the above findings, where H₂O₂ and osmotica pre-treatments could be used to induce stress acclimation in cryo-sensitive plant material such as recalcitrant zygotic germplasm prior to partial dehydration (which is a standard pre-treatment for the cryopreservation of such explants) and freezing, and so reduce their cryo sensitivity. The present study tested this hypothesis by investigating whether the exposure of recalcitrant Haemanthus montanus (Baker) zygotic embryos to low levels of oxidative stress (provoked by exogenously applied H_2O_2 , or recalcitrant *Amaryllis belladonna* (L.) zygotic embryos to low levels of osmotic stress (provoked by low water potential solutions of osmotica) reduces their sensitivity to subsequent partial dehydration and freeze-thawing. Solutions of sucrose or even sucrose pre-culture could have been used to expose embryos to low water potentials. In fact, sucrose pre-culture has been shown to increase freezing tolerance in vegetative and embryogenic tissues, however, sucrose when applied in solution or culture serves as a carbon substrate facilitating the *in vivo* synthesis of carbohydrates (Dumet *et al.*, 1993; Xu and Bewley, 1993). In the amaryllid embryos investigated here provision of a carbon substrate may have stimulated germinative events during pre-treatment and as shown by Sershen et al. (2008), amaryllid embryos become more desiccation-sensitive as germination progresses. So, to avoid the stimulation of germination, mannitol and polyethylene glycol were selected as osmotica here since solutions of these membrane impermeable compounds (Michel et al., 1983) were unlikely to have provided an additional carbon substrate for the embryos during pre-treatment.

To assess the role of antioxidant protection in dehydration and freezing tolerance, which is well documented in plant tissues (Green *et al.*, 1986; O'Kane *et al.*, 1996; Prasad, 1996; Xin and Browse, 2000; Touchell and Walters, 2000; Dussert *et al.*, 2003; Sung *et al.*, 2003; Odani *et al.*, 2003; Johnston *et al.*, 2007; Varghese and Naithani, 2008), *H. montanus* and *A. belladonna* post-drying and post-thaw viabilities were related to changes in total antioxidant activity (TAA) in the present study. Several total antioxidant assays have been employed to determine antioxidant scavenging activity (see Arnao *et al.*, 1999; Rice-Evans, 2000). One particular method, namely, the 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging assay has been used for a variety of applications (Rice-Evans, 2000; Johnston *et al.*, 2006), including the estimation of TAA in different types of plant germplasm (e.g. Johnston *et al.*, 2006, 2007) and was employed here. The ABTS assay is based on the scavenging of the

ABTS radical (ABTS•⁺; which occurs as a blue-green chromophore) generated from ABTS and $K_2S_2O_8$. More specifically, the assay is designed to measure the rate at which ABTS•⁺ is reduced back to ABTS by antioxidants; the rate of this decrease depends on the type and amount of antioxidants (Johnston *et al.*, 2006).

Additionally, studies described in Chapter 4 (section 4.3) showed post-thaw viability and enzymic antioxidant activity (of selected enzymes) to be maximised for both species when partial dehydration and cooling was preceded by glycerol (Gly) cryoprotection (CP). To distinguish between the effects of the osmotic and oxidative stress pre-treatment and CP, H_2O_2 pre-treatment + dehydration/freezing and osmotic stress pre-treatment + dehydration/freezing experiments were carried out with and without prior Gly CP.

Studies described in Chapter 2 (section 2.3) showed zygotic embryo WCs <0.4 g g⁻¹ to be superior to those >0.4 g g⁻¹, in promoting post-thaw viability for *A. belladonna*, while the reverse was true for *H. montanus*. Those studies also showed post-thaw viability in both species to be best when embryos were rapidly cooled. Based on those findings, zygotic embryos were cooled here, either at WCs in the optimum range (as in *A. belladonna*) or at WCs that are presumably 'sufficiently low' or 'deleteriously high' (as in *H. montanus*) for cryopreservation, but using rapid cooling rates only.

It would have been ideal to estimate total antioxidant activity immediately after H_2O_2 and osmotic stress exposure, as well as after $H_2O_2/osmotic$ stress pre-treatment + drying and H₂O₂/osmotic stress pre-treatment + freeze-thawing. However, as is often the case with recalcitrant seeds, seed numbers were limiting; since this study is primarily concerned with the ability of zygotic embryos to tolerate the oxidative stress associated with cryopreservation, it was decided to estimate TAA after the procedures shown to induce harmful free-radical production in A. belladonna and H. montanus zygotic embryos, viz. dehydration and freezethawing (see Chapter 4; section 4.3). Further to this, in desiccation-sensitive tissue the effect of a stress, particularly a mild stress, is unlikely to be instantaneous and if a stress induces a metabolic disorder, it takes time for the damage consequent upon that disorder to accumulate (Walters et al, 2001). In fact, the damage incurred may be evident only after the system has rehydrated fully, and metabolism has been reinitiated. In light of this, TAA was measured here, immediately after rehydration for cooled and partially dehydrated embryos, and immediately after excision for untreated (fresh) embryos. However, this does rely on the assumption that the metabolic status of cells during this early recovery phase is a reflection of damage incurred during the stress as well as mitigation against the stress.

5.2 Materials and Methods

Plant material

Mature *A. belladonna* and *H. montanus* fruits were harvested directly from parent plants and transported in plastic bags to the laboratory with minimum delay (1-2 d) or water loss. Upon arrival, the seeds were decontaminated and stored 'hydrated', as described in Chapter 2 (section 2.2).

Reagents

Ethylene diamine tetra-acetic acid (EDTA), 2,2'-azino-bis-3-ethylbenzthiazoline-6sulphonic acid (ABTS), polyethylene glycol (PEG) 8000, polyvinylpyrrolidone (PVP), mannitol (MAN), hydrogen peroxide (H₂O₂), potassium persulfate (K₂S₂O₈), sodium chloride (NaCl), calcium chloride (CaCl₂) potassium chloride (KCl), sodium di-hydrogen phosphate (NaH₂PO₄) and Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were analytical grade and locally available.

Embryo pre-treatments

Seeds were stored for between 7-10 d prior to experimentation. For these experiments zygotic embryos were excised and collected within closed Petri dishes on filter paper moistened with sterile calcium-magnesium solution (CaMg solution: 0.5 µM CaCl₂.2H₂O and 0.5 mM MgCl₂.6H₂O [Mycock 1999]), before exposure to any of the treatments described below. In order to minimise the potential variation in drying and/or cooling rate as a function of embryo size, only embryos of between 4-6 mm in length were used for these experiments.

Oxidative stress pre-treatment

As in others studies (e.g. Gadjev *et al.*, 2006) oxidative stress was provoked in *H. montanus* embryos by exogenous application of H_2O_2 and to optimise the concentration of H_2O_2 used in subsequent H_2O_2 pre-treatment + cryopreservation experiments, *H. montanus* embryos were initially immersed in solutions of 0.05, 0.10, 0.15 and 0.20 mM aqueous H_2O_2 for 30 min in the dark (with ten embryos at each concentration). Immediately after the exposure period, embryos were decontaminated and recovered *in vitro* to assess viability. Viability (based on the production of roots and shoots) was assessed daily for 7 d and based on these results (described in section 5.3), embryos were exposed to 0.1 mM H_2O_2 for 30, 60 and 120 min (with ten embryos at each time interval) to optimise the exposure time for all subsequent H_2O_2 pre-treatment + cryopreservation studies. These embryos were also decontaminated and recovered

in vitro immediately after the appropriate exposure period, and subsequently assessed for vigour and viability to identify the treatment with maximum exposure time, yet minimum vigour and viability loss.

Osmotic stress pre-treatment

A. belladonna embryos were exposed to sterile aqueous solutions of MAN and PEG with water potentials (Ψ) of -0.3, -0.6 and -1.2 MPa at temperatures of 6, 16 and 25°C, respectively. Solutions possessing these target water potentials (WPs) were prepared using equations 1 and 2 (after Michel *et al.*, 1983):

$$Ψ_{MAN} = -0.078 [MAN] T - 22.75 [MAN]$$
 [1]
 $Ψ_{PEG} = 1.29 [PEG]^2 T - 140 [PEG]^2 - 4 [PEG]$ [2]

where Ψ is WP (in bars); [MAN] is mannitol concentration in molal; [PEG] is polyethylene glycol concentration in g/g H₂O; and T is temperature in °C.

To optimise the WP × temperature × incubation time combination to be used in subsequent osmotic stress pre-treatment + cryopreservation studies, 15 embryos were exposed to each of the 27 possible WP × temperature × incubation time combinations. Here, freshly excised embryos were immersed in 20 ml osmoticum (of -0.3, -0.6 and -1.2 MPa) within glass vials and maintained at the desired temperature (6, 16 and 25°C) using water baths, in the dark. Polyethylene glycol solutions were continually aerated with an oxygen-enriched gas mixture containing 7.5 parts oxygen and 2.5 parts nitrogen (after Bujalski *et al.*, 1989). After incubation for the prescribed time, five embryos were used to determine average water content (WC) on a dry mass basis (as described below) while the remaining ten embryos were recovered *in vitro* to assess vigour and viability. Results of these studies were used to select four WP × temperature × incubation time combinations (two for each osmoticum) with maximum exposure time, yet minimum vigour and viability loss.

Osmotic stress pre-treatment + cryopreservation studies

Immediately after exposure to -0.3 MPa MAN at 16°C for 12 h, -0.3 MPa MAN at 6°C for 24 h, -0.3 MPa PEG at 16°C for 12 h, and -0.3 MPa PEG at 6°C for 12 h, 115 *A. belladonna* embryos from each treatment, as well as 115 freshly excised embryos, were rapidly dehydrated via flash drying to WCs in the range 0.36-0.29 g g⁻¹ (collectively referred to as '<0.4D' from here on), with and without prior Gly CP. For cryoprotection, embryos were immersed in a 5% solution of Gly (v/v) for 1 h, and thereafter transferred to a 10% Gly (v/v) solution for a further hour. Immediately after flash drying five embryos were assessed for WC while 55 embryos were rehydrated in CaMg solution at 25°C for 30 min, in the dark. Ten of these rehydrated

embryos were recovered *in vitro* to assess viability while 45 were immediately measured for TAA. The remaining 55 non-rehydrated embryos were immediately rapidly cooled (at hundreds of °C s⁻¹) and stored in liquid nitrogen (LN) as described in Chapter 2 (section 2.2). After 48 h, embryos were recovered from LN and immediately after thawing and rehydration (as described below), ten embryos were recovered *in vitro* to assess viability, while 45 embryos were immediately measured for TAA.

Oxidative stress pre-treatment + cryopreservation studies

Low levels of oxidative stress were provoked in freshly excised H. montanus embryos by exposing them to 0.1 mM H_2O_2 for 30 min. Immediately after exposure, 115 embryos were subjected to each of the following treatments: (1) flash drying to WCs < 0.4 g g⁻¹ (c. 0.35-0.30 g g⁻¹; referred to as '<0.4D' from here on); (2) flash drying to WCs >0.4 g g⁻¹ (c. 0.51-0.44 g g⁻¹; referred to as '>0.4D' from here on); and (3) flash drying to WCs > and <0.4 g g⁻¹ after Gly CP (referred to as '>0.4D-Gly' and '<0.4D-Gly' from here on). Freshly excised embryos that had not been exposed to H_2O_2 were also subjected to the above treatments. Cryoprotection was carried out as described for the osmotic stress pre-treatment + cryopreservation studies after exposure to H₂O₂. Immediately after flash drying five embryos were assessed for WC while 55 embryos were rehydrated in CaMg solution at 25°C for 30 min, in the dark. Ten of these rehydrated embryos were recovered *in vitro* to assess viability while 45 were immediately measured for TAA. The remaining 55 non-rehydrated embryos were immediately rapidly cooled (at hundreds of °C s⁻¹) and stored in LN as described in Chapter 2 (section 2.2). After 48 h, embryos were recovered from LN and immediately after thawing and rehydration, 10 embryos were recovered *in vitro* to assess viability, while 45 embryos were immediately measured for TAA.

Vigour and viability assessment for optimisation of oxidative/osmotic stress pre-treatments

Immediately after osmotic/oxidative stress pre-treatment, zygotic embryos were decontaminated and recovered *in vitro*, as described in Chapter 2 (section 2.2). Root and shoot production was scored daily across all treatments, for 7 d as in the H_2O_2 pre-treatments, or 30 d as in the osmotic stress pre-treatments. Viability was based on root and shoot production and daily germination records were used to calculate vigour, in terms of the two indices employed in the studies discussed in Chapter 2 (see section 2.2 for a description of these indices): (1) mean time to germinate (MTG; after Ellis and Roberts, 1981); and (2) germination index (GI; after Czabator, 1962).

Thawing, rehydration and in vitro viability assessment of cooled zygotic embryos

For H_2O_2 pre-treatment + cooled and osmotic stress pre-treatment + cooled treatments, embryos retrieved from LN were thawed in CaMg solution held at 40°C for 2 min and rehydrated in CaMg solution at 25°C for 30 min, in the dark. Ten of these rehydrated embryos were decontaminated and recovered *in vitro* for 40 d, as described in Chapter 2 (section 2.2), with one sub-culture after 20 d. *In vitro* grown embryos were assessed for viability daily for 40 d; viability was defined by root and shoot production.

Ten embryos (with shedding WCs of *c*. 4.67 g g⁻¹ for *A. belladonna* and 5.05 g g⁻¹ for *H. montanus*) exposed to none of the embryo treatments described above (i.e. fresh embryos) were also assessed for vigour and viability *in vitro*.

Water content determination

For WC determinations, embryos were weighed individually using a 6-place balance (Mettler, MT5; Germany) and dried in an oven at 80°C for 48 h before being re-weighed to determine the dry mass. Water content was expressed on a dry mass basis (dmb; g H₂O per g dry matter [g g⁻¹]).

Extraction and assay of total antioxidant activity (TAA)

Total antioxidant activity was measured via the ABTS radical cation decolorization assay, as described by Re *et al.* (1999). Immediately after rehydration, 45 embryos from each treatment for which antioxidant activity was to be estimated were divided into three replicate batches of 15 embryos and immediately extracted for total antioxidants. For this, the three embryo batches were homogenised separately in LN with 0.1 g insoluble PVP and suspended in 1.5 ml cold (4°C) 50 mM KH₂PO₄ buffer (pH 7) containing 1 mM CaCl₂, 1 mM KCl and 1 mM EDTA (Johnston *et al.*, 2006). Insoluble PVP was used to reduce phenolic-induced background interference and was removed along with cell debris at the first centrifugation step. Extracts were incubated on ice for 20 min, with gentle vortexing every 5 min. Samples were then centrifuged for 15 min at 4°C and 12,000 g, and the supernatant transferred to pre-cooled (4°C) microcentrifuge tubes on ice. Antioxidant assays were performed immediately. Three batches of freshly excised embryos that had not been exposed to any pre-treatment, dehydration or cooling were also analysed for TAA.

For the ABTS assay an aqueous solution of 7 mM ABTS and 2.45 mM $K_2S_2O_8$ was prepared and allowed to stand at room temperature for 14-16 h in the dark before first use. The resulting ABTS radical solution was diluted with phosphate buffered saline (5 mM NaH₂PO₄ + 37.5 mM NaCl, pH 7.4) until an absorbance of 0.70±0.02 at 734 nm (read using a UV-Vis spectrophotometer; Varian) was obtained. For both standards and samples, 1 ml of diluted ABTS radical solution was added to a 1.5 ml plastic cuvette, and the absorbance read at 734 nm for the 0 min reading. Then without delay, 10 μ l of aqueous tissue extraction or Trolox standard was added to the cuvette, the reaction mixed by inverting the cuvette three times, and the decrease in absorbance at 734 nm was read after 0.5, 1 and 2 min. Assays were performed twice on three different extracts, in the dark. A standard curve was created with a water-soluble ∞ -tocopherol analogue; 0.05-1.0 mM Trolox in phosphate antioxidant extraction buffer. Change in absorbance was calculated for samples and expressed as Trolox equivalents on a dry weight basis using the standard curve.

Statistical analysis

Total antioxidant activity data were tested for significant inter-treatment differences by Analysis of Variance (*ANOVA*; SPSS, Version 15). Multiple comparisons were then made using a Duncan's mean separation test. Correlations between TAA and viability were tested for using a Pearson correlation test (SPSS, Version 15). For all correlation analyses involving viability, viability percentages were arcsin transformed to conform data to parametric test assumptions. All viability data were tested for significant inter-treatment differences using null-model chisquared analyses (specifically designed to assess non-parametric data) (EcoSim Version 7.72 [developed by Gotelli and Entsminger, 2009]). Water content data were tested for significant differences using a Mann-Whitney-U test, or *ANOVA* where data were parametric (SPSS, Version 15). All statistical tests were performed at the 0.05 level of significance.

5.3 Results

Optimisation of osmotic stress pre-treatment for A. belladonna zygotic embryos Mannitol

Exposure to all MAN-based osmotic stress pre-treatment combinations decreased embryo WC, with exposure to WPs of -0.3 MPa resulting in WCs of 4.11-4.86 g g⁻¹; WPs of -0.6 MPa resulting in WCs of 4.07-4.61 g g⁻¹; and WPs of -1.2 MPa resulting in WCs of 3.97-4.54 g g⁻¹ (data not shown). Except for the fact that longer exposure times resulted in slightly lower WCs, within any particular WP category, there were no consistent trends among incubation time, temperature and embryo WC.

There were just three treatments (-0.6 MPa 25°C 1 h; -0.6 MPa 16°C 12 h; and -0.3 MPa 16°C 24 h) that did not reduce viability relative to fresh embryos (100% viability) (Table 5.1). Except for -1.2 MPa 16°C 1 h (10% viability loss), exposure to WPs of -1.2 MPa resulted in 20-70% viability loss. Except for one treatment (-0.6 MPa 16°C 24 h) in which viability loss was

equivalent to 50%, exposure to WPs of -0.3 and -0.6 MPa resulted in 10-30% viability loss. Viability loss across all three WPs was generally higher for longer incubation times (Table 5.1).

Exposure to WPs of -0.3 and -0.6 MPa generally enhanced vigour in terms of an increase in germination index (GI) relative to fresh embryos (Table 5.1). This increase was most often based on an increase in peak value (PV) and except for -0.6 MPa 25°C 1 h, generally occurred after exposure to MAN, at relatively lower temperatures (i.e. 6 and 16°C). While exposure to WPs of -1.2 MPa generally decreased GI relative to fresh embryos, -0.3 MPa 16°C 1 h resulted in the greatest increase in GI relative to fresh embryos.

An increase in mean time to germinate (MTG) relative to fresh embryos, indicative of a decline in vigour, was observed in a number of treatments across all three WPs but this increase was slightly more common at WPs of -0.6 and -1.2 MPa (Table 5.1). In the isolated cases where MTG was substantially reduced relative to fresh embryos at WPs of -0.6 and -1.2 MPa, viability loss was relatively high (e.g. -0.6 MPa 16°C 24 h = 50% viability). Except for -0.6 MPa 25°C 1 h, high GIs were generally accompanied by low MTGs.

There were indications that treatments involving incubation temperatures of 25°C led to a slightly more severe loss of vigour and viability than temperatures of 6 and 12°C (Table 5.1).

Based on the fact that they resulted in just 10% viability loss, increased vigour (in terms of an increase in GI and a decrease in MTG) relative to fresh embryos, and maximised the time available for stress acclimation, -0.3 MPa 16°C 12 h and -0.3 MPa 6°C 24 h were selected as the two MAN-based osmotic stress pre-treatments for subsequent cryopreservation studies.

Polyethylene glycol

Exposure to all PEG-based osmotic stress pre-treatment combinations decreased embryo WC relative to fresh embryos with exposure to WPs of -0.3 MPa resulting in WCs of 3.54-3.95 g g⁻¹; WPs of -0.6 MPa resulting in WCs of 3.21-3.55 g g⁻¹; and WPs of -1.2 MPa resulting in WCs of 3.27-3.65 g g⁻¹ (data not shown). As in the MAN treatments, longer exposure times resulted in slightly lower WCs within any particular WP, but there were no consistent trends among incubation time, temperature and embryo WC.

Treatment		Mannitol		Р	olyethylene g	glycol
	¹ MTG [*]	$^{2}\text{GI}^{*}$	³ Viability	MTG [*]	GI^*	Viability
Fresh	9.8	18.9	100	9.8	18.9	100
-0.3MPa 6°C 1h	9.1	4.6	90	7.1	74.1	90
-0.3MPa 16°C 1h	13.6	4.4	90	5.3	100.0	80
-0.3MPa 25°C 1h	15.1	6.6	80	14.6	20.7	100
-0.3MPa 6°C 12h	7.6	27.8	70	6.9	96.4	90
-0.3MPa 16°C 12h	5.8	140.6	90	6.3	41.7	90
-0.3MPa 25°C 12h	11.6	9.7	70	8.8	22.7	80
-0.3MPa 6°C 24h	8.6	38.6	90	9.8	16.5	80
-0.3MPa 16°C 24h	14.5	6.3	100	5.4	180.0	80
-0.3MPa 25°C 24h	10.4	4.7	60	6.3	49.4	80
-0.6MPa 6°C 1h	7.8	34.6	90	13.3	6.6	100
-0.6MPa 16°C 1h	6.9	33.6	80	7.8	13.7	100
-0.6MPa 25°C 1h	11.5	49.6	100	8.2	27.7	100
-0.6MPa 6°C 12h	7.0	51.8	70	14.5	7.7	100
-0.6MPa 16°C 12h	6.8	51.0	100	8.0	61.7	80
-0.6MPa 25°C 12h	12.0	10.5	70	6.3	82.6	100
-0.6MPa 6°C 24h	8.1	68.8	80	35.3	24.3	70
-0.6MPa 16°C 24h	1.0	25.0	50	8.0	17.4	100
-0.6MPa 25°C 24h	69.9	2.4	70	9.6	21.4	80
-1.2MPa 6°C 1h	14.4	9.3	80	10.3	14.8	100
-1.2MPa 16°C 1h	14.7	4.6	90	10.3	17.4	100
-1.2MPa 25°C 1h	11.5	2.4	80	6.7	69.4	100
-1.2MPa 6°C 12h	10.7	13.2	70	10.1	18.1	80
-1.2MPa 16°C 12h	8.4	24.6	70	6.0	55.6	80
-1.2MPa 25°C 12h	9.8	7.7	50	18.4	1.8	50
-1.2MPa 6°C 24h	8.5	10.4	40	11.0	0.8	20
-1.2MPa 16°C 24h	15.5	2.8	40	5.0	16.0	20
-1.2MPa 25°C 24h	22.0	0.3	30	5.0	4.0	10

 Table 5.1 Vigour and viability of A. belladonna zygotic embryos after exposure to various mannitol- and polyethylene glycol-based osmotic stress pre-treatments.

¹ mean time to germinate; ² germination index; ³ viability = root and shoot production. MTG and GI were based on root and shoot production. p < 0.05 when viability data were tested for inter-treatment differences (null-model chi-squared analysis, n = 10). ^{*}Experiment performed once, not subjected to statistical analysis. Treatments shaded in grey resulted in 10% viability loss, increased vigour relative to fresh embryos, and maximised time for stress acclimation; these pre-treatments were selected for all subsequent osmotic stress + cryopreservation studies.

A number of treatments did not decrease viability relative to fresh embryos (100% viability) and except for -0.3 MPa 6°C 1 h, -0.3 MPa 25°C 12 h and -0.6 MPa 25°C 12 h, these treatments most often involved the shortest exposure time (i.e. 1 h) (Table 5.1). Except for isolated treatments (e.g. -0.3 MPa 25°C 24 h and -0.6 MPa 16°C 24 h) exposure to WPs of -0.3 and -0.6 MPa led to 10-30% viability loss while exposure to WPs of -1.2 MPa generally resulted in higher viability loss (10-70%). The level of viability loss across all three WPs was generally higher for longer incubation times (Table 5.1).

Germination index was enhanced relative to fresh embryos in a number of treatments across all three WPs but the frequency of this increase (which was generally based on an increase in PV) was much higher in treatments involving WPs of -0.3 and -0.6 MPa (Table 5.1). Vigour was enhanced in terms of a decrease in MTG in >60% of the treatments; however, this increase in vigour was most often accompanied by a significant decline in viability relative to fresh embryos (Table 5.1). Except for -0.3 MPa 25°C 1 h, treatments that increased MTG relative to fresh embryos generally involved WPs of -0.6 or -1.2 MPa. When a decrease in MTG was observed, this was often most pronounced at incubation temperatures of 16°C.

Based on the fact that they resulted in just 10% viability loss, increased vigour (in terms of an increase in GI and a decrease in MTG) relative to fresh embryos, and maximised the time available for stress acclimation, -0.3 MPa 6°C 12 h and -0.3 MPa 16°C 12 h were selected as the two PEG-based osmotic stress pre-treatments for subsequent cryopreservation studies.

Viability of A. belladonna zygotic embryos after osmotic stress pre-treatment + dehydration and osmotic stress pre-treatment + freeze-thawing as related to changes in total antioxidant activity (TAA)

The selected osmotic stress pre-treatments (see Table 5.1) decreased embryo WC relative to fresh embryos, but these differences were not significant (Table 5.2). Irrespective of whether embryos were CP, osmotically stressed or osmotically stressed + CP before drying, embryos across treatments could be partially dried to statistically comparable WCs of *c*. 0.29-0.36 g g⁻¹ while still retaining viabilities of $\geq 60\%$ (Table 5.2).

Osmotic stress pre-treatment with PEG and MAN led to a slight decline in viability relative to fresh embryos (Table 5.2). Partial dehydration, irrespective of whether it was preceded by CP or osmotic stress pre-treatment + CP, always led to a significant decline in viability and TAA and compared to fresh embryos (Fig. 5.1). This decline in TAA was significantly more pronounced in osmotically stressed + dehydrated treatments and often (particularly in the non-CP treatments) accompanied by slightly higher viability loss than embryos dehydrated without osmotic stress pre-treatment. Within the osmotically stressed + partially dried treatments, TAA was always significantly higher in Gly CP treatments, while post-drying viability loss in non-CP treatments was often slightly higher than CP treatments.

Treatment	² Viability (%)	¹ WC
Fresh	100	4.67±0.57
M[-0.3MPa 16°C 12h]	90	4.47±0.35
M[-0.3MPa 6°C 24h]	90	4.15±0.97
P[-0.3MPa 16°C 12h]	90	3.82±0.85
P[-0.3MPa 6°C 12h]	90	3.95±0.76
D	80	0.31±0.05
Gly+D	80	0.34±0.04
M[-0.3MPa 16°C 12h]+D	70	0.35±0.06
M[-0.3MPa 6°C 24h]+D	60	0.30±0.07
P[-0.3MPa 16°C 12h]+D	70	0.29±0.08
P[-0.3MPa 6°C 12h]+D	70	0.32±0.06
M[-0.3MPa 16°C 12h]+Gly+D	80	0.36±0.03
M[-0.3MPa 6°C 24h]+Gly+D	70	0.32±0.05
P[-0.3MPa 16°C 12h]+Gly+D	80	0.31±0.07
P[-0.3MPa 6°C 12h]+Gly+D	70	0.34±0.05
M[-0.3MPa 16°C 12h]+D+C	0	0.35±0.06
M[-0.3MPa 6°C 24h]+D+C	0	0.30±0.07
P[-0.3MPa 16°C 12h]+D+C	0	0.29±0.08
P[-0.3MPa 6°C 12h]+D+C	0	0.32±0.06
D+C	0	0.32±0.10
Gly+D+C	60	0.31±0.07
M[-0.3MPa 16°C 12h]+Gly+D+C	0	0.36±0.03
M[-0.3MPa 6°C 24h]+Gly+D+C	10	0.32±0.05
P[-0.3MPa 16°C 12h]+Gly+D+C	30	0.31±0.07
P[-0.3MPa 6°C 12h]+Gly+D+C	20	0.34±0.05

Table 5.2 Viabilities and water contents for *A. belladonna* zygotic embryos exposed to various combinations of mannitol- and polyethylene glycol-based osmotic stress pre-treatment, glycerol (Gly) cryoprotection, partial dehydration, and rapid cooling.

¹ water content (g g⁻¹; dry mass basis); ² viability = root and shoot production. Gly = cryoprotected with glycerol; M = mannitol pre-treatment; P = polyethylene glycol pre-treatment; C = cooled at hundreds of °C s⁻¹; D = dried to water contents <0.4 g g⁻¹. p < 0.05 when viability (null-model chi-squared analysis, n = 10) and water content (Mann-Whitney U test, n = 10) data were tested for inter-treatment differences; p > 0.05 when WC data for osmotically stressed treatments were tested for inter-treatment differences (*ANOVA*, n = 10).

Cooling always led to a significant decline in viability and TAA and except for Gly+D+C this decline was always significantly greater than that observed after drying or osmotic stress pre-treatment + drying (Table 5.2; Fig. 5.1). This post-thaw decline in TAA was significantly more severe in treatments involving osmotic stress pre-treatment (Fig. 5.1). All four of the treatments that resulted in post-thaw viability involved Gly CP and within these, post-thaw viability was highest (60%) in the one treatment that excluded osmotic stress pre-treatment, lower (20 and 30%) in the two treatments that involved PEG-based osmotic stress pre-treatment and lowest (10%) in the single treatment that involved MAN-based osmotic stress pre-treatment

(Table 5.2). The treatment with the highest post-thaw viability namely, Gly+D+C, was also associated with the highest post-thaw TAA, and the two PEG-based osmotically stressed treatments that resulted in 20 and 30% post-thaw viability exhibited slightly (but not significantly) higher TAA than the other osmotically stressed + freeze-thawed treatments (Fig. 5.1). However, high post-thaw TAA was not always indicative of post-thaw viability retention; D+C for example, exhibited significantly higher TAA than many of the osmotically stressed + freeze-thawed treatments, yet resulted in no post-thaw viability retention (Fig. 5.1). Correlation analyses with all treatments pooled showed a significant relationship between TAA and viability (Fig. 5.2).

Optimisation of oxidative stress pre-treatment for H. montanus zygotic embryos

Exposure of *H. montanus* embryos to a range of H_2O_2 concentrations indicated that while concentrations >0.10 mM led to a decline in viability relative to fresh embryos (100% viability), concentrations of 0.10 and 0.05 mM had no adverse effect on viability (Table 5.3A). The higher of the two concentrations was selected for all subsequent studies, to maximise the potential effects of H_2O_2 exposure.

When embryos were exposed to $0.10 \text{ mM H}_2\text{O}_2$ for any longer than 30 min, viability was significantly reduced relative to fresh embryos while exposure for 30 min resulted in 100% viability (Table 5.3B). Irrespective of the duration of exposure, vigour (as indicated by a reduction in GI and an increase in MTG) declined relative to fresh embryos (Table 5.3B). The severity of this decline increased as the duration of exposure increased.

Based on the fact that exposure to 0.10 mM H_2O_2 for 30 min resulted in the least severe decline in vigour and was the only exposure time that did not result in viability loss, this H_2O_2 treatment combination was selected for all H_2O_2 pre-treatment + cryopreservation studies (Table 5.3B).

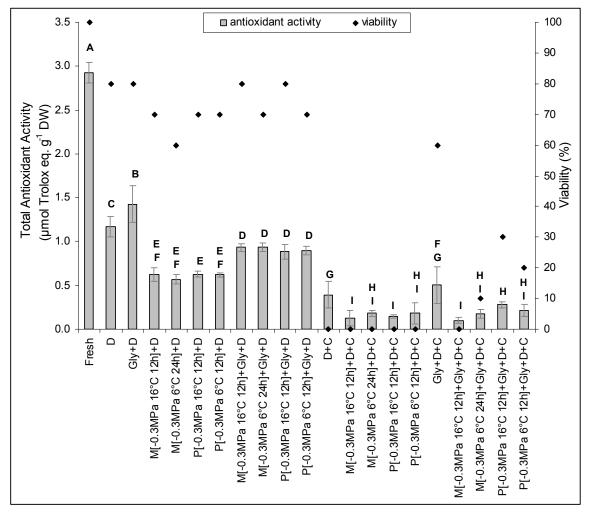


Figure 5.1 Total antioxidant activity and viability for *A. belladonna* zygotic embryos exposed to various combinations of mannitol- and polyethylene glycol-based osmotic stress pre-treatment, glycerol cryoprotection, partial dehydration, and rapid cooling. Viability = root and shoot production; M = mannitol pre-treatment; P = polyethylene glycol pre-treatment; Gly = cryoprotected with glycerol; D = dried to water contents <0.4 g g⁻¹; C = cooled at hundreds of °C s⁻¹. Values for antioxidant activity represent the mean±SD and are significantly different across treatments when followed by different letters (*ANOVA*, *n* = 6, *p* < 0.05). *p* < 0.05 when viability data were tested for inter-treatment differences (null model chi-squared analysis, *n* = 10).

[A]						
Treatment	¹ Viability (%)					
Fresh	100					
0.05 mM for 30 min	100					
0.10 mM for 30 min	100					
0.15 mM for 30 min	80					
0.20 mM for 30 min	70					
[B]						
Treatment	Viability (%)	² MTG [*]	³ GI [*]			
Fresh	100	8.2	69.4			
0.1 mM for 30 min	100	9.2	69.4			
0.1 mM for 60 min	70	10.0	59.2			
0.1 mM for 120 min	50	10.6	39.1			

Table 5.3 Vigour and viability of *H. montanus* zygotic embryos after [A] exposure to different concentrations of H_2O_2 and [B] exposure to 0.1 mM H_2O_2 for varying durations.

¹ viability = root and shoot production; ² mean time to germinate;

³germination index. MTG and GI were based on viability. p < 0.05 when viability data were tested for significant inter-treatment differences (null-model chi-squared analysis, n = 10). Experiment performed once, not subjected to statistical analysis. The treatment shaded in grey resulted in no viability loss and decreased vigour the least relative to fresh embryos; this treatment was selected for all subsequent H₂O₂ + cryopreservation studies.

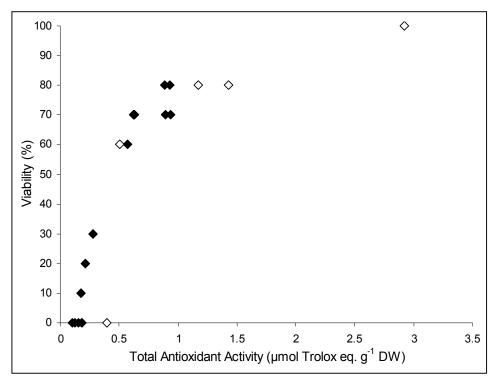


Figure 5.2 Relationship between total antioxidant activity and viability for *A. belladonna* zygotic embryos exposed to various combinations of mannitol- and polyethylene glycol-based osmotic stress pre-treatment, glycerol cryoprotection, partial dehydration and rapid cooling. Open symbols = non-osmotically stressed treatments and closed symbols = osmotically stressed treatments. r = 0.79 and p < 0.01 for Pearson correlation test.

Viability of H. montanus zygotic embryos after H_2O_2 *pre-treatment* + *dehydration and* H_2O_2 *pre-treatment* + *freeze-thawing as related to changes in total antioxidant activity (TAA)*

Drying and cooling significantly decreased viability and TAA relative to fresh embryos (Fig. 5.3). Except for <0.4D, this decline in TAA, within any particular drying or cooling treatment, was always more severe when the treatment was preceded by H_2O_2 exposure. Except for >0.4D-Gly-rapid+/-H₂O₂, these differences were always significant. Irrespective of whether drying or cooling was preceded by H_2O_2 exposure or not, the decline in TAA at <0.4 g g⁻¹ was always greater than that at >0.4 g g⁻¹. Within the drying treatments that did not involve H_2O_2 exposure there was a trend (not significant) for Gly CP embryos to exhibit relatively higher TAA than non-CP embryos but this was not necessarily accompanied by higher viability retention. Except for TAA in >0.4D, the decline in TAA and viability within any particular drying treatment was exacerbated by cooling; these differences were most often significant.

All four of the treatments that resulted in post-thaw viability involved Gly CP (Fig. 5.3). Within these treatments, post-thaw viability was highest in the two treatments that excluded H_2O_2 pre-treatment. Additionally, TAA across these four treatments was significantly higher in treatments dried to >0.4 g g⁻¹ and within the treatments that were not exposed to H_2O_2 , this was accompanied by relatively higher post-thaw viability retention. Gly+>0.4D+C resulted in the highest post-thaw viability and exhibited one of the highest antioxidant activities; however, high post-thaw TAA was not always indicative of post-thaw viability retention. >0.4D+C, for example, exhibited a post-thaw TAA that was statistically similar to Gly+>0.4D+C, but exhibited no post-thaw viability retention, while Gly+<0.4D+C exhibited a post-thaw TAA that resulted in no post-thaw viability retention, yet it exhibited a post-thaw viability of 20%. Nevertheless, correlation analyses with all treatments pooled showed a significant relationship between TAA and viability (Fig. 5.4).

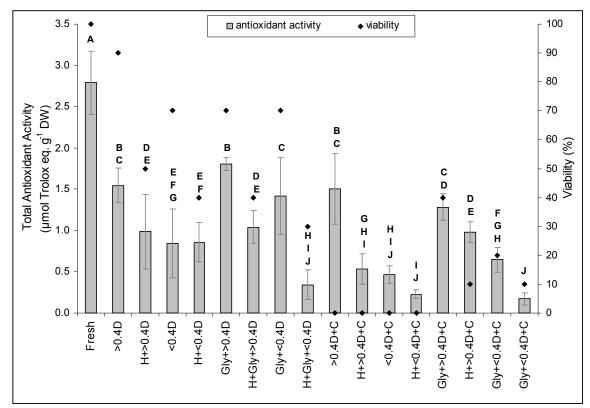


Figure 5.3 Total antioxidant activity and viability for *H. montanus* zygotic embryos exposed to various combinations of exogenous H_2O_2 exposure, glycerol cryoprotection, partial dehydration, and rapid cooling. Viability = root and shoot production; H = exposed to 0.1 mM H_2O_2 ; Gly = cryoprotected with glycerol; <0.4D = dried to water contents <0.4 g g⁻¹; >0.4D = dried to water contents >0.4 g g⁻¹; C = cooled at hundreds of °C s⁻¹. Values for antioxidant activity represent the mean±SD and are significantly different across treatments when followed by different letters (*ANOVA*, *n* = 6, *p* < 0.05). *p* < 0.05 when viability data were tested for intertreatment differences (null model chi-squared analysis, *n* = 10).

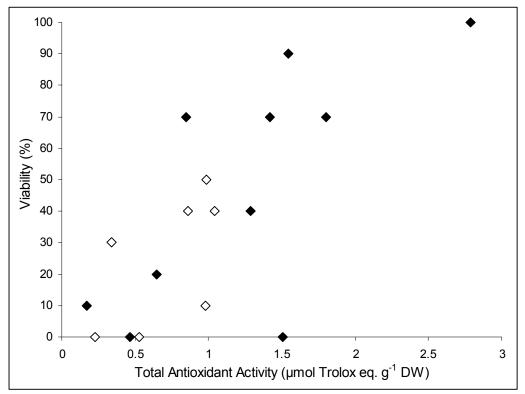


Figure 5.4 Relationship between total antioxidant activity and viability for *H. montanus* zygotic embryos exposed to various combinations of exogenous H_2O_2 exposure, glycerol cryoprotection, partial dehydration, and rapid cooling. Open symbols = H_2O_2 treated and closed symbols = H_2O_2 untreated. r = 0.72 and p = 0.001 for Pearson correlation test.

5.4 Discussion

This study investigated whether exposure of recalcitrant zygotic embryos to low levels of osmotic (in the case of *A. belladonna*) or oxidative (in the case of *H. montanus*) stress reduces their sensitivity to subsequent dehydration and freeze-thaw stresses associated with cryopreservation. Sensitivity, or rather viability, was also related to post-drying and post-thaw total antioxidant activity (TAA), since enhanced antioxidant levels have been correlated with increased stress tolerance in plants (Dhindsa and Matowe, 1981; Senaratna and McKersie, 1986; Leprince *et al*, 1990; Dhindsa, 1991; Price and Hendry, 1991; Anderson *et al.*, 1992; Seel *et al.*, 1992; Reuzeu and Cavalie, 1995; O'Kane *et al.*, 1996; Scebba *et al.*, 1999; Baek and Skinner, 2003), and pre-treatments that encourage stress acclimation can enhance antioxidant protection (Wahid *et al.*, 2007).

The osmotic stress pre-treatments used in this study are similar to those regularly employed to improve orthodox seed performance (Heydecker *et al.*, 1973; Heydecker, 1978; Job *et al.*, 2000). Termed 'osmopriming', this practice is generally defined as the uptake of sufficient water to initiate early germinative events, in previously dry, seeds but not sufficient to permit radicle protrusion (McDonald, 2000). In orthodox seeds the results of such priming include: increased vigour; more uniform germination (Tarquis and Bradford, 1992); and counteraction of the effects of lipid peroxidation in aged seeds by reversal of the loss of lipid-peroxidation-detoxifying enzymes (Bailly *et al.*, 1997, 1998, 2008; McDonald, 2000; Gallardo *et al.*, 2001; Posmyk *et al.*, 2001; Chiu *et al.*, 2002).

Unlike the dry orthodox seeds, to which osmopriming is usually applied (reviewed by McDonald, 2000), recalcitrant seeds are shed highly hydrated (0.4-4.0 g H₂O per g dry matter; Pammenter and Berjak, 1999) and grade into germinative metabolism on shedding, or even before (Berjak et al., 1989; Farrant et al., 1992). The application of osmopriming to recalcitrant seeds therefore constitutes an osmotic stress, since water is either removed or not provided in a sufficient amount to allow for radicle protrusion. The embryos of A. belladonna used in this study displayed typically high shedding WCs of c. 4.67 g g^{-1} and exposure to osmotica decreased embryo WC, and very often viability as well (Table 5.1). The variation in osmotic stress-induced viability loss across the different WP \times temperature \times incubation time combinations was considerable for both osmotica but there was a trend for this loss to be higher for WPs of -1.2 MPa and longer incubation times (within particular WPs) (Table 5.1). The negative correlation between WC and viability is widely documented for physically (Pammenter et al., 2000; Walters et al., 2001; reviewed by Kermode and Finch-Savage, 2002) and osmotically (e.g. Probert and Longley, 1989; Dussert et al., 1999; Sun and Liang, 2007) dried recalcitrant seeds. Dehydration to intermediate hydration levels induces uncoordinated metabolism in recalcitrant seeds (reviewed by Côme and Corbineau, 1996; Pammenter et al., 1998; Walters *et al.*, 2001) and the free-radical generation associated with such metabolism can have lethal consequences, most especially during slow dehydration (Smith and Berjak, 1995; Côme and Corbineau, 1996; Walters et al., 2002). This brings us to the important point of 'drying rate'. In this regard, recalcitrant embryos, including those of A. belladonna in this study (see Fig. A2, Appendix A), can be rapidly dried (via flash drying) to WCs of $0.3-0.4 \text{ g s}^{-1}$ with little to no viability loss (Wesley-Smith et al., 1992; Kioko et al., 1998; Sershen et al., 2008); however, osmotically stressed embryos in this study exhibited as much as 90% viability loss at WCs >3.0 g g^{-1} (Table 5.1). Desiccation damage in recalcitrant seeds is a function of two interrelated parameters: the rate and duration of dehydration (e.g. Pammenter *et al.*, 1998, 2002, 2003; Walters *et al.*, 2001) and when desiccation-sensitive seed tissues spend a longer period at intermediate WCs, the time for aqueous-based deleterious processes to occur is extended, promoting viability loss (Pammenter *et al.*, 1998; reviewed by Pammenter and Berjak, 1999; Walters *et al.*, 2001). The high viability losses at relatively high WCs in osmotically stressed embryos in this study may therefore be attributed to them spending hours at intermediate hydration levels (i.e. an increase in the duration of the stress). Consistent with this suggestion, the severity of viability loss within particular WPs, generally increased with an increase in incubation time (Table 5.1).

Slight dehydration has been shown to stimulate vigour in some recalcitrant seeds (Eggers *et al.*, 2007), and this may explain the enhanced vigour observed across a few of the osmotic stress pre-treatments here (mainly those involving WPs of -0.3 and -0.6 MPa); however, this enhancement was almost always accompanied by some viability loss (Table 5.1). While emergence rate is often increased by priming, percentage emergence can be decreased (e.g. onion [Haigh *et al.*, 1986]). There were indications that MAN-based treatments were more detrimental than PEG-based treatments, at similar WPs and incubation times (Table 5.1), and this may be related to the fact that MAN unlike PEG (which has a relatively higher molecular weight) can be absorbed by axial tissue (Michel and Kaufmann, 1973), often with toxic effects (Bradford, 1995).

Despite the lethal effects of osmotic stress pre-treatment, four WP \times temperature \times incubation time combinations, viz. PEG[-0.3 MPa 6°C 12 h]; PEG[-0.3 MPa 16°C 12 h]; MAN[-0.3 MPa 6°C 24 h]; and MAN[-0.3 MPa 6°C 24 h] (two for each osmoticum), were shown to result in 90% viability and increase vigour relative to fresh embryos (Table 5.1). Exposure of a variety of plant tissues to non-lethal osmotic stress can enhance their tolerance to a wide range of abiotic stresses (e.g. Bueno et al., 1998; Guan and Scandalios, 1998; Guan et al., 2000). However, when rapid drying was preceded by osmotic stress pre-treatment in this study, viability loss and TAA reduction, relative to fresh embryos, was consistently more severe than that in flash-dried non-pre-treated embryos (Table 5.2 & Fig. 5.1). Dehydration of recalcitrant embryonic axes and embryos to even relatively high WCs (>0.25 g g^{-1}) is often characterised by the failure of antioxidant systems (e.g. Hendry et al., 1992; Chaitanya and Naithani, 1994, 1998; Côme and Corbineau, 1996; Varghese and Naithani, 2002; Pukacka and Ratajczak, 2006) and the relatively greater decline in TAA in osmotically stressed + partially dried, compared to non-stressed + partially dried, embryos in this study may have been a consequence of embryos exposed to the former spending more time at intermediate hydration levels. Priming of orthodox seeds has been shown to be an effective approach to overcome

abiotic stresses (e.g. Wahid and Shabbir, 2005; Ashraf and Foolad, 2005) but, as observed here, the combination of osmopriming and dehydration can be detrimental (includes a loss of membrane integrity), even in orthodox seeds (e.g. soyabean [Armstrong and McDonald, 1992]). Interestingly, the post-drying decline in TAA observed here was significantly less severe in Gly CP embryos, irrespective of whether the embryos were osmotically stressed or not (Fig. 5.1). These data are in agreement with the results of studies described in Chapter 4 (section 4.3), which showed Gly cryoprotection to limit the decline in enzymic antioxidant capacity associated with partial dehydration. The possible basis of this protection of the antioxidant system by Gly has already been addressed in Chapter 4 (section 4.4).

Enhanced antioxidant activity can increase freezing tolerance in non-orthodox seeds by alleviating the effects of freezing-thaw-induced oxidative stress (Touchell and Walters, 2000; Dussert et al., 2003; Varghese and Naithani, 2008). In the present study, post-thaw TAA was always significantly lower than in fresh embryos, irrespective of the combination of preconditioning treatments (i.e. osmotic stress pre-treatment, CP or osmotic stress pre-treatment + CP) (Fig. 5.1). These results corroborate other reports that antioxidant activity may decline in plant tissues following freezing (Guy, 1990; Thomas *et al.*, 1999; Varghese and Naithani, 2008). Contrary to the hypothesis proposed for this study, osmotic stress pre-treatment did not decrease the sensitivity of A. belladonna zygotic embryos to subsequent dehydration and freeze-thaw stresses. Further to this, post-thaw TAAs in osmotically stressed + partially dried embryos were always (and often significantly) lower than non-stressed + partially dried embryos (in CP and non-CP embryos; Fig. 5.1). As explained for the results of the osmotic stress pre-treatment + partial dehydration studies earlier, osmotic stress pre-treatment, extended the time spent by embryos at intermediate WCs, compared to partial dehydration simply by flash drying. This, could have lead to a relatively greater accrual of metabolism-induced damage in osmotically stressed + partially dried embryos, compared to non-stressed + partially dried embryos, predisposing the former to a greater degree of freeze-thaw damage. In recalcitrant seeds prolonged periods of dehydration, while not lethal in themselves, can act synergistically and lethally with freezing (Pritchard et al., 1995; Kioko et al., 1998; Berjak et al., 1999; Sun, 1999; Wesley-Smith et al., 2001, 2004). This may also explain why within the four cryoprotected treatments in which post-thaw viability retention was observed here (Fig. 5.1), post-thaw viability retention and TAA was lower in the three treatments that involved osmotic stress pre-treatment.

Finally, TAA in this study may not always have been indicative of post-thaw viability retention (e.g. <0.4D-rapid) but TAA and viability were positively correlated (Fig. 5.2), lending support to other suggestions that antioxidant protection has a role in low temperature and freezing tolerance in plant tissues (e.g. Green *et al.*, 1986; Prasad, 1996; Fryer *et al.*, 1998;

Fleck *et al.*, 2000; Touchell and Walters, 2000; Xin and Browse, 2000; Dussert *et al.*, 2003; Odani *et al.*, 2003; Sung *et al.*, 2003; Johnston *et al.*, 2007; Varghese and Naithani, 2008).

Effects of H_2O_2 pre-treatment on dehydration and cryo-sensitivity of H. montanus zygotic embryos as related to changes in total antioxidant activity

The major components of the generic pathway of stress response include the stimulus, signals, transducers, transcription regulators, target genes and finally, a morphological, physiological or biochemical stress response, which ultimately confers stress resistance (Monroy and Dhindsa, 1995; Netting, 1999; Pearce, 2004; for a review see Verslues and Zhu, 2005). In addition to triggering the expression of genes directly involved in protection against a particular stress, transducers may also trigger the activation of genes whose products regulate gene expression and secondary signal transduction. Secondary signal molecules such as H_2O_2 (and other active oxygen species), abscisic acid (a phytohormone widely accepted as a stress hormone), and inositol-1,4,5-triphosphate, initiate different response pathways from the primary signals and may be shared by different stress pathways; this is possibly the underlying factor governing cross tolerance (Xiong et al., 2002). Hydrogen peroxide can also lead to lethal levels of hydroxyl radicals (in the presence of metal catalysts; i.e. the Fenton reaction) and related lipid peroxidation (MacRae and Fergusion, 1985; Foyer et al., 1994; Samuilov et al., 2001; Sairam et al., 2002). However, there is increasing evidence for a role for H_2O_2 as an intermediate signal molecule and/or second messenger (when produced internally or applied externally at low concentrations) in signal transduction pathways that elicit plant stress responses and ultimately stress acclimation (Foyer et al., 1997; Bowler and Fluhr, 2000; Dat et al., 2000; Desikan et al., 2004; Hung et al., 2005). This has encouraged its (exogenous) application to a variety of plant tissues (e.g. Azevedo Neto et al., 2005; Hung et al., 2005; Wahid et al., 2007) as a pre-treatment to enhance tolerance to abiotic stresses such as chilling (Prasad et al., 1994b; Murphy et al., 2002) and freezing (Mora-Herrera et al., 2005).

In line with its well-documented toxicity in plant cells (MacRae and Fergusion, 1985; Foyer *et al.*, 1994; Samuilov *et al.*, 2001; Sairam *et al.*, 2002) exposure of *H. montanus* embryos to H_2O_2 concentrations >0.10 mM led to a decline in viability relative to fresh embryos in the present study. However, at a concentration of 0.10 mM, which was the concentration used for all H_2O_2 pre-treatment + cryopreservation studies here, H_2O_2 had no adverse effect on viability, provided that the duration of exposure was no longer than 30 min (Table 5.3A). These data also indicated that *H. montanus* embryo tolerance of oxidative stress was limited by both the intensity and duration of the stress. Considering that the seeds of a number of orthodox-seeded species tolerate exposure to *c*. 0.10 mM, or even higher, concentrations of H_2O_2 for hours

without lethal consequences (e.g. *Orobanche aegyptica* [Nun *et al.*, 2003] and *Triticum aestivum* [Wahid *et al.*, 2007]), it was interesting to note that the antioxidant system within *H. montanus* embryos was over-whelmed by H_2O_2 exposure (i.e. H_2O_2 became lethal) after just 30 min (Table 5.3B). There are many reports of H_2O_2 breaking dormancy in orthodox seeds (Wanga *et al.*, 1998; Hsiao and Quick, 2006) and improving germination (Breusegem *et al.*, 2001; Ogawa and Iwabuchi, 2001; Schopfer *et al.*, 2001) by stimulating radicle protrusion (Morohashi, 2002). However, exposure *of H. montanus* embryos to 0.10 mM H_2O_2 reduced vigour relative to fresh embryos (Table 5.3B), irrespective of the duration of exposure. These data may well be a reflection of the suggestion that the induction of certain components of the antioxidant system usually present in orthodox seeds, may occur to a lesser extent, or not at all, in recalcitrant seeds (Pammenter and Berjak 1999; Walters *et al.*, 2008).

Hydrogen peroxide has been variably implicated in the signalling mechanism involved in polar growth, hormone transduction and stress signalling (reviewed by Mori and Schroeder, 2004). The mechanism(s) by which H₂O₂ enhances stress tolerance are still elusive but two suggestions, in particular, have gained widespread support: (1) H₂O₂ accumulation leads to modifications in Ca²⁺ channels and subsequent fluxes in Ca²⁺ (McAinsh *et al.*, 1996), a key component of stress responses (Gao *et al.*, 2004); and (2) H₂O₂ directly regulates the expression of genes involved in defense (Kovtun *et al.*, 2000), antioxidants (Prasad *et al.*, 1994a & b; Azevedo Neto *et al.*, 2005; Wahid *et al.*, 2007), and/or cell rescue/defense proteins (Murphy *et al.*, 2002; Robert and David, 2004; Hung *et al.*, 2005). Most interestingly, Roach *et al.* (2010) have recently shown that the exogenous application of H₂O₂ to recalcitrant *Castanea sativa* seeds may mimic the transient ROS burst at the onset of desiccation, counteracting viability loss of sub-lethally desiccation-stressed seeds and of excised embryonic axes grown in tissue culture. Those authors suggest that extracellular ROS produced by embryonic axes may be important signalling components involved in wound response, regeneration and growth.

Hydrogen peroxide pre-treatment did not decrease *H. montanus* embryo sensitivity to dehydration and freeze-thaw stresses in this study though, since the post-drying and post-thaw decline in viability and TAA, relative to fresh embryos, was generally (except for <0.4D) more severe when the drying or cooling treatment was preceded by H_2O_2 exposure (Fig. 5.3). Dehydration (Hendry *et al.*, 1992; Chaitanya and Naithani, 1994, 1998; Côme and Corbineau, 1996; Varghese and Naithani, 2002; Pukacka and Ratajczak, 2006) and freezing (Touchell and Walters, 2000; Dussert *et al.*, 2003; Varghese and Naithani, 2008) can lead to the failure of antioxidant systems and concomitant viability loss in recalcitrant seeds. In *H. montanus* embryos the post-drying and post-thaw decline in TAA was always relatively more severe at WCs <0.4 g g⁻¹, irrespective of whether the treatment involved H_2O_2 exposure or not (Fig. 5.3).

The greater degree of pre-cooling desiccation stress (i.e. longer drying times and lower WCs) inflicted on *H. montanus* embryos dried to WCs $<0.4 \text{ g g}^{-1}$, compared to those dried to WCs $>0.4 \text{ g g}^{-1}$, may have pre-disposed the antioxidant system in the former to a relatively greater degree of freeze-thaw damage since dehydration damage in recalcitrant seed tissues is often exacerbated by freezing (Pritchard *et al.*, 1995; Kioko *et al.*, 1998; Sun, 1999; Wesley-Smith *et al.*, 2001, 2004). Consistent with this, except for TAA in >0.4D, the decline in TAA and viability within any particular drying treatment, appeared to be exacerbated by cooling.

As in the osmotic stress pre-treatment + cryopreservation studies, Gly CP generally enhanced post-thaw viability retention and TAA, relative to non-CP embryos (Fig. 5.3), but across the four Gly CP treatments in which post-thaw viability was recorded, viability and TAA were significantly higher in the two treatments (namely, >0.4D-Gly-rapid and <0.4D-Gly-rapid) that excluded H_2O_2 exposure. The concentration of H_2O_2 employed in these studies was not lethal but it led to a reduction in vigour, relative to fresh embryos (Table 5.3B). This implies that *H. montanus* embryos may well have incurred some damage (allbeit not lethal) during H_2O_2 exposure, since an increase in the lag before the first germination or a decrease in the rate of germination is usually indicative of ongoing repair of damage (Pammenter *et al.*, 2002). This implies that even when oxidative stress is not lethal in itself, it may act synergistically and lethally with freeze-thawing.

As in the osmotic stress pre-treatment + cryopreservation studies, high post-thaw TAA was not always accompanied by post-thaw viability retention (e.g. >0.4D-rapid), reinforcing the fact that the antioxidant system is an internal system integrated into a number of cellular and physiological regulatory systems (Blagojević, 2007), making it just one of the many determinants of post-thaw survival. Correlation analyses did, however, indicate a significant relationship between TAA and viability, even when H_2O_2 exposed and unexposed treatments were analysed separately (Fig. 5.4). These results complement the existing evidence for the role of antioxidant protection in low temperature and freezing tolerance in plant tissues (e.g. Green *et al.*, 1986; Prasad, 1996; Fryer *et al.*, 1998; Fleck *et al.*, 2000; Touchell and Walters, 2000; Xin and Browse, 2000; Dussert *et al.*, 2003; Odani *et al.*, 2003; Sung *et al.*, 2003; Johnston *et al.*, 2007; Varghese and Naithani, 2008).

5.5 Concluding remarks

Exogenous use of various chemicals to alleviate the adverse effects of abiotic stresses has great implications both from theoretical and practical perspectives (Uchida *et al.*, 2002; Sivritepe *et al.*, 2003, 2005). In this study, pre-treatment of *A. belladonna* embryos with MAN and PEG and *H. montanus* embryos with H_2O_2 failed to decrease embryo sensitivity to

dehydration and freeze-thaw stresses. While damage accrued in *A. belladonna* embryos in the sub-imbibed state appears, at best, to negate any possible benefits of osmotic treatments, the failure of H_2O_2 pre-treatment to decrease cryo-sensitivity may be based on a number of factors, including: (1) the genes coding for enzymes involved in cell defense (e.g. those involved in ROS degradation) that are usually induced by H_2O_2 in orthodox-seeded systems (Desikan *et al.*, 2001; Neill *et al.*, 2002) may be absent, poorly expressed, or reliant on different induction pathways and/or signal molecules in recalcitrant types; and (2) the free-radical-mediated damage incurred during H_2O_2 pre-treatment and the subsequent dehydration- and freeze-thawing-induced enhancements in free-radical production (shown to occur in these embryos [see Chapter 4; section 4.3]), may have simply been additive. Finally, these results tie in with two prevailing ideas on cryo-related stresses in plant tissues: (1) stresses associated with the different procedures involved in cryopreservation are additive in their effect/s (Pammenter *et al.*, 2010); and (2) tissues tolerant of stresses must possess efficient antioxidant systems (Touchell and Walters, 2000; Benson and Bremner, 2004).

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CHAPTER SIX: Effects of partial dehydration of recalcitrant *Haemanthus montanus* zygotic embryos on vigour of recovered seedlings

Based on

Sershen, Berjak, P., Pammenter, N.W. 2011. Effects of partial dehydration of recalcitrant *Haemanthus montanus* zygotic embryos on vigour of recovered seedlings. South African Journal of Botany 77, 193-202.

Abstract

Cryopreservation is the most promising route for the long-term conservation of recalcitrant seed germplasm. Partial dehydration is a standard pre-treatment for the cryopreservation of zygotic embryos or embryonic axes excised from recalcitrant seeds since it reduces the likelihood of lethal ice-crystal generation during cooling. However, there is presently little to no understanding of how pre-conditioning treatments such as partial dehydration imposed at the embryonic stage are translated or manifested during subsequent *in* and *ex vitro* seedling growth. The present study assessed the vigour of seedlings recovered from partially dried (D) zygotic embryos, excised from recalcitrant Haemanthus montanus (Baker) seeds. Seedlings recovered from fresh (F) and partially dried (D) embryos in vitro, were hardened-off ex vitro, and subsequently subjected to either 42 d of watering (W) or 42 d of water deficit (S). The adverse effects of partial dehydration on seedling dry mass accumulation observed after 60 d in vitro growth did not disappear with an extension of the *in vitro* growth period but did appear to be reversible during ex vitro growth. A water stress during ex vitro growth dominated over the effects of embryo pre-treatment with relative growth rates in FS-seedlings (recovered from fresh embryos and subsequently stressed) and DS-seedlings (recovered from dried embryos and subsequently stressed) being statistically comparable. D- and F-seedlings responded typically to the water stress but DS-, compared with FS-seedlings, appeared to have incurred permanent damage to their photosynthetic machinery, attained lower predawn water potentials, were less efficient at adjusting leaf water potential to meet transpirational demands, did not exhibit signs of osmotic adjustment, failed to adopt growth patterns that reduce transpirational water loss, and were more susceptible to persistent turgor loss. It was therefore not surprising that ex vitro seedling mortality occurred in more DS- than FS-seedlings. These results suggest that partial dehydration of recalcitrant *H. montanus* zygotic embryos, even when not followed by cooling, can reduce the vigour and drought tolerance of recovered seedlings.

6.1 Introduction

Partial dehydration, in reducing explant heat capacitance and mass, can facilitate faster cooling rates and reduce the formation of lethal ice-crystals during the cryopreservation of seed tissues (Wesley-Smith et al., 2001, 2004), and has become a standard pre-treatment for the cryopreservation of zygotic embryos or embryonic axes excised from recalcitrant seeds (e.g. Pritchard and Prendergast, 1986; Pence, 1992; Pritchard et al., 1995; Sershen et al., 2007; Steinmacher et al., 2007). Seedlings recovered from partially dehydrated, cryopreserved orthodox zygotic embryos or embryonic axes have been reported to be morphologically similar to those generated from control axes, going on to produce flowers and fruit in the field (e.g. Gagliardi et al., 2002). However, partial dehydration represents a source of physicochemical damage in recalcitrant zygotic germplasm (Walters et al., 2001), with a number of studies showing seedlings recovered from partially dried and/or cryopreserved recalcitrant zygotic germplasm to exhibit abnormal phenotype (e.g. Pence, 1992; Berjak et al., 1996; Dumet et al., 1997; Wesley-Smith et al., 2001, 2004) and/or reduced growth (e.g. Fu et al., 1993; Sershen et al., 2007; Steinmacher et al., 2007). Similarly, qualitative observations made in studies described in Chapter 2 (section 2.3; see Fig. 6.1A) suggested seedlings recovered from partially dried (D) zygotic embryos of Haemanthus montanus (Baker), a recalcitrant-seeded wild geophyte, to be less vigorous than those from fresh (F) embryos, even after 60 d in vitro growth. The present study involved a quantitative assessment of the effects of partial dehydration of excised *H. montanus* embryos on subsequent seedling vigour. Also, based on reports that losses of phenotypic fidelity in plants recovered from cryopreserved samples are often temporary (e.g. sugarcane callus [Martínez-Montero et al., 2002]; oil palm polyembryonic cultures [Konan et al., 2007]), this study investigated whether the effects of partial dehydration on subsequent in vitro seedling growth disappear with an extension of the *in vitro* growth period.

There is at present little to no understanding of how stresses imposed at the embryonic stage are translated or manifested during subsequent *ex vitro* seedling growth, in recalcitrant-seeded species. Reports in orthodox-seeded species suggest that there exists within developing embryos (zygotic and somatic) some 'memory' based mechanism that senses environmental signals such as duration of imbibition (Forsyth and van Staden, 1983) and temperature during embryogenesis (Kvaalen and Johnsen, 2007), which in turn influences adaptive traits in the seedlings they give rise to. If such a mechanism were to exist within recalcitrant zygotic germplasm it would have implications on the design of future plant germplasm cryopreservation protocols, the ultimate aim of which is to re-introduce healthy, vigorous, seedlings back into the wild. Since the success of such re-introduction will depend on the ability of recovered seedlings to tolerate abiotic stresses such as drought, most especially during the early seedling establishment phase, the

current contribution assessed the effects of *H. montanus* zygotic embryo partial dehydration on subsequent *ex vitro* seedling vigour. Seedling vigour was assessed in terms of physiological and growth responses to an *ex vitro* water stress.

6.2 Materials and Methods

Plant material

Mature *H. montanus* fruits were harvested directly from parent plants and transported in plastic bags to the laboratory with minimum delay (1-2 d) or water loss. Upon arrival, the seeds were decontaminated and stored 'hydrated', as described in Chapter 2 (section 2.2).

Embryo partial dehydration, in vitro recovery and growth assessment

All experiments were carried out on seeds stored for between 7-10 d, never longer. Zygotic embryos were excised and collected within closed Petri dishes on filter paper moistened with sterile calcium-magnesium solution (CaMg solution: 0.5 µM CaCl₂.2H₂O and 0.5 mM MgCl₂.6H₂O [Mycock, 1999]). In order to minimise the potential variation in drying rate as a function of embryo size, only embryos of between 4-6 mm in length were used for all the experiments described below. Excised embryos were rapidly dehydrated via flash drying (devised by Berjak et al., 1990) for various times. At each drying interval, water content (WC) of 10 embryos was determined gravimetrically and expressed on a dry mass basis (as described in Chapter 2, section 2.2) while 20 embryos were rehydrated, decontaminated with 1% (w/v) aqueous calcium hypochlorite for 3 min, washed with sterile CaMg solution (3 times) and then set to germinate with 5 embryos per Petri dish on full-strength Murashige and Skoog medium (Murashige and Skoog, 1962), containing 3% (w/v) sucrose. All Petri dishes were initially placed in the dark, and transferred upon signs of root and shoot development to a growth room with cool fluorescent lights (52 μ E s⁻¹ m⁻²) and a 16 h photoperiod, at ~25°C. Embryos were grown *in vitro* for 60 d with one sub-culture at 30 d and a sample of those that subsequently produced seedlings with normal (i.e. callus free) roots and shoots were assessed for dry mass accumulation. For this, 5 seedlings from each drying interval were oven-dried individually for 72 h at 80°C to a constant weight, for dry mass estimation.

For recalcitrant amaryllid zygotic germplasm, optimum WCs for successful cryopreservation are generally in the range of 0.40-0.25 g g⁻¹ (Sershen *et al.*, 2007). So, for subsequent experiments 250 zygotic embryos were rapidly dehydrated to *c*. 0.28 g g⁻¹, rehydrated, decontaminated and, together with a second batch of 250 freshly excised embryos (WC of *c*. 6.63 g g⁻¹), recovered *in vitro*. These embryos were recovered *in vitro* for 90 d with two subcultures (one at 30 d and one at 60 d). Of those that subsequently produced seedlings with

normal (i.e. callus free) roots and shoots, 6 from each treatment were separated into bulb, leaves and roots and oven-dried for 72 h at 80°C, to a constant weight for dry mass estimation. The remaining seedlings were directed towards the ex vitro studies; however, to validate the comparison of physiological measurements between F- and D-seedlings in these studies, we had to ascertain whether D- and F-seedlings were at the same developmental, as opposed to culture, age after 90 d in vitro growth. This was challenging since germination across different embryos from the same seed lot is not synchronous, even when they are not dried. Further to this, as explained in Chapter 2 (section 2.3; see Table 6.1B) partial dehydration of *H. montanus* embryos to WCs < 0.4 g g⁻¹ decreased germination speed relative to fresh ones. Nevertheless, "ageing" plants by their morphology rather than chronological time using the leaf plastochron index (LPI) (originally described by Erickson and Michelini, 1957) provides an effective, nondestructive, method of estimating leaf age under normal stable growth conditions (Groot and Meicenheimer, 2000; Chen et al., 2009) and was employed here to compare leaf age between Dand F-seedlings after 90 d in vitro growth. H. montanus seedlings generally produced two leaves by day 85 of *in vitro* recovery. Leaf 1 and 2 (numbered in the order that the leaves were produced) length was measured for five D- and F-seedlings on day 85 using calipers, and then again on day 90. These data suggested that growth had not leveled-off and leaf length in both treatments was still actively increasing during this period (data not shown). With this mind, leaf lengths recorded on day 90 were used to calculate the plastochron index (PI) for each of five Dand F-seedlings, according to Erickson and Michelini (1957) using Equation 1:

$$PI = n + (\ln L_n(t) - \ln \lambda) / [\ln L_n(t) - \ln L_{n+1}(t)]$$
[1]

where *n* is the position number for the smallest leaf (which in this case was leaf 2) that is greater than or equal to the reference length λ ; λ is designated as a leaf length of 1.0 cm during the exponential growth stage, and $L_n(t)$ is the length of leaf *n* at time *t*. The mean leaf plastochron index (LPI) for leaf *I* was then calculated using Equation 2:

$$LPI_{l} = PI - l = n - l + (\ln L_{n}(t) - \ln \lambda) / [\ln L_{n}(t) - \ln L_{n+1}(t)]$$
[2]

When calculated, mean LPI₁ in F-seedlings (-10.99±1.81) was statistically comparable to that of D-seedlings (-10.24±1.63) (p > 0.05, *ANOVA*; data not shown), suggesting that D- and F-seedlings were at approximately the same developmental age upon introduction to *ex vitro* conditions (i.e. day 91).

Ex vitro experimental design

After 90 d *in vitro* growth, 190 randomly selected seedlings generated from partially dehydrated embryos, and a further 190 randomly selected seedlings that had been recovered from freshly excised (F) embryos, were transplanted independently into plastic inserts, filled

with a mixture of 1 part pine bark, 1 part potting soil, and 1 part coarse river sand, and placed in a misthouse for 14 d to harden-off. Seedlings subjected to hardening-off were then directed towards the *ex vitro* studies described in Figure 6.1. Here, seedlings (still within plastic inserts) were transferred to natural conditions of illumination in a polycarbonate-clad greenhouse. After 7 d (111 d since embryo excision) D- and F-seedling groups were sub-divided in two batches of 95 seedlings each, with one batch being subjected to a water stress for 42 d, by with-holding water, while the second batch were watered daily (with 5 ml of water) for 42 d. From here on, when the labels 'F' or 'D' are followed by the letter 'S' it refers to seedlings exposed to a water stress while the letter 'W' indicates the absence of this stress. CO₂-assimilation, potential photochemical efficiency as well as leaf water, osmotic and pressure potential were measured across all embryo pre-treatment × watering regimes (referred to as 'all treatments' from here on) on days 0, 12, 29 and 39, while growth responses (seedling dry mass and biomass partitioning) and leaf chlorophyll content were assessed after 90 d *in vitro* growth and after 63 d *ex vitro* growth (that is, 42 days after the initiation of the stress treatment).

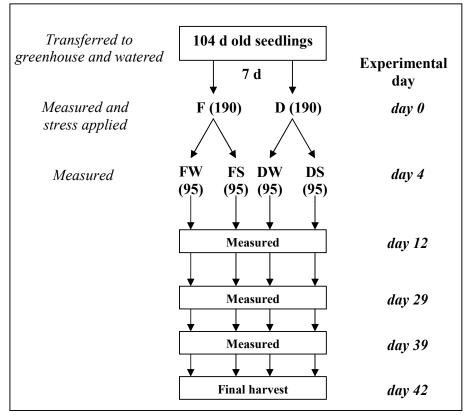


Figure 6.1 *Ex vitro* experimental design. Measured: CO₂-assimilation, potential photochemical efficiency, leaf water, osmotic and pressure potential measured on this day. Seedlings were generated from (F) fresh and (D) partially dried zygotic embryos. Recovered seedlings were either (S) water stressed by with-holding water for 42 d or (W) watered daily for 42 d. The number of replicates associated with each treatment combination is given within brackets.

Photosynthetic capacity

Chlorophyll content

Chlorophyll (chl) content was measured at the final harvest (day 42) using one leaf from each of 4 seedlings (only living leaf material sampled), across all treatments. After determining their fresh weight (FW) individual leaves were ground in a pestle and mortar with LN and chlorophyll immediately extracted in 5 ml of 80% acetone, in the dark (after Arnon, 1949). After 6 h, the leachate was filtered and its absorbance read at 663 nm for *chl a* and 645 nm for *chl b* (after Arnon, 1949). Chlorophyll a, b and total *chl* content were thereafter expressed on a FW basis. The chlorophyll assay employed here may seem outdated in light of Ultra Performance Liquid Chromatography- and High Performance Liquid Chromatography-based methods (e.g. Rodrigues-Amaya and Kimura, 2004) presently used for qualitative and quantitative measurement of caretenoids in plant tissue. However, rather than aiming to identify and quantify individual caretenoids present, the current study simply aimed to quantify changes in leaf *chl* content in response to drought stress. In such cases, spectrophotometric pigment assays such as the one used here (after Arnon, 1949) are sufficient (Bulda et al., 2008), but stability of the pigment extract should be checked since extraction with acetone solutions can lead to *chl* degradation to phaeophytin by co-extracted acids (Rodrigues-Amaya and Kimura, 2004). This was unlikely to have been the case here, since the absorbance of acetone *chl* extracts of H. montanus leaves remained stable (to the third decimal) for as long as 6 h after filtration (when kept in the dark).

Steady-state gas exchange

All gas exchange measurements were carried out using the Li-Cor 6400 portable photosynthesis measuring system, fitted with an *Arabidopsis* chamber and configured as an open system (Li-Cor, Nebraska, U.S.A.). On experimental days 0, 4, 12, 29 and 39, instantaneous (spot) measures of leaf-based CO₂-assimilation rates (*A*) were carried out at carbon dioxide concentration (C_a): 400 µmol. mol⁻¹ and then at above ambient C_a: 600 µmol. mol⁻¹, across all treatments. Measurements were carried out around midday, on non-senescing leaves and under natural illumination. For each treatment, three consecutive measurements were taken for one leaf from each of 7 seedlings, when the total percentage coefficient of variance [% Δ H₂O; Δ CO₂; Δ flow rate] was <1%. The mean of these three measurements yielded the final reading for that leaf. Only values measured at a photosynthetic photon flux density (PPFD) >800 µmol m⁻² s⁻¹ (i.e. light saturated) were used for subsequent analyses.

The ratio between A at C_a: 600 and 400 μ mol. mol⁻¹, referred to as 'A@600 : A@400' from here on, was also calculated. This ratio has been used to assess the effects of water stress on A in

other studies (e.g. Osmond *et al.*, 1980), since the response of A to changing C_a is believed to be a reliable indication of stomatal limitation of A (Cornic *et al.*, 1983); a higher ratio being indicative of greater stomatal limitation.

Potential photochemical efficiency

On experimental days 0, 4, 12, 29 and 39, a Plant Efficiency Analyser (Hansatech Instruments Ltd., Kings Lynn, U.K.) was used to measure chlorophyll fluorescence transients on one fully expanded, non-senescing, mature leaf from each of 6 seedlings, across all treatments. After samples were dark adapted for 20 min, transients were induced by red light of 1500 μ mol m⁻² s⁻¹ generated by six light-emitting diodes (peak 650 nm). These diodes covered the exposed area of the leaf (4 mm in diameter) in homogenous illumination and fluorescence signals were recorded within a time span of 10 μ s to 1 s with a data acquisition rate of 10 μ s for the first 2 ms and 1 ms thereafter. F_v/F_m, the ratio of variable (F_v) to maximum fluorescence (F_m), is a measure of potential photochemical efficiency of photosystem II (PSII) and was calculated from the fluorescence data using Biolyzer 3.0 software (developed by Maldonado-Rodriguez, 2002).

Leaf water status

Leaf water, osmotic and pressure potential

On experimental days 0, 4, 12, 29 and 39, leaf water potential (Ψ_w) measurements were carried out at predawn (pd) and 5-6 h into the light period (md) using thermocouple psychrometers (Model C-52; Wescor, Logan, U.S.A.) in combination with a microvoltmeter. For this, leaf discs were excised from the middle of one fully expanded, non-senescing, mature leaf from each of 4 seedlings, across all treatments, and Ψ_w recorded after a pre-determined equilibration period. The microvoltmeter was calibrated against NaCl standards, at 25°C, and a cooling period of 20 s was used to measure the dew point. The difference between pd Ψ_w and md Ψ_w , referred to as 'pd-md', was also calculated. pd-md Ψ_w reflects the extent to which md Ψ_w is lowered from that at pd, to generate the Ψ_w gradient between soil and leaf to drive water through the plant and hence meet the transpirational demand during the light period.

After Ψ_w was determined, leaf samples were wrapped in at least four layers of ParafilmTM (American National Can) covered with aluminium foil and plunged repeatedly into LN for 30 s intervals, over a period of 5 min. After thawing, samples were unwrapped, placed immediately into C52 sample chambers and Ψ_w recorded. If apoplastic water is negligible, Ψ_w after cooling and thawing of tissue in this way is generally regarded as the 'osmotic' potential (Ψ_s), or water activity (Jones and Rawson, 1979). Pressure potential (Ψ_p) was calculated as the difference between osmotic and water potential.

Ex vitro growth response

After the *in vitro* and *ex vitro* growth periods, six seedlings from each treatment were separated into bulb, leaves and roots and oven-dried for 72 h at 80°C to a constant weight. Relative growth rate (RGR) was calculated via Equation 3 below:

RGR =

[individual seedling DM after 63 d *ex vitro* growth (g) - mean seedling DM after 90 d *in vitro* growth (g)] mean seedling dry mass after 90 d *in vitro* growth (g) 63 d

where DM refers dry mass.

Statistical analysis

Leaf plastochron and *in vitro* biomass data were compared between treatments by one-way Analysis of Variance (*ANOVA*; SPSS, Version 15). All other variables were tested for differences across treatments, within time intervals, by two-way *ANOVA*. Two-way *ANOVAs* were factorial in design, testing for the main-effects of embryo pre-treatment (referred to as 'Embryo') and water stress (referred to as 'Stress'), as well as the interaction between these (referred to as 'Embryo×Stress') (STATISTICA Version 6.1, StatSoft Inc. Tulsa, U.S.A.). Multiple comparisons were made using a Scheffe's mean separation test. Where the original data were expressed as a proportion (%) these values were arcsin transformed to conform data to *ANOVA* assumptions. At the end of the *in vitro* and *ex vitro* growth period's embryo/seedling viability (%) was compared across all treatments using null-model chi-squared analyses (EcoSim Version 7.72 [developed by Gotelli and Entsminger, 2009]). All statistical tests were performed at the 0.05 level of significance.

6.3 Results

Zygotic embryo water content vs. seedling biomass after 60 d in vitro growth

Freshly excised embryos had a shedding WC of *c*. 6.13 g g⁻¹ and seedlings generated from such embryos (F-seedlings) exhibited a biomass of *c*. 0.033 g after 60 d *in vitro* growth (Fig. 6.2). With dehydration to an embryo WC of 0.79 g g⁻¹, subsequent seedling biomass was significantly reduced and the severity of this reduction increased with a further decline in embryo WC. For instance, embryo dehydration to 0.28 g g⁻¹ reduced subsequent seedling biomass by 48% to *c*. 0.017 g, while further dehydration to 0.11 g g⁻¹, reduced seedling biomass by 63% to *c*. 0.012 g.

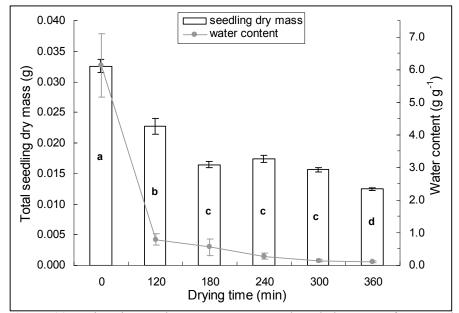


Figure 6.2 Drying time, embryo water content and total dry mass of recovered seedlings. Dry mass was assessed for individual seedlings after 60 d *in vitro* growth. Water content (n = 10) and biomass values (n = 5) represent mean±SD. Columns labelled with different letters are significantly different across treatments (p < 0.05, *ANOVA*, n = 5).

Growth responses after 90 d in vitro growth

While 84% of zygotic embryos dehydrated to *c*. 0.28 g g⁻¹ produced seedlings after 90 d *in vitro* growth, 96% of F-embryos (i.e. undried embryos) produced seedlings after a similar *in vitro* growth period (data not shown). Total biomass of D-seedlings was significantly lower than F-seedlings after 90 d *in vitro* growth (Fig. 6.3A). Bulb, leaf and root dry mass were relatively lower in D-seedlings (significant for leaves and roots; Fig. 6.3A) but biomass partitioning to different organs did not differ significantly between treatments (Fig. 6.3B).

Ex vitro growth responses

Except for FS-seedlings, all treatments exhibited positive relative growth rate (RGRs) (Table 6.1). Relative growth rate was greater in unstressed seedlings but this trend was significant for D-seedlings only. Within the stressed treatments RGR was higher in D-seedlings, but not significantly so.

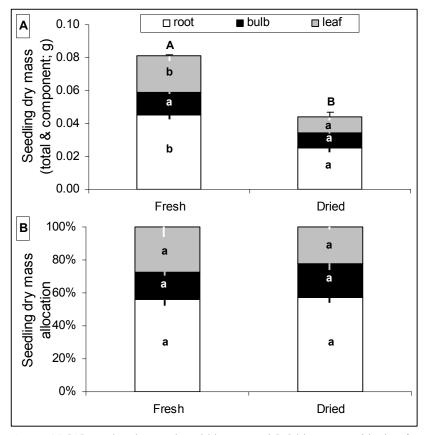


Figure 6.3 [A] Total and organ-based biomass and [B] biomass partitioning for seedlings generated from fresh (F) and partially dried (D) embryos after 90 d *in vitro* growth. Blocks labelled with different lower-case letters are significantly different when compared within organs, while upper-case letters indicate intertreatment differences in total seedling dry mass (p = 0.01, *ANOVA*, n = 6). Bars represent SD.

FW	0.018 ± 0.024^{ab}			
DW	0.032 ± 0.017^{a}			
FS	-0.001 ± 0.008^{b}			
DS	0.009 ± 0.016^{b}			

Table 6.1 *Ex vitro* seedling relative growth rate (RGR; g g⁻¹ DM d⁻¹).

RGR = relative growth rate ([Individual seedling dry mass after 63 d *ex vitro* growth (g) - Mean seedling dry mass after 90 d *in vitro* growth (g) / Mean seedling dry mass after 90 d *in vitro* growth (g)] / 63 d) for seedlings generated from fresh (F) and partially dried (D) embryos, subjected to watering (W) or water deficit (S). Values represent mean±SD and are significantly different when followed by different letters (p < 0.05 for Stress and Embryo, *ANOVA*, n = 6). Total seedling dry mass and bulb dry mass in unstressed seedlings were greater than stressed ones but this trend was significant for F-seedlings only (Fig. 6.4A). Leaf biomass in FW-seedlings was also significantly higher than FS-seedlings (Fig. 6.4A). Within the unstressed treatments, biomass partitioning to leaves was significantly lower in D-seedlings, however, biomass partitioning to leaves in DS-seedlings was significantly higher than DW-seedlings (Fig. 6.4B). Biomass partitioning to bulbs in D-seedlings was also slightly, but not significantly, lower when stressed (Fig. 6.4B).

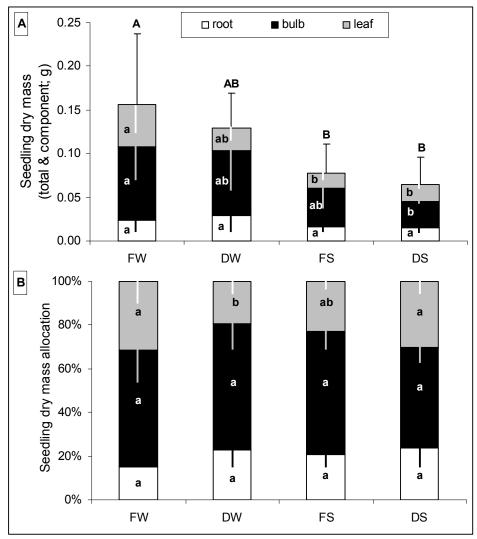


Figure 6.4 [A] Total and organ-based biomass and [B] biomass partitioning for seedlings generated from fresh (F) and partially dried (D) embryos, subjected to watering (W) or water deficit (S). Blocks labelled with different lower-case letters are significantly different when compared within organs, while upper-case letters indicate inter-treatment differences in total seedling biomass. For [A]: total dry mass: p = 0.001 for Stress; bulb: p = 0.002 for Stress; leaves: p = 0.003 for Stress and for [B]: leaves: p = 0.02 for Embryo×Stress (*ANOVA*, n = 6).

Plant water relations

Leaf water, osmotic and pressure potential

Water, osmotic and pressure potential data were tested for differences across treatments, within a measurement day. On day 0, embryo pre-treatment had no significant effect on predawn (pd) Ψ_w , the difference between pd and midday (md) Ψ_w (pd-md Ψ_w) (Fig. 6.5), Ψ_s (Fig. 6.6) or Ψ_p (Fig. 6.7).

After day 12 pd Ψ_w in DW-seedlings was consistently, but not significantly, slightly lower than FW-seedlings (Fig. 6.5A). Water stress depressed pd Ψ_w in stressed seedlings, relative to their respective unstressed controls, but this trend was significant on day 39 only (Fig. 6.5A, B). Pre-dawn Ψ_w in DS-seedlings was lower than FS-seedlings on day 39 (Fig. 6.5A). In FSseedlings, pd-md Ψ_w was often higher than DS- and FW-seedlings but this trend was not significant (Fig. 6.5B).

Except that pd Ψ_s in FS-seedlings was consistently lower than FW-seedlings throughout the experimental period, Ψ_s displayed no consistent trends across or within treatments throughout the experimental period (Fig. 6.6).

Within the unstressed treatments, Ψ_p exhibited no consistent trends (Fig. 6.7). During the stress pd Ψ_p in DS-seedlings was progressively depressed relative to DW-seedlings (Fig. 6.7A), suggesting the onset of permanent turgor loss. This trend was also observed at md, but significant on day 29 only (Fig. 6.7B). During this time, Ψ_p in FS-seedlings was occasionally slightly, but never significantly, lower than FW-seedlings. Predawn and md Ψ_p in DS-seedlings was also significantly lower than FS-seedlings.

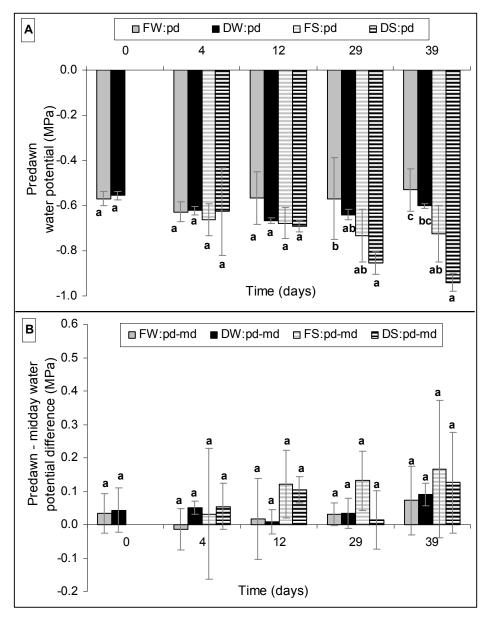


Figure 6.5 [A] Predawn (pd) leaf water potential and [B] Predawn - midday water potential difference (pd-md) of seedlings recovered from fresh (F) and partially dried (D) embryos, subjected to watering (W) or water deficit (S). Columns labelled with different letters are significantly different when compared within experimental days (pd Ψ_w : p = 0.01 for Stress and p = 0.004 for Embryo on day 39, *ANOVA*, n = 4). Bars represent ±SD.

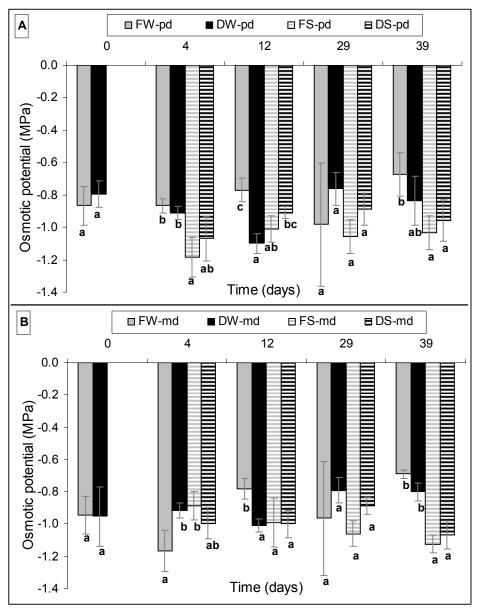


Figure 6.6 [A] Predawn (pd) and [B] midday (md) leaf osmotic potential of seedlings recovered from fresh (F) and partially dried (D) embryos, subjected to watering (W) or water deficit (S). Columns labelled with different letters are significantly different when compared within experimental days (pd Ψ_s : p < 0.01 for Stress on days 4 and 39 and for Embryo×Stress on day 12, *ANOVA*, n = 4). Bars represent ±SD.

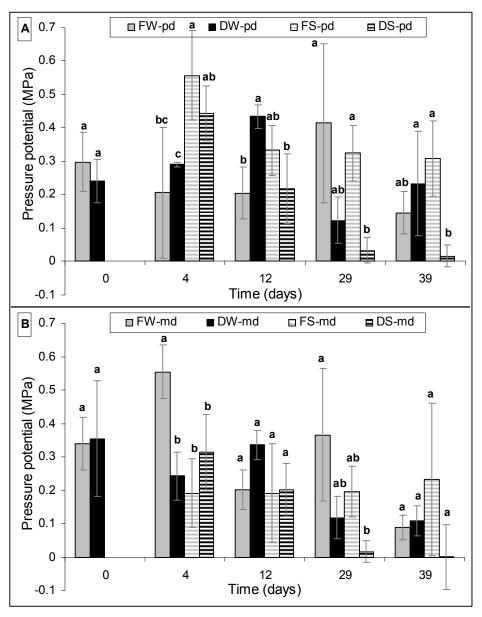


Figure 6.7 [A] Predawn (pd) and [B] midday (md) leaf pressure potential of seedlings recovered from fresh (F) and partially dried (D) embryos, subjected to watering (W) or water deficit (S). Columns labelled with different letters are significantly different when compared within experimental days (pd Ψ_p : p < 0.01 for Embryo×Stress on days 12 and 39 and for Embryo on day 29; md Ψ_p : p = 0.03 for Stress and < 0.01 for Embryo on day 29, *ANOVA*, n = 4). Bars represent ±SD.

Photosynthetic characteristics

Leaf chlorophyll content

Embryo pre-treatment did not affect leaf *chl* content significantly but *chl a*, *b* and total leaf *chl* content in stressed seedlings were significantly lower than their respective unstressed controls, at the final harvest (Fig. 6.8).

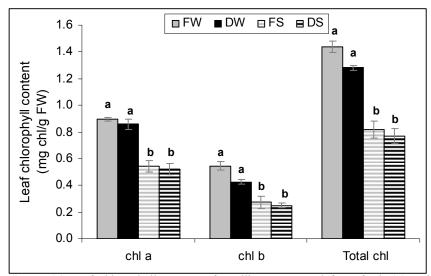


Figure 6.8 Leaf chlorophyll content of seedlings recovered from fresh (F) and partially dried (D) embryos, subjected to watering (W) or water deficit (S). Chlorophyll content was measured only at the end of the *ex vitro* growth period. Columns labelled with different letters are significantly different when compared within categories (p = 0.04 for Stress, *ANOVA*, n = 4). Bars represent ±SD.

CO₂-assimilation

On day 0, and throughout the experimental period leaf-based CO₂-assimilation rates (*A*) at C_a: 400 µmol. mol⁻¹ in D-seedlings were significantly lower than F-seedlings, for both stressed and unstressed treatments (Fig. 6.9). Water stress significantly depressed *A* in DS- and FS-seedlings, relative to their respective unstressed controls, throughout the experimental period. The ratio *A* at C_a: 600 µmol. mol⁻¹: *A* at C_a: 400 µmol. mol⁻¹ (A@600:A@400), and hence the degree of stomatal limitation of *A*, in D-seedlings was significantly higher than F-seedlings in the absence of a stress but consistently significantly lower when stressed (Table 6.2). A@600:A@400 in FS-seedlings was also consistently significantly higher than FW-seedlings (Table 6.2).

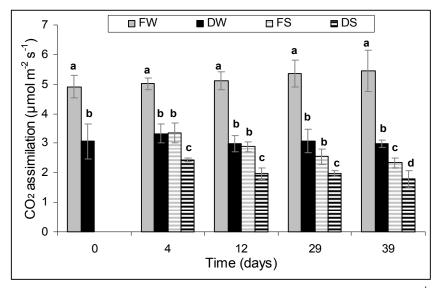


Figure 6.9 Instantaneous leaf-based CO₂-assimilation rates (at C_a: 400 µmol. mol⁻¹) of seedlings recovered from fresh (F) and partially dried (D) embryos, subjected to watering (W) or water deficit (S). Columns labelled with different letters are significantly different when compared within experimental days (p < 0.01 for Embryo on days 0, 4, 12, 29 and 39; p < 0.01 for Embryo on days 4, 12, 29 and 39, ANOVA, n = 7). Bars represent ±SD.

Table 6.2 Ratio of CO₂-assimilation rate at C_a: 600 µmol. mol⁻¹ to CO₂-assimilation rate at C_a: 400 µmol. mol⁻¹.

-				-
Day	FW	DW	FS	DS
0	$2.04{\pm}0.24^{a}$	2.76 ± 0.64^{a}	NA	NA
4	1.70 ± 0.12^{b}	2.11±0.14 ^a	2.06±0.25 ^a	1.61±0.06 ^b
12	1.59±0.11 ^b	2.03±0.15 ^a	2.32±0.16 ^c	1.91±0.24 ^a
29	1.59±0.34 ^a	2.13 ± 0.30^{b}	2.48 ± 0.28^{b}	1.65 ± 0.06^{a}
39	1.05±0.41 ^a	2.04±0.23 ^b	2.46±0.30 ^b	1.58±0.26 ^a

Ratio was calculated for seedlings recovered from fresh (F) and partially dried (D) embryos, subjected to watering (W) or water deficit (S). Values followed by different letters are significantly different when compared within a measurement day (p < 0.01 for Embryo×Stress on days 4, 12, 29 and 39, *ANOVA*, n = 7). Values represent mean±SD. NA = not applicable.

Chlorophyll fluorescence

Within the unstressed treatments there was little, to no difference in potential photochemical efficiency of PSII (F_v/F_m ; Fig. 6.10A), maximal fluorescence intensity (F_m ; Fig. 6.10B) and fluorescence intensity at 50 µs (F_o ; Fig. 6.10C). Values of F_v/F_m in DS-seedlings were consistently lower than DW-seedlings (significant on day 39 only) and on day 39 this was accompanied by a significant decline in F_m . F_v/F_m in DS-seedlings was also relatively lower than FS-seedlings on days 4, 12, 29 and 39 (significant on day 39 only). F_v/F_m in FS-seedlings was occasionally slightly, but not significantly, lower than FW-seedlings.

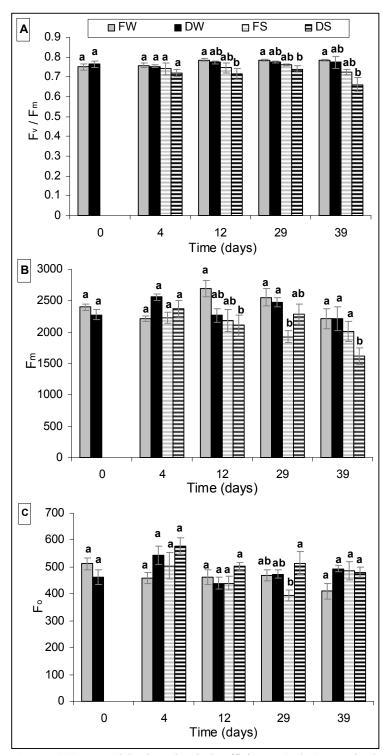


Figure 6.10 Potential photochemical efficiency (F_v/F_m) , maximal fluorescence intensity (F_m) and fluorescence intensity at 50 µs (F_o) of seedlings recovered from fresh (F) and partially dried (D) embryos, subjected to watering (W) or water deficit (S). Columns labelled with different letters are significantly different when compared within experimental days $(F_v/F_m: p < 0.01 \text{ for Stress on day 39}; F_m: p < 0.01 \text{ for Stress on day 39}, ANOVA, <math>n = 6$). Bars represent ±SD.

Seedling mortality

When unstressed, seedling mortality did not differ by much between embryo pre-treatments (Table 6.3). Mortality was significantly higher in stressed seedlings; within the stressed treatments mortality was significantly higher in DS-seedlings (Table 6.3).

Table 6.3 Total seedling mortality over the entire ex vitro growth period.

Treatment	FW	DW	FS	DS
% Mortality mbryos and subj	ected to wate	ring (W) o	and partial	$\frac{1}{10}$
0.05 when values v squared test, $n = 95$	*	d across trea	atments (nu	ll model chi-

6.4 Discussion

There are differences among species in growth and developmental responses of plantlets or seedlings recovered from partially dried zygotic embryos or embryonic axes with some, particularly those of orthodox-seeded species, showing normal phenotype whilst others, particularly those of recalcitrant-seeded species, exhibit reduced growth and development rates (see Introduction for details). Even though partial dehydration is widely used as a preconditioning treatment in cryopreservation protocols for zygotic germplasm (see Engelmann, 2004), information on how dehydration stresses imposed at the embryonic stage are translated or manifested during subsequent seedling growth is scarce. There are a few studies (e.g. Gagliardi et al., 2002; Steinmacher et al., 2007) in which the ex vitro growth of plants derived from partially dried zygotic germplasm has been reported; however, those studies were almost always based on phenotypic descriptions and there are presently few, if any published reports on the physiological performance of seedlings or plantlets recovered from partially dried or cryopreserved zygotic embryos or embryonic axes. The present contribution reports on the in and ex vitro growth of seedlings recovered from partially dried embryos of the wild species H. *montanus*. Since seedlings derived from cryopreserved zygotic germplasm of such species are unlikely to be carefully tended upon re-introduction to the wild and will be subject to the vagaries of the weather, the effects of embryo partial dehydration on subsequent ex vitro seedling vigour are important. This was assessed here by observing physiological and growth responses of recovered seedlings to an *ex vitro* water stress.

Growth responses

The poor *in vitro* vigour of *H. montanus* seedlings recovered from D- compared with Fembryos (Fig. 6.2) is in accordance with the results of other studies (e.g. Sershen *et al.*, 2007; Steinmacher *et al.*, 2007) which suggest that partial dehydration of recalcitrant zygotic germplasm, can induce morphological abnormalities and/or reduced vigour in recovered seedlings. The adverse effects of partial dehydration on seedling biomass accumulation observed after 60 d in vitro growth (Fig. 6.2) did not disappear with an extension of the in vitro growth period (Fig. 6.3). These effects were not isolated to any particular organ but were most apparent at the shoot level, possibly compromising light-harvesting capacity of D-seedlings upon introduction to the ex vitro environment. The final biomass achieved during the ex vitro growth period (Fig. 6.4) could be a consequence of either or both ex vitro growth rates or the amount of material at the start of this growth phase. To assess this, RGRs (relative to the dry mass at the end of the *in vitro* growth period) were calculated. The adverse effect of embryo partial drying observed during the *in vitro* growth phase appeared reversible during subsequent ex vitro growth (Table 6.1). However, a water stress during this period dominated over the effects of embryo pre-treatment, with RGRs in DS- and FS-seedlings being statistically comparable. Reduced growth in droughted seedlings (e.g. Hsiao, 1973; Frensch and Hsiao, 1994) has been variably attributed to a reduction in leaf Ψ_w , turgor loss (e.g. Hsiao, 1973; Frensch and Hsiao, 1994) and/or a reduction in the photosynthetic uptake of carbon (Katul et al., 2003); all of which were observed here (see Figs 6.5 and 6.7 for $\Psi_{\rm w}$ and $\Psi_{\rm p}$ respectively and Fig. 6.9 for A). Interestingly, FS- unlike DS-seedlings exhibited a negative RGR. This was most likely a consequence of drought-induced root and leaf senescence in FS-seedlings, since final values for root and shoot dry mass in this treatment (leaf: 0.018 g; root: 0.016 g) were both lower than initial values (leaf: 0.022 g; root: 0.045 g). The leaf senescence observed in FSseedlings is consistent with typical geophytic growth strategy which selects for bulb increment during a stress event, since survival in the season following dormancy is determined by the reserves stored in this organ, while leaf area is progressively reduced via shoot die-back to reduce the transpirational demand on the roots (e.g. von Willert et al., 2000). Additionally, roots produced in vitro are often dysfunctional and usually senesce, being replaced with new roots during ex vitro growth (Barry-Etienne et al., 2002). This replacement process may have been more a feature of FS- than DS-seedling growth strategy since FS-seedlings entered the ex vitro growth phase with considerably more root dry matter than DS-seedlings (Fig. 6.3A).

Plant water relations

Water stress is usually quantified in terms of the extent to which tissue Ψ_w falls below that at full turgor (Fitter and Hay, 2002) and pd Ψ_w was indiscriminately reduced in stressed seedlings in this study (Fig. 6.5A). Such a decrease is often initiated to increase the Ψ_w gradient between soil and plant and drive water through the plant (Barker *et al.*, 1993; Galmés *et al.*, 2007). The decrease in Ψ_w in stressed seedlings was significantly greater in D-seedlings, and DS- unlike FS-seedlings did not exhibit any signs of long-term osmotic adjustment, reflected in a decrease in Ψ_s in FS-seedlings relative to their well watered controls (Fig. 6.6). Many species exhibit a decrease in Ψ_s during a water stress to maintain turgor at low Ψ_w (Barker *et al.*, 1993; Galmés *et al.*, 2007). However, such an adjustment demands considerable metabolic investment (Barker *et al.*, 1993) and the absence or poor expression of this response in DS-seedlings may have been a consequence of the relatively lower *A* (Fig. 6.9) in D-seedlings. Pd-md Ψ_w values in FS-seedlings were often higher than FW- and DS-seedlings, suggesting that FS-seedlings may have been more efficient than DS-seedlings at generating a gradient between soil and leaf Ψ_w to meet transpirational demands. Failure to equilibrate with soil Ψ_w overnight can severely depress Ψ_w in droughted seedlings (Fitter and Hay, 2002) and prolonged exposure to such low Ψ_w can be extremely stressful in juvenile plants (Hsiao, 1973). This may explain why DS-seedlings exhibited greater and more prolonged turgor loss than FS-seedlings (Fig. 6.7); prolonged turgor loss is indicative of permanent leaf-wilting (Leopold *et al.*, 1981; Galmés *et al.*, 2007).

In the absence of a stress there were no significant differences in the measured water relations parameters between F- and D-seedlings, indicating that the effects of partial drying of excised embryos were reversible in the long-term (Figs 6.5 and 6.7).

The estimates of osmotic and turgor potential were variable within treatments though and this may have been due to non-negligible apoplastic water content. The use of a vapor-pressure osmometer to measure leaf osmotic potential (e.g. Ball and Oosterhuis, 2005) may have overcome the limitations of the present approach. Pressure chamber techniques may represent an alternate option for estimating turgor potential in the seedlings investigated here but it must be noted that such techniques have been shown to over-estimate leaf water potential relative to thermocouple psychrometer measurements (Wright *et al.*, 1988).

Photosynthetic characteristics

The light-energy transducing system of a plant is sensitive to tissue water status and a decrease in *chl* content after a drought often results in a decrease in light harvesting capacity (Alberte *et al.*, 1974). In *H. montanus chl* content decreased indiscriminately in stressed seedlings (Fig. 6.8); this may explain the drought-induced reduction in *A* observed in these seedlings (Fig. 6.9). Additionally, a reduction in *A* during a drought event (e.g. Hsiao, 1973; Tezara *et al.*, 1999; Flexas *et al.*, 2006) is generally attributed to a restriction of leaf gas exchange, mediated by the closure of stomata during turgor loss (Björkman and Powles, 1984; Galmés *et al.*, 2007; McDowell *et al.*, 2008). As discussed earlier, stressed seedlings in this study did exhibit a significant decrease in Ψ_p (Fig. 6.7).

Interestingly, A in D-seedlings was significantly lower than F-seedlings, irrespective of whether they were stressed or not (Fig. 6.9). These differences were unlikely to have been related to differences in leaf *chl* content (Fig. 6.8). In the absence of a stress the ratio A@600: A@400 (taken as a measure of stomatal limitation) in D-seedlings was significantly greater than F-seedlings (Table 6.2), suggesting that the significantly lower A in D-seedlings may have been a consequence of stomatal limitation. However, when subjected to a water stress stomatal limitation was greater in F-seedlings. These data suggest that the reduction of A in DS-seedlings was, to some degree, a consequence of damage to the photosynthetic machinery. Plants can incur permanent damage to their photosynthetic machinery during a water stress (Flexas et al., 2006) and as in other studies (Burke and Hatfield, 1987; Govindjee et al., 1981), DS-seedlings may have incurred such damage as a consequence of metabolic disruption and membrane rupture associated with prolonged turgor loss (Leopold *et al.*, 1981). Such damage depresses photosynthesis (Radin and Ackerson, 1981) and phytochemical efficiency (Govindjee et al., 1981) and consistent with this, F_v/F_m values in DS-seedlings declined significantly towards the latter stages of the stress (Fig. 6.10). Other studies (e.g. Havaux and Lannoye, 1983; Rolando and Little, 2003) have also reported a depression in F_v/F_m in droughted seedlings and such a decrease is believed to be a reliable indicator of a reduction in seedling vigour (Maxwell and Johnson, 2000). A depression in F_v/F_m is often related to a rise in F_o (fluorescence intensity at 50μ s) and/or a decrease in F_m (maximal fluorescence intensity) (e.g. Rolando and Little, 2003). The former was not observed here, but F_m did decline significantly on day 39 in DS-seedlings (Fig. 6.10B).

Seedling mortality

R

In the absence of a water stress D-seedlings did not appear to be more susceptible to mortality than F-seedlings (Table 6.3) and under such ideal conditions the significantly higher RGRs in D-seedlings (Table 6.1) may assist D-seedlings in overcoming the adverse effects of dehydration, aiding successful seedling establishment. The decrease in Ψ_w in stressed seedlings was typically (see McDowell *et al.*, 2008) accompanied by greater seedling mortality than their respective well watered controls (Table 6.3). Drought-induced seedling mortality was, however, significantly higher in DS-seedlings, possibly due to the combination of insufficient adjustment of Ψ_w to meet transpirational demands (Fig. 6.5), a failure to adopt growth patterns that reduce transpirational water loss (Fig. 6.4), exposure to significantly lower pd Ψ_w than FS-seedlings (Fig. 6.5), poor osmotic adjustment (Fig. 6.6), and the onset of permanent leaf wilting (Fig. 6.7); all of which promote hydraulic-failure (see McDowell *et al.*, 2008) in juvenile plants (e.g. Hsiao, 1973). The influence of carbon-starvation (i.e. stomatal closure to prevent hydraulic failure) cannot be ruled out, since F_v/F_m (Fig. 6.10A) also declined in DS-seedlings, but carbonstarvation is after all believed to be brought about by hydraulic-failure (McDowell *et al.*, 2008).

6.5 Concluding remarks

The results obtained here suggest that the exposure of recalcitrant *H. montanus* embryos to a dehydration stress can compromise the vigour and drought tolerance of recovered seedlings. While certain adverse effects of partial dehydration may be reversed during *ex vitro* growth (e.g. higher RGR) the adverse effects on physiological components, such as the photosynthetic machinery, may be carried through to the early *ex vitro* stage. An extended period of *ex vitro* acclimatization before re-introduction of such seedlings into the wild may alleviate such effects, but this remains to be tested. The results obtained here warrant an investigation of the effects of recalcitrant embryo cryopreservation on the vigour of recovered seedlings; this forms the basis of studies described in Chapter 7, where the study was extended to another species.

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CHAPTER SEVEN: Effects of cryopreservation of recalcitrant *Amaryllis belladonna* zygotic embryos on vigour of recovered seedlings

Based on

Sershen, Berjak, P., Pammenter, N.W. 2010. Effects of cryopreservation of recalcitrant *Amaryllis belladonna* zygotic embryos on vigour of recovered seedlings: a case of stress 'hangover'? Physiologia Plantarum 139, 205-219.

Abstract

Cryopreservation is the most promising long-term storage option for recalcitrant (i.e. desiccation-sensitive) seed germplasm, however, its effects on the vigour of recovered seedlings is unclear. This study looked at the vigour of seedlings recovered from partially dried (D) and cryopreserved (C) recalcitrant zygotic embryos of Amaryllis belladonna (L). Seedlings recovered from fresh (F), D- and C-embryos were recovered in vitro, hardened-off ex vitro and then exposed to 12 d of watering (W) or 8 d of water deficit (S), followed by 3 d of re-watering. Seedling vigour was assessed in terms of physiological and growth responses to the imposed water stress. Compared with F-embryos, partial dehydration and cryopreservation reduced the number of embryos that produced seedlings, as well as the subsequent in vitro biomass of these seedlings. DW- and CW-seedlings (recovered from dried and cryopreserved embryos and watered for 12 d) exhibited lower CO₂-assimilation rates and abnormal root growth. Stomatal density was also lower in C-seedlings. DS- and CS-seedlings (recovered from dried and cryopreserved embryos and stressed) were exposed to persistent low leaf water and pressure potentials and unlike FS-seedlings, displayed signs of having incurred damage to their photosynthetic machinery. Consistent with this, leaf chlorophyll content was lower in D- and Cseedlings than F-seedlings, and potential photochemical efficiency was significantly reduced in CS-seedlings. CS-seedlings were less efficient at adjusting leaf water potential to meet transpirational demands and more susceptible to persistent turgor loss than DS- and FSseedlings. DS-seedlings performed slightly better than CS-seedlings but drought-induced seedling mortality in both these treatments was higher than FS-seedlings. These results suggest that seedlings recovered from partially dried and cryopreserved embryos were less vigorous and more susceptible to hydraulic-failure than those from fresh embryos.

7.1 Introduction

Cryopreservation (i.e. storage at ultra-low temperatures, usually -196°C) is the most promising long-term storage option for recalcitrant seed germplasm, which otherwise cannot be stored for any useful period of time (Berjak and Pammenter, 2004). Unlike the success achieved with somatic (e.g. Mycock *et al.*, 1995) and nucellar embryos (e.g. Kartha, 1985), and even zygotic

embryos from orthodox seeds (e.g. Gagliardi *et al.*, 2002), recovery after cryopreservation of recalcitrant zygotic embryos or embryonic axes very seldom results in the production of callus free plants, rid of morphological abnormalities (for reviews see Engelmann, 2004; Berjak *et al.*, 2010). In fact, a number of studies (e.g. Pence, 1992; Dumet *et al.*, 1997; Wesley-Smith *et al.*, 2001, 2004; Sershen *et al.*, 2007; Steinmacher *et al.*, 2007), have observed abnormal phenotype in seedlings recovered from recalcitrant zygotic germplasm exposed to partial dehydration or the combination of partial dehydration and cooling. Further to this, the cryopreservation tissue-culture regeneration process may also expose recovered seedlings to the effects of somaclonal variation, resulting in changes to their genotypic and/or phenotypic profiles (reviewed by Harding, 2004; Panis and Lambardi, 2006).

It has been known for some time now that exposure to different types of stress can alter subsequent plant responses (Bruce *et al.*, 2007) but there is, at present, little to no understanding of how stresses imposed at the embryonic stage are translated or manifested during subsequent *in* and *ex vitro* seedling growth. A few reports suggest that there exists within developing embryos (zygotic and somatic) some 'memory' based mechanism that senses environmental signals such as duration of imbibition (Forsyth and van Staden, 1983) and temperature during embryogenesis (Kvaalen and Johnsen, 2007), which in turn influence adaptive traits in the seedlings they give rise to. If such a mechanism were to exist within recalcitrant zygotic embryos or embryonic axes, the dehydration and freezing stresses imposed on these explants during cryopreservation, together with the morphological abnormalities that often characterise recovered seedlings (reviewed by Engelmann, 2004; Steinmacher *et al.*, 2007), may compromise the aim of plant cryopreservation - to regenerate true-to-type seedlings.

There have been reports of no observed differences in morphological characters between plants recovered from control or cryopreserved: shoot apices (e.g. Helliot *et al.*, 2002), meristems (e.g. Bajaj, 1983; Matsumoto *et al.*, 1994; Caswell and Kartha, 2009), somatic embryos (Aronen *et al.*, 1999), embryogenic cell suspensions (e.g. Côte *et al.*, 2000), polyembryonic cultures (Konan *et al.*, 2007), shoot tips (e.g. Benson *et al.*, 1996; Wang *et al.*, 2005), non-orthodox whole seeds (e.g. Potts and Lumpkin, 2000; Popov *et al.*, 2004), and even zygotic embryos from recalcitrant seeds (Assy-Bah and Engelmann, 1992). Also, there are an increasing number of reports indicating no phenotypical, biochemical, chromosomal or molecular modifications of thawed material attributed to cryopreservation (for reviews see Harding, 2004; Engelmann, 2004). Many of those observations have been made very soon after cryopreservation and on a small number of individuals, often using material still cultured *in vitro* or after a short period of growth *ex vitro*. However, there are cases (e.g. Engelmann, 1991; Benson *et al.*, 1996; Côte *et al.*, 2000; Martínez-Montero *et al.*, 2002; Konan *et al.*, 2007;

Caswell and Kartha, 2009) where many plants (often hundreds) derived from cryopreserved germplasm have been grown and assessed in the field for many months, to years. Those studies have shown recovered plantlets of a number of (mainly crop) species (including: potato, banana, sugarcane and apple and oil palm) to exhibit normal phenotype and even flower, fruit and produce seed. These observations were based on a comparison of a wide range of developmental and morphological growth characteristics of plants recovered from frozen and unfrozen samples, however, except for one report on the photochemical activities of two photosystems in frozen-thawed Bratonia orchid protocorms during in vitro recovery (Bukhov et al., 2006) there are, at present, no published reports on the in or ex vitro physiological performance and/or stress tolerance of plants recovered from cryopreserved samples. Ex vitro physiological performance and stress tolerance of seedlings recovered from cryopreserved germplasm is of special interest to programs concerned with the cryopreservation of the germplasm of endangered wild species, which ultimately aim to re-introduce plants or seedlings recovered from cryopreserved samples back into the wild. Since the successful re-introduction of recovered seedlings will depend on their ability to tolerate abiotic stresses such as drought, most especially during the early seedling establishment phase, the current contribution looked at the *in* and *ex vitro* vigour of seedlings recovered from partially dried and cryopreserved, recalcitrant zygotic embryos. The studies were undertaken on the recalcitrant zygotic embryos of the wild geophyte Amaryllis belladonna (L.) and seedling vigour was assessed in terms of physiological and growth responses to an ex vitro water stress

7.2 Materials and methods

Plant material

Mature *A. belladonna* fruits were harvested directly from parent plants and transported in plastic bags to the laboratory with minimum delay (1-2 d) or water loss. Upon arrival, the seeds were decontaminated and stored 'hydrated', as described in Chapter 2 (section 2.2).

Embryo pre-treatment, in vitro seedling regeneration and ex vitro hardening-off

All experiments were carried out on seeds stored for between 7-10 d, never longer. Zygotic embryos were excised and collected within closed Petri dishes on filter paper moistened with sterile calcium-magnesium solution (CaMg solution: 0.5μ M CaCl₂.2H₂O and 0.5 mM MgCl₂.6H₂O [Mycock, 1999]). In order to minimise the potential variation in drying and/or cooling rate as a function of embryo size, only embryos of between 4-6 mm in length were used for all the experiments described below. Excised embryos were subjected to one of the following treatment combinations: (*a*) no cryoprotection, no dehydration and no cooling (i.e.

freshly excised embryos possessing water content of c. 4.65 g g^{-1} ; (b) cryoprotection with glycerol (Gly; a combination of 5 and 10% [v/v]), rapid dehydration to c. 0.31 g g⁻¹ (via flash drying [devised by Berjak et al., 1990]) and rapid rehydration in CaMg solution; and (c) cryoprotection with Gly, rapid dehydration to c. 0.31 g g⁻¹, rapid cooling (hundreds of °C s⁻¹) of naked embryos in nitrogen slush (liquid nitrogen [LN] sub-cooled to -210°C [Echlin, 1992]), transfer under LN into LN-containing cryovials and storage in LN for 24 h; followed by thawing in CaMg solution held at 40°C for 2 min and rehydration in CaMg solution at 25°C for 30 min. For cryoprotection (CP), embryos were immersed in 5% (v/v) aqueous Gly and thereafter transferred to a 10% (v/v) aqueous solution of the cryoprotectant, for 1 h at each concentration. Embryos subjected to each of these three treatment (300 embryos for each) combinations (a to c) were then decontaminated with 1% (w/v) aqueous calcium hypochlorite for 3 min, washed with sterile CaMg solution (3 times) and then set to germinate with 5 embryos per Petri dish on full-strength Murashige and Skoog medium (Murashige and Skoog, 1962), containing 3% (w/v) sucrose. All Petri dishes were initially placed in the dark, and transferred upon signs of root and shoot development to a growth room with cool fluorescent lights (52 μ E s⁻¹ m⁻²) and a 16 h photoperiod, at ~25°C.

Embryos were grown *in vitro* for 150 d with two sub-cultures (at 50 and 100 d) and those that subsequently produced seedlings with callus free roots and shoots were transplanted independently into plastic inserts (5-cm-wide; 15-cm-deep), filled with a mixture of 1 part pine bark and 1 part coarse river sand, and placed in a misthouse for 14 d to harden-off.

Ex vitro experimental design

From here on, seedlings generated from fresh embryos will be labelled 'F', dried 'D' and cryopreserved 'C'. Where any of these labels is followed by the letter 'S' it refers to seedlings exposed to a water deficit, imposed by with-holding water, while the letter 'W' indicates the absence of this stress (i.e. seedlings that were watered daily). To validate the comparison of physiological measurements among F-, D- and C- seedlings during the *ex vitro* studies, we had to ascertain that seedlings recovered from such pre-treated embryos were at the same developmental stage after the 90 d *in vitro* growth period. As described for *Haemanthus montanus* seedlings in Chapter 6 (section 6.2), *A. belladonna* seedlings in this study were 'aged' by their morphology rather than chronological time using the leaf plastochron index (LPI) (originally described by Erickson and Michelini, 1957). *A. belladonna* seedlings generally produced two leaves by day 85 of *in vitro* regeneration. Leaf *1* and *2* (numbered in the order that the leaves were produced) length was measured for five D-, F- and C-seedlings on day 85 using calipers, and then again on day 90. These data suggested that growth had not leveled-off and

leaf length across all treatments was still actively increasing during this period (data not shown). With this mind, leaf lengths recorded on day 90 were used to calculate LPI₁ for each of five D-, F-, and C-seedlings, according to Erickson and Michelini (1957) (see Chapter 6, section 6.2 for detailed description of calculations). When calculated, mean LPI₁ was statistically comparable across F- (-11.95±1.88), D- (-12.01±1.72) and C- (-11.82±1.68) seedlings (*ANOVA*, p > 0.05; data not shown), suggesting that seedlings across these treatments were at approximately the same developmental age upon introduction to *ex vitro* conditions (i.e. day 91).

After hardening-off, plants were transferred to natural conditions of illumination in a polycarbonate-clad greenhouse. Plants were fertilised upon transfer to the greenhouse and after 7 d D-, F- and C-seedling groups, now composed of 174 (randomly selected) seedlings each, were further sub-divided into two batches of 87. Within each group, one seedling batch was subjected to a water stress by with-holding water for 8 d, followed by 3 d of re-watering while the second batch was watered daily for 12 d (see Fig. C1, Appendix C). Instantaneous leaf-based CO_2 -assimilation rate, potential photochemical efficiency as well as leaf water, osmotic and pressure potential, were measured across all embryo pre-treatment × watering regimes (referred to as 'all treatments' from here on) on days 0, 8 and 12, while growth responses (seedling biomass, biomass partitioning) and leaf chlorophyll content were assessed after the *in* and *ex vitro* growth period. Root morphology and stomatal density were assessed across all treatments at the end of the *ex vitro* growth period.

Photosynthetic capacity

Chlorophyll content

Chlorophyll (*chl*) content was measured on experimental day 0 (*ex vitro*) and at the final harvest (day 12) using one leaf from each of 4 seedlings (only living leaf material sampled), across all treatments. Chlorophyll was extracted and *chl a*, *b* and total *chl* was measured spectrophotometically according to a method described by Arnon (1949) (see Chapter 6; section 6.2). As in studies based on the vigour of *H. montanus* seedlings (described in Chapter 6), stability of the pigment extract was checked since extraction with acetone solutions can lead to *chl* degradation to phaeophytin by co-extracted acids (Rodrigues-Amaya and Kimura, 2004). In this regard, the absorbance of acetone *chl* extracts of *A. belladonna* leaves remained stable (to the third decimal) for as long as 6 h after filtration (when kept in the dark). This suggested that *chl* degradation to phaeophytin was unlikely to have affected the results obtained in subsequent studies.

Steady-state gas exchange

All gas exchange measurements were carried out on experimental days 0, 8 and 12 using the Li-Cor 6400 portable photosynthesis measuring system fitted with an *Arabidopsis* chamber (Li-Cor, Nebraska, U.S.A.). Instantaneous (spot) measures of leaf-based CO₂-assimilation rates (*A*) were carried out at carbon dioxide concentration (C_a): 400 µmol. mol⁻¹ and then at above ambient C_a : 600 µmol. mol⁻¹, across all treatments, according to the methods described for *H. montanus* in Chapter 6 (section 6.2). The ratio between *A* at C_a : 600 and 400 µmol. mol⁻¹, referred to as '*A*@600 : *A*@400' from here on, was also calculated. This ratio is a reflection of the response of *A* to changing C_a and is believed to be a reliable indication of stomatal limitation of *A* (Osmond *et al.*, 1980; Cornic *et al.*, 1983); a higher ratio is indicative of greater stomatal limitation.

Potential photochemical efficiency

On experimental days 0, 8 and 12 a Plant Efficiency Analyser (Hansatech Instruments Ltd., Kings Lynn, U.K.) was used to measure chlorophyll fluorescence transients on one fully expanded, non-senescing, mature leaf from each of 6 seedlings, across all treatments. Measurements were carried out according to the methods described for *H. montanus* in Chapter 6 (section 6.2) and F_v/F_m , the ratio of variable (F_v) to maximum fluorescence (F_m), which is a measure of potential photochemical efficiency of photosystem II (PSII), was calculated from the fluorescence data using Biolyzer 3.0 software (developed by Maldonado-Rodriguez, 2002). Fluorescence intensity at 50 µs (F_o) and maximal fluorescence intensity (F_m) were also calculated in this way.

Leaf water status

On experimental days 0, 8 and 12 leaf water potential (Ψ_w) measurements were carried out at predawn (pd) and 5-6 h into the light period (md), on one fully expanded, non-senescing, mature leaf from each of four seedlings across all treatments, using thermocouple psychrometers (Model C-52; Wescor, Logan, U.S.A.) in combination with a microvoltmeter. The difference between pd Ψ_w and md Ψ_w , referred to as 'pd-md', was also calculated. pd-md Ψ_w reflects the extent to which md Ψ_w is lowered from that at pd, to generate the Ψ_w gradient between soil and leaf, to drive the water flow through the plant to meet the transpirational demand during the light period. After Ψ_w was measured, 'osmotic' potential (Ψ_s) or water activity was measured on the same samples and pressure potential (Ψ_p) was calculated (as the difference between osmotic and water potential). The methods of measurement and calculations used for these studies were the same as those described for *H. montanus* in Chapter 6 (section 6.2).

Growth and biomass

After the *in vitro* growth period (150 d) six seedlings were separated into bulb, leaves and roots, on a per individual basis, and oven-dried for 72 h at 80°C to a constant weight, for dry mass estimates. Dry mass of these organs was similarly determined for six seedlings at the end of the *ex vitro* growth period and used to calculate relative growth rate (RGR) via Equation 1:

Individual seedling DM after 33 d *ex vitro* growth (g) - Mean seedling DM after 150 d *in vitro* growth (g) Mean seedling dry mass after 150 d *in vitro* growth (g) 33d

where DM refers to dry mass.

Stomatal density

Only fully expanded leaves were used to investigate the effect of embryo pre-treatment and subsequent *ex vitro* growth (with and without an imposed water stress) on stomatal density. In *A. belladonna* leaves the region of highest density and most regular stomatal distribution was found to lie in a 1 cm wide band across the middle portion of the adaxial surface, so this portion of the leaf was excised and used for all subsequent estimates. One leaf from each of 10 seedlings across all treatments was sampled early in the morning, on day 12 and fixed in gluteraldehyde immediately after excision. Samples were dehydrated using an ethanol series after fixation, critical point dried and directly sputter-coated with gold in a Polaron E5100 sputter coater. Surface structure was viewed using a Leo 1450 Scanning Electron Microscope (SEM).

Stomatal density on the left and right-hand side of the selected leaf portion was captured at a constant magnification and field of view. The left- and right-hand side of each leaf sample was further sub-divided into a top and bottom half, such that each sample was represented by four sectors: left-top and -bottom and right-top and bottom. The number of stomata (opened and closed) in each sector was counted keeping the sample area constant (μ m²). The counts for the four sectors of each leaf were then averaged to yield a mean stomatal density for each leaf.

Root morphology

Roots from each of 10 seedlings across all treatments were excised after the *ex vitro* growth period. Roots of individual seedlings were divided into tips, middle (portion between the bulb base and root tip) and bulb-base (portion immediately beneath the bulblet), prior to fixation in gluteraldehyde. After fixation, samples were dehydrated using an ethanol series, critical point dried, directly sputter-coated with gold and viewed using a Leo 1450 SEM.

[1]

Statistical analysis

Leaf plastochron and *in vitro* biomass data were compared across treatments by one-way Analysis of Variance (*ANOVA*; SPSS, Version 15). All other variables were tested for differences across treatments, within time intervals, by two-way *ANOVA*. Two-way *ANOVAs* were factorial in design, testing for the main-effects of embryo pre-treatment (referred to as 'Embryo') and water stress (referred to as 'Stress'), as well as the interaction between these (referred to as 'Embryo×Stress') (STATISTICA Version 6.1, StatSoft Inc. Tulsa, USA). Multiple comparisons were made using a Scheffe's mean separation test. Where the original data were expressed as a proportion (%) these values were arcsin transformed to conform data to *ANOVA* assumptions. At the end of the *in vitro* and *ex vitro* growth period's embryo/seedling viability (%) was compared across all treatments using null-model chi-squared analyses (EcoSim Version 7.72 [developed by Gotelli and Entsminger, 2009]). All statistical tests were performed at the 0.05 level of significance.

7.3 Results

In vitro growth responses

While 98% of freshly excised embryos developed into seedlings, significantly (p < 0.05) fewer embryos produced seedlings after partial dehydration (83%) and the combination of partial dehydration and cooling (72%) during *in vitro* recovery (data not shown).

After the *in vitro* growth period leaf, bulb, root and total dry mass were highest in F-seedlings and lowest in C-seedlings (Fig. 7.1A). While biomass partitioning to bulbs and roots in C-seedlings was significantly lower than F- and D-seedlings, biomass partitioning to leaves was significantly higher (Fig. 7.1B). Biomass partitioning to roots in D-seedlings was also significantly lower than F-seedlings.

Ex vitro growth responses

Relative growth rate (RGR) in CW-seedlings was significantly lower than DW- and FWseedlings (Table 7.1). In DS- and FS-seedlings, RGR was significantly reduced relative to their respective unstressed controls but stress did not further affect the already low RGR of Cseedlings significantly.

In the absence of a stress total seedling dry mass in F-seedlings was significantly higher than D- and C-seedlings while water stress significantly reduced dry mass accumulation across all embryo pre-treatments (Fig. 7.2A). Within the stressed treatments, dry mass accumulation was significantly higher in FS- and lower in CS-seedlings. Irrespective of whether the seedlings were stressed or not, bulb and root dry mass was significantly higher in F- and lower in C-

seedlings. Water stress decreased bulb and root dry mass across all embryo pre-treatments; these differences were significant for F- and D-seedlings for bulb, and for F- and C-seedlings for root. While leaf dry mass in DW- and CW-seedlings was significantly lower than FW-seedlings, water stress significantly reduced leaf dry mass across all embryo pre-treatments.

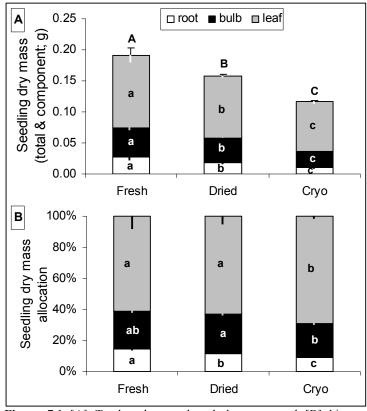


Figure 7.1 [A] Total and organ-based dry mass and [B] biomass partitioning of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) embryos, after 150 d *in vitro* growth. Blocks labelled with different lower-case letters are significantly different across treatments when compared within organs, while upper-case letters indicate inter-treatment differences in total dry mass (p < 0.05, *ANOVA*, n = 6). Bars represent SD.

Table 7.1 *Ex vitro* seedling relative growth rate (RGR; g g⁻¹ DM d⁻¹).

FW	$0.049{\pm}0.008^{a}$	FS	0.017 ± 0.002^{b}
DW	0.041 ± 0.009^{a}	DS	0.019 ± 0.001^{b}
CW	0.023 ± 0.007^{b}	CS	0.023 ± 0.001^{b}

RGR = relative growth rate ([Individual seedling dry mass after 33 d ex vitro growth (g) - Mean seedling dry mass after 150 d in vitro growth (g) / Mean seedling dry mass after 150 d in vitro growth (g)] / 33 d) of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) embryos, subjected to watering (W) or water deficit (S). Values represent mean±SD and are significantly different when followed by different letters (p < 0.01 for Embryo, Stress and Embryo×Stress, ANOVA, n = 6).

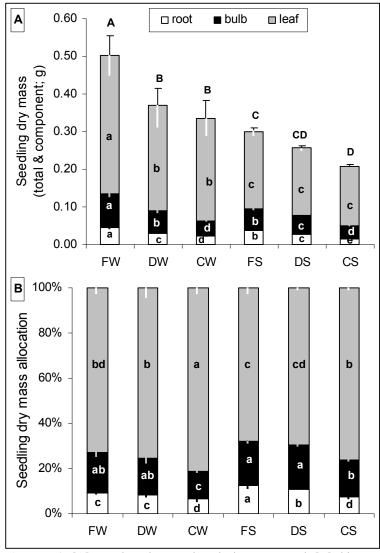


Figure 7.2 [A] Total and organ-based dry mass and [B] biomass partitioning of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) embryos, subjected to watering (W) or water deficit (S). Blocks labelled with different lower-case letters are significantly different across treatments when compared within organs, while upper-case letters indicate inter-treatment differences in total dry mass (for [A]: p < 0.01 for Embryo and Stress and = 0.01 for Embryo×Stress; for [B]: p < 0.01 for Embryo; roots: p < 0.01 for Stress and Embryo×Stress; leaves: p < 0.01 for Stress, *ANOVA*, n = 6). Bars represent SD.

Leaf stomatal density

Since there were no significant differences in stomatal density between stressed and unstressed leaves, stomatal density data were pooled based on embryo pre-treatment for all subsequent analyses, which showed stomatal density of C-seedlings to be significantly lower than F- and D-seedlings (Table 7.2; also see Fig. C2, Appendix C).

Table 7.2 Adaxial stomatal density.				
Treatment	Stomatal density (no. of stomata mm ⁻²)			
Fresh	36.43±5.21 ^a			
Dried	35.75±8.34 ^a			
Cryopreserved	15.56±5.11 ^b			

Estimated from SEM images of individual leaves of seedlings recovered from fresh, partially dried and cryopreserved embryos after 33 d *ex vitro* growth. Values represent mean \pm SD (data for stressed and unstressed treatments were pooled within embryo pretreatments) and are significantly different across treatments when followed by different letters (p < 0.01, *ANOVA*, n = 10).

Root morphology

This was assessed at the end of the *ex vitro* growth period across all treatments; however, within embryo pre-treatments there were no obvious differences between stressed and unstressed seedlings so root morphology is presented only for non-stressed F-, D- and C-seedlings. Results of these studies were not quantitative and in light of their descriptive nature only features that occurred in more than five out of the ten replicates for each embryo pre-treatment are shown.

Scanning electron micrographs of roots produced by unstressed F-, D- and C-seedlings revealed root hair density to be greatest (qualitative assessment) in F-seedlings (Fig. 7.3A, D, G). Root hairs in F-seedlings occurred across all three sections of the root (i.e. tip, middle and bulb base) but were most abundant in the middle portion (Fig. 7.3D). In D-seedlings, root hair density was also greatest in the middle portion of the root but unlike F-seedlings, root hairs were sporadically distributed across the three portions and clearly absent in some areas (Fig. 7.3B, E, H). Root tips in D-seedlings were characterised by root hairs that were relatively shorter (qualitative assessment) than those associated with the root tips of F-seedlings (Fig. 7.3G and H, respectively). In C-seedlings large areas across all three root portions were devoid of root hairs (Fig. 7.3C, F, I) while root hair density in the middle portion, the site of the greatest root hair density in other treatments, was also far lower (qualitative assessment) than F- and D-seedlings (Fig. 7.3D, E, F).

The roots of D- and F-seedlings tapered towards the tip while the root tips of C-seedlings were almost nodular and often exhibited a tuft of relatively long root hairs (Fig. 7.3G, H, I). In C-seedlings the section of the root immediately beneath the bulb was almost always abnormally thick, when compared with F- and D-seedlings (Fig. 7.3A, B, C).

Plant water relations

On day 0, Ψ_w , Ψ_s and Ψ_p in CW-seedlings were significantly lower than FW- and DWseedlings at pd, but not at md (Figs 7.4A, 7.5A, B and 7.6A, B) (note md Ψ_w data not shown). While pd-md Ψ_w was close to zero in CW-seedlings on day 0, this parameter was significantly higher in DW- and FW-seedlings (Fig. 7.4B), suggesting that DW- and FW-seedlings were more efficient at adjusting Ψ_w to meet transpirational demands than CW-seedlings. Within the unstressed treatments pd Ψ_w and pd-md Ψ_w in C-seedlings remained significantly (on days 8 and 12) lower than D- and F-seedlings throughout the experimental period (Fig. 7.4A, B) but pd-md Ψ_w values did become more positive (relative to day 0) in CW-seedlings as the experimental period progressed.

By day 8, water stress had depressed pd Ψ_w across all embryo pre-treatments and even with re-watering (i.e. day 12) pd Ψ_w across all stressed treatments never recovered to levels comparable with their respective unstressed controls (Fig. 7.4 A, B). On day 8, pd-md Ψ_w across all stressed treatments was relatively lower than their respective unstressed controls (significant for D-seedlings only), while within the stressed treatments pd-md Ψ_w was significantly lower in CS-seedlings (Fig. 7.4B). When stressed plants were re-watered pd-md Ψ_w in these treatments remained significantly lower than their respective unstressed controls.

Irrespective of whether they were stressed or not, Ψ_s in C-seedlings was significantly lower than D- and F-seedlings at pd and md on days 8 and 12 (Fig. 7.5A, B). On days 8 and 12, Ψ_s in FS- and DS-seedlings was significantly lower than their respective unstressed controls at pd, and at pd and md for CS-seedlings.

On day 8, water stress depressed Ψ_p across all embryo pre-treatments but this trend was significant at md only (Fig. 7.6A, B). Even though Ψ_p in FS-seedlings was slightly higher than DS- and CS-seedlings at pd and md on day 8, these differences were not significant. With rewatering Ψ_p in DS- and FS-seedlings recovered to levels comparable with their respective unstressed controls on day 12 (being more pronounced at pd); however, Ψ_p in CS-seedlings remained relatively lower than in CW-seedlings (significant at md only), and DS- and FSseedlings.

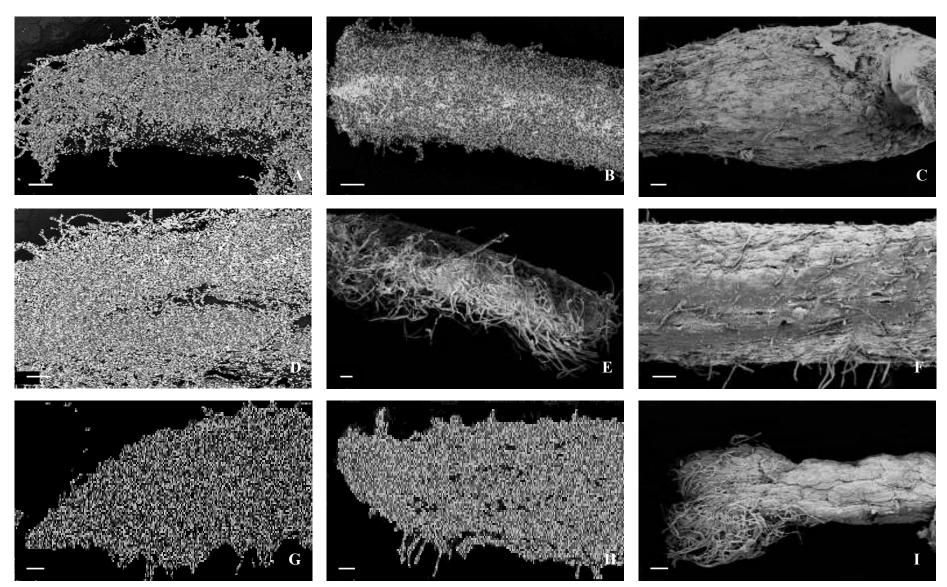


Figure 7.3 Scanning electron micrographs of roots of seedlings derived from [A, D, G] fresh, [B, E, H] partially dried and [C, F, I] cryopreserved embryos after 33 d *ex vitro* growth. [A, B, C] = root portion immediately beneath bulb; [D, E, F] = middle portion of root; [G, H, I] = root tip. Bar = $200 \,\mu\text{m}$.

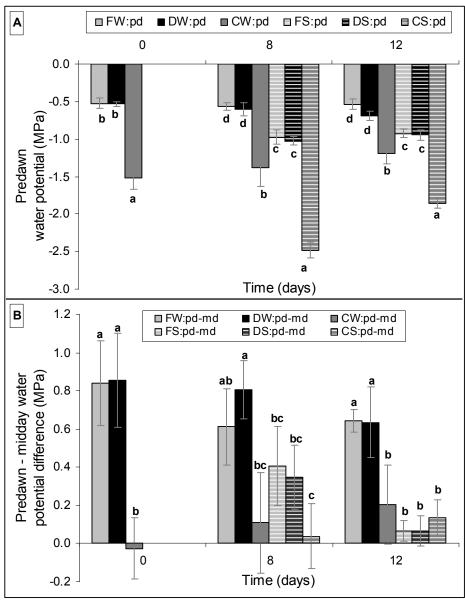


Figure 7.4 [A] Predawn (pd) leaf water potential and [B] Predawn – midday water potential difference (pd-md) of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) embryos, subjected to watering (W) or water deficit (S). Columns labelled with different letters are significantly different when compared within experimental days (p < 0.01 for Embryo, Stress and Embryo×Stress, *ANOVA*, n = 4). Bars represent ±SD.

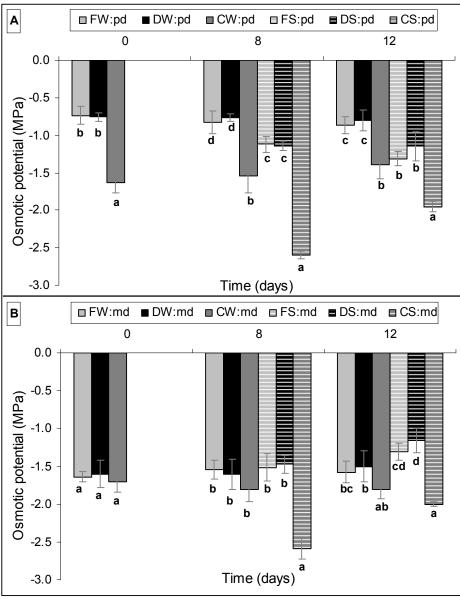


Figure 7.5 [A] Predawn (pd) and [B] midday (md) leaf osmotic potential of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) embryos, subjected to watering (W) or water deficit (S). Columns labelled with different letters are significantly different when compared within experimental days (p < 0.01 for Embryo and Embryo×Stress, *ANOVA*, n = 4). Bars represent ±SD.

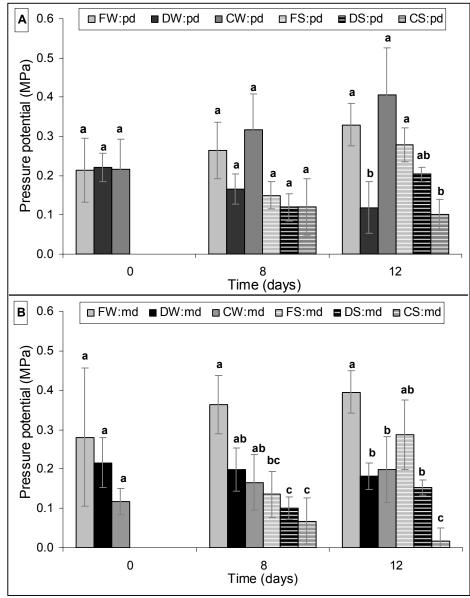


Figure 7.6 [A] Predawn (pd) and [B] midday (md) leaf pressure potential of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) embryos, subjected to watering (W) or water deficit (S). Columns labelled with different letters are significantly different when compared within experimental days (p < 0.01 for Embryo, Stress and Embryo×Stress, *ANOVA*, n = 4). Bars represent ±SD.

Photosynthetic characteristics

Leaf chlorophyll content

After 150 d *in vitro* growth, *chl a*, *b* and total leaf *chl* content did not differ significantly with embryo pre-treatment (data not shown). With *ex vitro* growth, *chl a* and total *chl* content in F-leaves were significantly higher than D- and C-leaves, while *chl b* content in F- and D-leaves was significantly higher than C-leaves (Fig. 7.7). Total *chl* content in D-leaves was also relatively higher than C-leaves but this trend was significant for unstressed material only. DS- and CS-seedlings exhibited a decline in *chl a*, *b* and total *chl* content, relative to their respective unstressed controls (significant for total *chl* in D-seedlings only) but this decline was not observed in FS-seedlings.

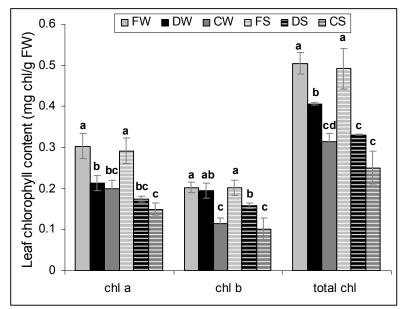


Figure 7.7 Leaf chlorophyll content of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) embryos, subjected to watering (W) or water deficit (S). Chlorophyll content was measured at the end of the *ex vitro* growth period only. Columns labelled with different letters are significantly different when compared within categories (p < 0.01 for Embryo and Stress, *ANOVA*, n = 4). Bars represent ±SD.

CO₂-assimilation rates

On day 0 and throughout the experimental period leaf-based CO₂-assimilation rates (*A*) at C_a: 400 μ mol. mol⁻¹ were significantly higher in FW-seedlings (Fig. 7.8). On days 8 and 12, *A* in DW-seedlings was relatively higher than CW-seedlings (significant on day 12 only). On day 8, water stress depressed *A* across all embryo pre-treatments (significant for F-seedlings only). Within the stressed treatments A was highest in FS-seedlings and lowest in CS-seedlings (not significant). With re-watering, A in stressed treatments did not recover to levels comparable with their respective unstressed controls on day 12.

Within the unstressed treatments, the ratio of *A* at C_a : 600 µmol. mol⁻¹ : *A* at C_a : 400 µmol. mol⁻¹ (*A*@600 : *A*@400), and hence the degree of stomatal limitation of *A*, did not differ significantly across embryo pre-treatments throughout the experimental period (Table 7.3). However, *A*@600 : *A*@400 in FS-seedlings was significantly higher than DS- and CS-seedlings on days 8 and 12. Interestingly, *A*@600 : *A*@400 in FS-seedlings was also significantly greater than FW-seedlings on days 8 and 12.

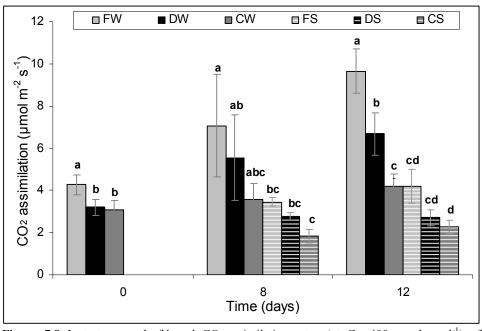


Figure 7.8 Instantaneous leaf-based CO₂-assimilation rates (at C_a: 400 µmol. mol⁻¹) of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) embryos, subjected to watering (W) or water deficit (S). Columns labelled with different letters are significantly different when compared within experimental days (p < 0.01 for Embryo on days 0 and 12 and = 0.01 on day 8; p = 0.04 for Stress and = 0.01 for Embryo×Stress on day 8, *ANOVA*, n = 7). Bars represent ±SD.

Day	FW	DW	CW	FS	DS	CS
0	2.29 ± 0.60^{a}	2.11 ± 0.55^{a}	1.85 ± 0.35^{a}	NA	NA	NA
8	1.68 ± 0.44^{b}	1.66 ± 0.70^{b}	1.81 ± 0.35^{b}	2.48±0.23 ^a	1.57 ± 0.15^{b}	1.76 ± 0.47^{b}
12	1.68 ± 0.10^{b}	1.61 ± 0.17^{b}	1.51 ± 0.15^{b}	2.36±0.53 ^a	1.66 ± 0.45^{b}	1.43 ± 0.20^{b}

Table 7.3 Ratio of CO₂-assimilation rate at C_a: 600 µmol. mol⁻¹: CO₂-assimilation rate at C_a: 400 µmol. mol⁻¹.

Ratio was calculated for seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) embryos, subjected to watering (W) or water deficit (S). Values followed by different letters are significantly different when compared within a measurement day (p < 0.01 for Embryo on days 8 and 12 and for Stress and Embryo×Stress on day 12; p < 0.05 for Stress and Embryo×Stress on day 8, *ANOVA*). Values represent mean±SD (n = 7). NA = not applicable.

Chlorophyll fluorescence

On days 0 and 8, potential photochemical efficiency (F_v/F_m), fluorescence intensity at 50 µs (F_o) and maximal fluorescence intensity (F_m) did not differ significantly with embryo pretreatment in unstressed material (Fig. 7.9A, B, C). On day 8 and after re-watering (i.e. day 12), F_v/F_m in CS-seedlings was significantly lower than CW-seedlings and DS- and FS-seedlings (Fig. 7.9A). On day 12, F_m in CS-seedlings was relatively lower (Fig. 7.9B), while F_o was relatively higher, than CW-, DS- and FS-seedlings (not significant; Fig. 7.9C).

Ex vitro seedling mortality

In the stressed treatments *ex vitro* seedling mortality was higher than in their respective unstressed controls (Table 7.4). Within the stressed treatments mortality was highest in CS-seedlings and lowest in FS-seedlings. Mortality in DS-seedlings was only slightly lower than CS-seedlings, while within the unstressed treatments mortality in DW- and CW-seedlings was marginally higher than FW-seedlings.

Table 7.4 Total ex vitro seedling mortality (%) over the entire ex vitro growth period.

Treatment	FW	DW	CW	FS	DS	CS
% Mortality	5	11	9	26	41	48

Seedlings were recovered from fresh (F), partially dried (D) and cryopreserved (C) embryos and subjected to watering (W) or water deficit (S). p < 0.05 when values were compared across treatments (null-model chi-squared test, n = 87).

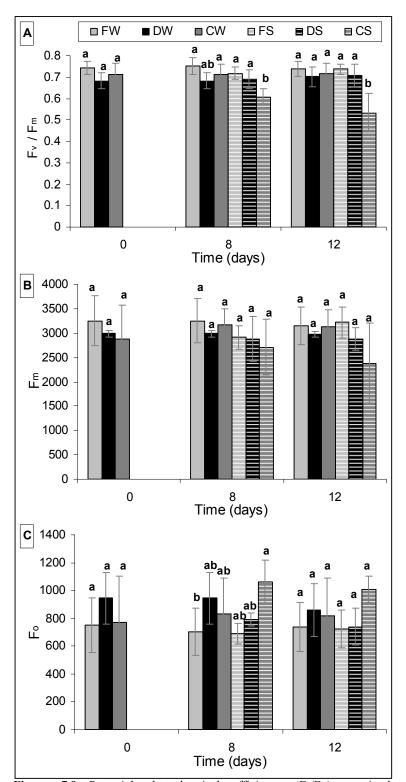


Figure 7.9 Potential photochemical efficiency (F_v/F_m) , maximal fluorescence intensity (F_m) and fluorescence intensity at 50 µs (F_o) of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) embryos, subjected to watering (W) or water deficit (S). Columns labelled with different letters are significantly different when compared within experimental days (p < 0.05 for Stress and Embryo×Stress on days 8 and 12 and p < 0.01 for Embryo on day 8, *ANOVA*, n = 5). Bars represent ±SD.

7.4 Discussion

There are differences among species and explants in growth and morphological responses of recovered plantlets or seedlings following cryopreservation, with some studies showing no effects whilst others have demonstrated morphological abnormalities and reduced development rates (see Introduction for details). Of the studies cited there are a few examples (e.g. Côte *et al.*, 2000; Martínez-Montero *et al.*, 2002; Konan *et al.*, 2007) where plants (of mainly crop species) derived from cryopreserved germplasm have been grown in the field for a long period. However, those studies were almost always based on phenotypic descriptions and there are presently few, if any published reports on the physiological performance of seedlings or plantlets recovered from cryopreserved material. The present contribution reports on the growth and physiological performance of seedlings derived from cryopreserved zygotic embryos of the wild species *A. belladonna*. The ultimate aim of cryopreservation of the germplasm of endangered wild species is to re-introduce plantlets or seedlings back into the wild. Under such conditions the seedlings are unlikely to be carefully tended after planting and will be subject to the vagaries of the weather, and so the vigour of such seedlings is important; this was assessed by observing the response of such seedlings to an *ex vitro* water stress.

Growth characteristics

Phenotypic variation in *in vitro* recovery times, plant heights and modes of regeneration in plants recovered from cryopreserved germplasm have been previously reported (Harding and Benson, 1994; Harding, 1996). While seedlings recovered from cryopreserved orthodox embryonic axes have been reported to be similar to those generated from partially dehydrated and control axes, going on to produce flowers and fruit in the field (Gagliardi et al., 2002), Steinmacher et al. (2007) showed partial dehydration and cryopreservation of recalcitrant peach palm embryos to result in plantlets with significantly lower plant height and deficient haustorium development. A number of studies have also observed a reduction in the actual number of embryos that produce roots and shoots (compared with the control) following dehydration and/or cryopreservation (e.g. Pence, 1992; Dumet et al., 1997; Wesley-Smith et al., 2001, 2004; Sershen et al., 2007). In the case of excised embryos of A. belladonna, both partial drying alone, and drying followed by exposure to cryogenic temperatures, reduced dry matter accumulation (Fig. 7.1A) and partitioning to roots during the *in vitro* growth phase, relative to fresh embryos (Fig. 7.1B). The final biomass achieved after the ex vitro growth period (Fig. 7.2A) could be a consequence of either or both ex vitro growth rates or the amount of material at the start of this growth phase. To assess this RGRs (relative to the dry mass at the end of the *in* vitro growth period) were calculated. The adverse effect of embryo partial drying observed during the *in vitro* growth phase was reversible during subsequent *ex vitro* growth, but the adverse effect of exposure to cryogenic temperatures as well, was carried through to early ex vitro growth (Table 7.1). However, a water stress during this period dominated over the effects of embryo pre-treatment and RGRs for all embryo pre-treatments were comparable. Growth limitation during a water stress is not uncommon (e.g. Hsiao, 1973; Frensch and Hsiao, 1994) and has been correlated with a reduction in leaf water and pressure potential (e.g. Hsiao, 1973) and/or a reduction in the photosynthetic uptake of carbon (Katul et al., 2003). Water stress depressed $\Psi_{\rm w}$ (Fig. 7.4), $\Psi_{\rm p}$ (Fig. 7.6), A (Fig. 7.8) and dry mass accumulation (Fig. 7.2A) across all embryo pre-treatments. The lack of response to the stress by C-seedlings, in terms of RGR (Table 7.1), is probably because the combination of partial dehydration and cryopreservation impaired their ability to acquire resources to such an extent that a withdrawal of resources (i.e. water) had no effect on overall performance. The significantly lower ex vitro light and water harvesting capacity in D- and C-seedlings, compared with F-seedlings (Fig. 7.2A), may have also compromised their physiological performance and/or stress tolerance. There have been suggestions that cryopreservation of recalcitrant zygotic germplasm may compromise vigour in recovered seedlings (e.g. Dumet *et al.*, 1997; Steinmacher *et al.*, 2007); however, none of those studies reported RGRs for recovered seedlings.

Not only did partial drying and cryopreservation affect partitioning of biomass to roots (Fig. 7.2B), they also resulted in abnormal roots (Fig. 7.3). These phenotypic and morphological responses could be expected to reduce the abilities of plants derived from D- and C-embryos to acquire water, particularly under water limited conditions. Additionally, unlike FS- and DS-seedlings, CS-seedlings failed to significantly increase biomass partitioning to roots relative to their unstressed control (Fig.7.2B).

Shoot growth is usually most severely affected by drought (e.g. Frensch and Hsiao, 1994) and was so across all embryo pre-treatments here (Fig. 7.2A, B). Typical geophytic growth strategy selects for below-ground (generally bulb) increment during a water stress (since survival in the season following dormancy is determined by the reserves stored below-ground) while leaf area is progressively reduced via shoot dieback (e.g. von Willert *et al.*, 2000). It was therefore interesting to note that unlike FS- and DS-seedlings, CS-seedlings failed to significantly increase biomass partitioning to roots relative to their unstressed control; rather, CS-seedlings invested significantly more biomass in leaves than FS- and DS- seedlings (Fig. 7.2B). This preferential investment in shoot biomass during a drought could have further increased the transpirational demand on the already compromised root system in CS-seedlings and may explain why Ψ_w (Fig. 7.4) and Ψ_p (Fig. 7.6) generated in CS-seedlings were significantly lower than DS- and FS-seedlings. Also, partial dehydration and cryopreservation

resulted in abnormalities in *ex vitro* root morphology in recovered seedlings (Fig. 7.3). These phenotypic and morphological responses could be expected to reduce the abilities of plants derived from D- and C-embryos to acquire water, particularly under water limited conditions.

Plant water relations

The generation of relatively low pd $\Psi_{\rm w}$ as a consequence of a water stress is not uncommon (Petrie and Hall, 1992), while turgor loss is generally the first sign of water stress (Leopold et al., 1981; Galmés et al., 2007) and can develop during even a relatively mild stress (Hsiao, 1973). Many species also exhibit a decrease in Ψ_s during a drought to maintain turgor at a low $\Psi_{\rm w}$ and when rapidly initiated, osmotic adjustment can prolong the life of a leaf under water stress (Galmés et al., 2007). In the present study, within the unstressed seedlings there were no significant differences in the measured water relations parameters between F- and D-seedlings (except for $\Psi_{\rm p}$), indicating that the effects of partial drying of excised embryos were reversible in the long term (Figs 7.4, 7.5, 7.6). C-seedlings had lower pd Ψ_w and did not develop lower md Ψ_{w} , indicating that little transpiration was occurring, in keeping with the relatively lower partitioning of biomass to roots and the observed root abnormalities. There were declines in both pd Ψ_w and pd-md Ψ_w (Fig. 7.4), and an indication of long-term osmotic adjustment, reflected in a decrease in Ψ_s in both FS- and DS-seedlings relative to their well watered controls (Fig. 7.5). There also appeared to be some diurnal osmotic adjustment with md Ψ_s being lower than pd Ψ_s , maintaining positive (although reduced) Ψ_p within FS- and DS-seedlings (Fig. 7.6), permitting some transpiration and hence positive pd-md Ψ_w . CS-seedlings demonstrated further reductions in $\Psi_{\rm w}$ and $\Psi_{\rm s}$, but no diurnal osmotic adjustment and also appeared to be less efficient than DS- and FS-seedlings at adjusting Ψ_w to meet transpirational demands (i.e. exhibited lower pd-md Ψ_w values [Fig. 7.4B]). Failure to equilibrate with soil Ψ_w overnight can severely depress $\Psi_{\rm w}$ in droughted seedlings (Fitter and Hay, 2002) and prolonged exposure to such low Ψ_w can be extremely stressful in juvenile plants (Hsiao, 1973). This may explain why permanent wilting was observed in CS-seedlings (Fig. 7.6): cell plasmolysis and membrane rupture are associated with permanent leaf-wilting (Leopold et al., 1981; Galmés et al., 2007), and the decrease in Ψ_s observed in CS-seedlings on day 8 (Fig. 7.5) may have been a consequence of tissue dehydration, rather than osmotic adjustment. By three days after rewatering there was no recovery in pd Ψ_w in any of the stressed treatments and no development of substantial differences between pd and md Ψ_{w} . Positive turgor is a requirement for cell expansion and the generally lower values of pd Ψ_p for stressed seedlings, although not significant using a factorial ANOVA design, are consistent with the lower RGRs measured in these seedlings (Table 7.1). The generally lower values of pd-md Ψ_w (Fig. 7.4B) of stressed seedlings are also consistent with the reduced RGRs associated with these seedlings (Table 7.1).

As in the experiments described in Chapter 6, estimates of osmotic and turgor potential were variable within treatments though and may have been due to non-negligible apoplastic water content. Alternate approaches to measuring these parameters have already been discussed in Chapter 6 (section 6.4).

Photosynthetic characteristics

Although not always statistically significant there was a consistent trend of D- and Cseedlings having lower A than F-seedlings (Fig. 7.8), possibly because of lower leaf *chl* content compared with F-seedlings (Fig. 7.7). The significantly lower stomatal density of leaves of Cseedlings, compared with F- and D-seedlings (Table 7.2), may have also contributed to the relatively lower A in C-seedlings, by reducing the influx of CO_2 for photosynthesis.

A drought-induced reduction in A, as observed across all embryo pre-treatments here (Fig. 7.8), has been widely documented (e.g. Hsiao, 1973; Tezara *et al.*, 1999) and can be a consequence of restriction of CO₂ supply, through reduction in stomatal conductance, or of the rate of mesophyll processes (these could be the result of either damage or a controlled down-regulation of the biochemical and/or photochemical components of CO₂ fixation) (see McDowell *et al.*, 2008). Except for one report of photosynthetic electron transport being strongly inhibited in freeze-treated *Bratonia* protocorms, examined immediately after thawing (Bukhov *et al.*, 2006), there is little by way of studies on the photosynthetic characteristics of plants recovered from cryopreserved germplasm. Bukhov *et al.* (2006) suggested that in *Bratonia* protocorms freeze-thawing caused partial disorders in linear electron transport between PSII and PSI, with the functional interactions among carriers in the electron-transport chain being disturbed between the plastiquinone pool and the PSI reaction centre, resulting in a reduction in photosynthetic capacity during *in vitro* culture.

In the case of *A. belladonna* the ratio A@600 : A@400, taken as a measure of stomatal limitation, did not differ among embryo pre-treatments in unstressed plants (Table 7.3), suggesting that differences in *A* were not a consequence of stomatal limitation (despite the reduced stomatal density of C-seedlings). When subjected to a water stress, stomatal limitation was greater in F-seedlings (A@600 : A@400 was higher for FS-than FW-seedlings), than in D-and C-seedlings (A@600 : A@400 was similar for DS- and DW-, and for CS- and CW-seedlings). These data suggest that the reduction in *A* experienced by D- and C-seedlings was, to some degree, a consequence of damage to the photosynthetic machinery. Consistent with this, leaf *chl* content was lower in D- and C-seedlings than F-seedlings, and F_v/F_m was significantly

reduced in CS-seedlings. A drought-induced decline in *chl* content has been correlated with a loss of photosynthetic capacity in other studies (e.g. Alberte *et al.*, 1974) while a reduction of F_v/F_m is generally indicative of a decrease in seedling vigour (e.g. Strasser *et al.*, 2000; Rolando and Little, 2003) and often precedes seedling mortality (Havaux and Lannoye, 1983). When observed in droughted seedlings (e.g. Havaux and Lannoye, 1983; Rolando and Little, 2003) this decrease is usually induced by a reduction in F_m (e.g. Rolando and Little, 2003) but this was not the case in CS-seedlings here (Fig. 7.9B). The lower F_v/F_m values in CS-seedlings were also sustained after re-watering, suggesting the onset of hydraulic failure in CS-seedlings; plasmolysis and membrane rupture associated with permanent leaf-wilting (Leopold *et al.*, 1981) can depress photosynthesis (Radin and Ackerson, 1981) and phytochemical efficiency (Govindjee *et al.*, 1981). As was the case with leaf water potential, within 3 d of re-watering leaf *chl* and *A* did not recover to levels similar to their respective unstressed control values, but recovery from a water stress can be slow, from days to weeks (Flexas *et al.*, 2006).

Seedling mortality

R

Steinmacher *et al.* (2007) showed *ex vitro* mortality in seedlings generated from cryopreserved recalcitrant zygotic embryos to be almost 50% higher than seedlings recovered from control embryos. Similarly, *ex vitro* seedling mortality within the unstressed treatments was slightly higher in DW- and CW-seedlings here (Table 7.4). Water stress led to mortality in significantly more seedlings across all embryo pre-treatments (Table 7.3), being more severe in DS- and CS-seedlings (Table 7.4). Seedling mortality was highest in CS-seedlings (Table 7.4), possibly due to the combination of insufficient adjustment of Ψ_w to meet transpirational demands (Fig. 7.4), a failure to adopt growth patterns that reduce transpirational water loss (Fig. 7.2), exposure to significantly lower pd Ψ_w than DS- and FS-seedlings (Fig. 7.4), and the onset of permanent leaf wilting (Fig. 7.6); all of which promote hydraulic-failure (see McDowell *et al.*, 2008) in juvenile plants (e.g. Hsiao, 1973). The signs of hydraulic-failure were not as pronounced in DS-seedlings but, D-, like C-seedlings exhibited abnormalities in root morphology and when stressed incurred a loss of light harvesting capacity (i.e. reduction in *chl* content; Fig. 7.7) and developed relatively lower Ψ_w and Ψ_p than FS-seedlings (Figs 7.4 and 7.6 respectively).

7.5 Concluding remarks

Partial dehydration and cryopreservation of recalcitrant *A. belladonna* zygotic embryos can compromise vigour and drought tolerance of recovered seedlings. The effect of partial drying alone did appear to be reversible (in terms of RGR) within the time-frame of this study, and so

an extended period of *ex vitro* acclimatization before re-introduction of such seedlings into the wild may alleviate the adverse effects of cryopreservation on seedling vigour; but this remains to be tested. Cryopreservation studies involving explants other than recalcitrant zygotic germplasm "indicate a clear 'consensus' for plants displaying morphological normality after cryopreservation" (Harding, 2004) but in seedlings recovered from cryopreserved recalcitrant zygotic germplasm, the retention of 'morphological normality' may not necessarily be equated to stress-related physiological responses. The results of this study highlight the need to investigate the potential impacts of cryoinjury on the genome, transcriptome, proteome and metabolome of recovered plants (see Harding and Benson, 1994; Harding *et al.*, 2009), which in disrupting established patterns of growth and reproduction, may impact on the re-introduction of such plants into natural environments.

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CHAPTER EIGHT: Concluding Remarks and Recommendations for Future Studies

8.1 Introduction

The purpose of the present work was to investigate the factors influencing vigour and viability after cryopreservation of excised zygotic embryos from recalcitrant seeds of two indigenous geophytes, *viz. Amaryllis belladonna* (L.) and *Haemanthus montanus* (Baker). This encompassed an investigation of some of the physico-chemical consequences of the procedural steps in the cryopreservation of these explants: cryoprotection; partial dehydration; exposure to cryogenic temperatures; subsequent thawing; and *in vitro* recovery. In doing so, the present study aimed to understand the fundamental basis of the successes and failures of current cryoprotocols for recalcitrant seed germplasm, and identify physiological and biochemical markers of cryo-related stresses that may be used to better optimise future cryopreservation protocols for these explants. Lastly, in investigating the *ex vitro* vigour of seedlings recovered from partially dried and cryopreserved recalcitrant zygotic embryos, the current contribution aimed to inform re-introduction programmes involving seedlings recovered from cryopreserved germplasm of wild recalcitrant-seeded species. The salient points from studies described in Chapters 2 through 7, the difficulties encountered during the experimental phase of this work, and recommendations for future studies are discussed below.

8.2 Partial dehydration as a pre-treatment for cryopreservation

The cryopreservation of zygotic germplasm from recalcitrant seeds requires that all metabolic activities be halted without injury, followed by storage at water contents (WCs) and ultra-low temperatures that preclude degradative reactions (reviewed by Walters *et al.*, 2008). Unlike orthodox seeds which can tolerate the loss of most or all freezable water, facilitating their storage at cryogenic temperatures without freezing injury (e.g. Stanwood, 1985; Vertucci, 1989a & b; reviewed by Pritchard and Nadarajan, 2008), desiccation-sensitive (recalcitrant) propagules are shed hydrated (generally at 0.4-4.0 g g⁻¹ [Berjak and Pammenter, 2004) and therefore require partial dehydration to increase the likelihood of survival after cryogenic exposure (Wesley-Smith *et al.*, 1992; reviewed by Normah and Makeen, 2008; Walters *et al.*, 2008). The benefits conferred by partial drying are many-fold: it reduces the thermal mass of the axes/embryos, allowing these to traverse rapidly the critical range of temperatures supporting ice-crystal growth (Wesley-Smith *et al.*, 1992); it reduces the range of the critical temperatures supporting crystallisation, as the freezing point is lowered (Rasmussen *et al.*, 1975; Wesley-Smith *et al.*, 1993; Pritchard *et al.*, 1995); it raises the glass transition temperature (Williams *et al.*, 1993;

Buitink *et al.*, 1996); and increases cytoplasmic viscosity, hindering the process of ice-crystal growth by reducing the intracellular mobility of water (Luyet *et al.*, 1962; Leprince and Hoekstra, 1998; Leprince *et al.*, 1999).

The present work (Chapter 2) confirmed the findings of Sershen et al. (2008) in showing that the zygotic embryos of A. belladonna and H. montanus are recalcitrant, and as suggested by those authors, there were inter-species differences in drying kinetics: A. belladonna zygotic embryos could be dried to target WCs (i.e. > and <0.4 g g⁻¹), more than ten times faster than those of H. montanus (Fig. A2 and Table A1, Appendix A). Studies on the effects of dehydration rate on desiccation-sensitivity in recalcitrant seeds suggest that when seed tissues spend a longer period of time at intermediate WCs, the time for the accumulation of damage associated with aqueous-based deleterious processes is extended, promoting viability loss (Berjak et al., 1990, 1993; Vertucci and Farrant, 1995; Pammenter et al., 1998; Walters et al., 2001). Post-thaw viabilities in plant tissues depend to a large extent on the length of the drying period (Niino and Sakai, 1992). Results of studies discussed in Chapters 2 through 4 suggest that the extended drying times required to achieve target WCs in H. montanus embryos reduced vigour, which probably pre-disposed the tissues to damage associated with exposure to cryogenic temperatures. This brings us to one of the most important recommendations to come out of the present work: to optimise cryopreservation protocols for recalcitrant zygotic germplasm attention must be paid to pre-cooling dehydration stress, which appears to be the product of both the 'intensity' and 'duration' of the stress.

The present work has provided some of the first quantitative evidence that partial dehydration of recalcitrant zygotic gemplasm, even when not followed by cooling, can reduce the vigour and drought tolerance of recovered seedlings (see Chapters 6 and 7). When partial dehydration was followed by exposure to cryogenic temperatures and subsequent thawing, seedling vigour and drought tolerance were further compromised. The post-drying and post-cooling loss of cytomatrical organisation and organellar integrity within the meristematic cells of the embryos presently investigated (see Chapter 3; section 3.3), may underlie their abnormal growth and poor vigour following recovery from cryostorage (see Chapters 2 and 7). However, the biological basis of these differences, and their permanence (discussed further in section 8.8), demands further investigation since there are an increasing number of reports indicating no phenotypical, biochemical, chromosomal or molecular modifications of thawed material attributed to cryopreservation (Bajaj, 1983; Assy-Bah and Engelmann, 1992a; Matsumoto *et al.*, 1994; Benson *et al.*, 1996; Aronen *et al.*, 1999; Côte *et al.*, 2000; Potts and Lumpkin, 2000; Helliot *et al.*, 2002; reviewed by Harding, 2004; reviewed by Engelmann, 2004; Wang *et al.*, 2005; Konan *et al.*, 2007; Caswell and Kartha, 2009). Further investigation of the post-cryo

growth of seedlings recovered from cryopreserved recalcitrant zygotic germplasm will also improve our currently poor understanding of how stresses imposed at the embryonic stage are translated or manifested during subsequent *ex vitro* seedling growth in recalcitrant-seeded species. Based on the gaps in the present work, some of the things that future studies should compare between cryopreserved and control recalcitrant zygotic germplasm include: (a) cytoskeletal architecture, which has crucial functions in a number of cellular processes that are essential for cell morphogenesis, organogenesis and development (reviewed by Kost *et al.*, 1999); (b) the levels of the various plant growth regulators, the effects and balance among which have been shown to change in stressed material (Cavusoglu and Kabar, 2007); and given the pivotal and multi-faceted role that reactive oxygen species (ROS) play in seed physiology (reviewed by Bailly *et al.*, 2008), (c) germination related oxidative signaling (reviewed by El-Maarouf-Bouteau and Bailly, 2008).

8.3 Cryoprotection and post-thaw survival

Cryoprotectant additives are frequently employed in cryopreservation procedures and 'traditionally' (Benson, 1999) involve the use of both penetrating and non-penetrating additives (reviewed by Fuller, 2004). The ability of this heterogeneous group of compounds to depress the kinetic freezing point of water (often referred to as the 'supercooling point' in biological solutions [Wilson et al., 2003]), and so reduce the likelihood of lethal ice-crystal formation during cooling and subsequent thawing is widely documented (Kartha and Engelmann, 1994; Santarius and Franks, 1998; reviewed by Fuller, 2004; Muldrew et al., 2004). The potential reasons for the superiority of glycerol (Gly; penetrating) over sucrose (Suc; non-penetrating) cryoprotection in promoting post-thaw survival in both the species presently investigated has been discussed at length in Chapters 2 through 4. However, a major short-coming of the present work is that it did not allow for an assessment of intracellular ice-crystal formation (e.g. via freeze-fracture electron microscopy [Wesley-Smith et al, 1992]), nor the thermal properties of tissue water (e.g. via differential scanning calorimetry [Vertucci et al., 1991; Wesley-Smith et al., 1992). It was therefore impossible to interpret the differences between Suc and Gly cryoprotection in terms of the biophysical changes brought about by the transition of water to ice during cooling, which are the main causes of damage, rather than low temperatures per se (Mazur, 1990, 2004; Karlsson and Toner, 1996). Nevertheless, results of ultrastructural studies involving conventional transmission electron microscopy suggest that the superiority of glycerol over sucrose in promoting post-thaw survival in the embryos presently investigated may have been based on its presumed ability to extend protection to internal organelles (reviewed by Fuller, 2004). Additionally, cryoprotecting the embryos in this study with radiolabeled glycerol (e.g. Zheng *et al.*, 2003; Neves *et al.*, 2004) may have aided in elucidating the uptake and the extent and nature of the interaction between glycerol and intracellular constituents, as this would have allowed traceability of glycerol at the ultrastructural level.

The ability of cryoprotectants like Gly to confer biochemical protection, aside from their osmotic effects, has been documented (Polge *et al.*, 1949; Smirnoff and Cumbes, 1989; Benson and Bremner, 2004) and may explain why partially dried Gly cryoprotected embryos were associated with higher post-thaw and post-drying respiratory and antioxidant activities. However, it must be stressed that even though the mechanisms via which Gly conferred cryoprotection were effective enough to promote post-thaw viability in both species, these mechanisms may not necessarily have been the same and/or equally effective in both species.

Although the zygotic germplasm of a number of temperate species (e.g. *Camellia sinensis* [Wesley-Smith *et al.*, 1992; Kim *et al.*, 2002]; *Aesculus hippocastanum* [Wesley-Smith *et al.* 2001a; Pence, 2004]; *Quercus suber* and *Quercus ilex* [González-Benito *et al.*, 2002]; *Poncirus trifoliata* [Wesley-Smith *et al.*, 2004]) have been successfully cryopreserved, similar success has not been achieved with tropical species (Walters *et al.*, 2008; Engelmann, 2009; Pammenter *et al.*, 2010; Whitaker *et al.*, 2010). The use of cryoprotectants followed by rapid partial drying and cooling of axes/embryos from seeds of tropical species has been tried, but with variable success (e.g. Pence, 1991; Assy-Bah and Engelmann, 1992a & b; Kioko *et al.*, 1998; Thammasiri, 1999; Sershen *et al.* 2007). The present work endorses other suggestions that cryoprotection can improve post-thaw recovery in zygotic germplasm of non-orthodox-seeded species (Engelmann, 1997; Walters *et al.*, 2002; Normah and Makeen, 2008), including those of tropical provenance (Walters *et al.*, 2008). However, in some tropical species cryoprotectants may be injurious (e.g. *Trichilia dregeana* [Berjak *et al.*, 1999a] and *Boophane disticha* [Sershen *et al.*, 2007]). At the moment there is no explanation for these differing responses to cryoprotectants, even for species within the same genus (e.g. Sershen *et al.*, 2007).

8.4 The problem of uneven drying

Irrespective of how rigorously the pre-freezing WC of recalcitrant embryos/axes is optimised, if such explants are dried rapidly using the flash drying technique, the possibility of uneven drying should not be ignored (Pammenter *et al.*, 1998, 2002; Wesley-Smith *et al.*, 2001b). The concern here is that axis WCs in such studies are generally taken as representative of all the tissues, when they are actually a mass-weighted average of the different tissues in the axis. Wesley-Smith *et al.* (2001b) for instance, showed microscopically that cells of the root cortex of recalcitrant *Artocarpus heterophyllus* axes dried more rapidly than those of the procambial cylinder, i.e. that an uneven distribution of water resulted when axes were dehydrated rapidly.

Consequently the meristem would have been exposed to relatively less intense drying than the cortical cells and so suffered less damage. In contrast, upon slow dehydration (days vs. hours), the WC of the various tissues would have been close to equilibrium; however, the meristematic cells would have been exposed to a relatively greater degree of drying stress (i.e. longer and more injurious) (Wesley-Smith *et al.*, 2001b). This uneven distribution of water is likely to have implications on the response of recalcitrant embryos/axes to exposure to cryogenic temperatures, with the more hydrated tissue being more susceptible to ice-crystal formation. This may be one reason why post-thaw survival for some of the treatments in this study was high (i.e. 50-70%), but never 100%.

Additionally, for both species, post-thaw survival was best achieved when embryos were rapidly cooled at WC that minimised dehydration and freezing damage. However, the benefits of this optimal WC appeared to have been realised only when pre-conditioning involved the combination of (Gly) cryoprotection with partial dehydration. It would be interesting to look at whether the post-thaw success achieved with solution-based cryoprotectants, such as Gly in this study, are based on the presumed ability of such additives to increase water permeability and therefore effectively improve the movement of water through samples (Walters *et al.*, 2008); thus avoiding large water potential gradients, which can be harmful in both large and very small samples for different reasons (Wesley-Smith *et al.*, 2003). In this regard, future studies should look at whether methods such as magnetic resonance imaging (Matsushima *et al.*, 2009) and phase-contrast X-ray micro-imaging (Lee and Kim, 2008), which are presently used to visualise water *in vivo* in plants, can be used to investigate whether cryoprotectants alter the behaviour of water in tissues such as those composing the embryos presently investigated, during drying and freezing.

8.5 The balance between dehydration and freezing damage

Rapid cooling rates may restrict intracellular ice-crystallisation below lethal levels by minimising the time spent by the tissue at temperatures favouring ice formation and growth (generally taken to be 0 to -80°C [Moor, 1973]), and have been successfully applied to the zygotic germplasm of a number of recalcitrant-seeded species (e.g. *Camellia sinensis* [Wesley-Smith *et al.*, 1992]; *Aesculus hippocastanum* [Wesley-Smith *et al.* 2001a]; *Quercus suber* and *Quercus ilex* [González-Benito *et al.*, 2002]; *Poncirus trifoliata* [Wesley-Smith *et al.*, 2004]; *Ekebergia capensis* [Perán *et al.*, 2006]; and a number of amaryllid species [Sershen *et al.*, 2007]). Consistent with this, rapid, as opposed to slow, cooling resulted in significantly better post-thaw survival in both species presently investigated but as discussed above, one of the short-comings of the present work was that it did not employ techniques that allow for an

appreciation of the relationships among ice-crystal formation, cooling rate and post-thaw viability. Nevertheless, with reference to the parameters measured in studies discussed in Chapters 2 through 4, the following suggestions can be made with regards to the cryopreservation of *A. belladonna* and *H. montanus* zygotic embryos:

- post-thaw viabilities are maximised when partially dried embryos are rapidly, as opposed to slowly cooled, possibly due to the freeze-induced dehydration usually associated with slow cooling;
- (2) post-thaw viabilities are best when embryos are cooled after Gly (penetrating), as opposed to Suc (non-penetrating) cryoprotection + partial dehydration, possibly because Suc cryoprotection exacerbates the injurious effects of dehydration while Gly cryoprotection alleviates some of these;
- (3) the optimum WC-range for cryopreservation is species-specific and based on the 'intensity' and 'duration' of the dehydration stress associated with particular target WCs (note: the duration of the dehydration stress is dependent on the drying kinetics of the embryonic tissues, which are species-specific) and;
- (4) while post-thaw survival depends on the balance between dehydration and freezing damage, the benefits of relatively lower WCs are negated at extended drying times.

Future studies should look at whether the benefits of partial drying, cryoprotection and rapid cooling in the embryos presently investigated included the avoidance of lethal intracellular icecrystallisation. In this regard, the processing of embryos for transmission electron microscopy by freeze-substitution (e.g. Wesley-Smith, 2003) immediately after cooling may reveal the contrasting degree and distribution of intracellular ice-crystals across the various treatments investigated. Such studies may also help in elucidating whether it was the avoidance of intracellular ice *per se* that promoted survival in Gly cryoprotected + partially dried + rapidly cooled embryos in the species presently investigated, or whether this was influenced by the actual *localisation* of ice crystals within different intracellular compartments.

Additionally, it is now widely accepted that the higher the final embryo/axis WC after drying, the more rapid the rate of cooling should be to restrict ice-crystallisation and associated freezing damage (Vertucci, 1989b; Wesley-Smith *et al.*, 1992; Walters *et al.*, 2008). The possibility that more rapid cooling rates than those presently used (hundreds of °C s⁻¹) could improve post-thaw viability in *H. montanus* embryos at the relatively high WCs (>0.4 g g⁻¹) shown to be optimum for this species, should therefore be investigated.

8.6 Oxidative stress as a determinant of post-thaw survival

An increasing number of studies have suggested oxidative stress to be a major component of cryoinjury in recalcitrant seed tissues (Touchell and Walters, 2000; Dussert et al., 2003; Normah and Makeen, 2008; Varghese and Naithani, 2008; Pammenter et al., 2010; Whitaker et al., 2010). Results of the present work endorse these suggestions. In this regard, studies discussed in Chapter 4 showed enzymic antioxidant activities and viability to often decline relative to fresh embryos after partial dehydration and freezing in both species; however, this decline was consistently less severe in Gly cryoprotected (CP), as opposed to non-CP, embryos. These results are encouraging in that they provide some evidence for previous suggestions that cryoprotectants such as Gly (Polge et al., 1949) and dimethyl sulphoxide (Benson and Withers, 1987; Fleck et al., 2000) may confer 'biochemical' as well as 'physical' protection during cryopreservation (Benson and Bremner, 2004; Fuller, 2004). Those authors suggested that this biochemical protection is mainly based on the scavenging of damaging free-radicals. In the present study Gly CP failed to reduce post-drying and post-freezing extracellular superoxide (O_2) production (relative to non-CP embryos), but Gly cryoprotection was associated with the maintenance or enhancement of post-drying and post-cooling antioxidant activity (see Chapters 4 and 5). The exact mechanism/s upon which this protection of the antioxidant system by Gly was based are at present unknown and it must be stressed that the sustained or enhanced postdrying and post-thaw antioxidant activities in Gly CP embryos observed in this study should be confirmed via an investigation of antioxidant gene expression levels (e.g. Yang et al., 2003; Jitesh et al., 2006) - an objective of studies that have already been planned for the immediate future. All that can be suggested at present is that Gly CP may have promoted the retention of ultrastructural integrity (Chapter 3; section 3.3) by conserving metabolic integrity, e.g. antioxidant protection. Also, hydrogen peroxide levels (H₂O₂) were not measured here but results of studies discussed in Chapter 4 suggest that Gly CP embryos may have been more efficient than non-CP embryos at the enzymatic detoxification of H₂O₂. Studies planned for the immediate future will therefore involve the measurement of post-drying and post-thaw H_2O_2 production in Gly CP and non-CP A. belladonna and H. montanus embryos, as well as an assessment of the gene expression levels of the antioxidant enzymes involved in the detoxification of H₂O₂ (e.g. catalase and ascorbate peroxidase [reviewed by Scandalios, 1997]).

Although exposure of *A. belladonna* (exposed to osmotic stress pre-treatment) and *H. montanus* (exposed to oxidative stress pre-treatment) embryos to stress acclimation pre-treatments failed to decrease their cryo-sensitivity, it was encouraging to note that Gly CP promoted post-thaw viability retention in the embryos of both species, even after exposure to the apparently injurious stress acclimation treatments (see Chapter 5). These data reinforced

earlier indications that Gly CP promoted post-thaw viability in partially dried, rapidly cooled embryos by maintaining or enhancing enzymic antioxidant protection (see Chapter 4).

Chemical pre-treatments have been used to induce embryo desiccation- and cryo-tolerance in temperate recalcitrant-seeded species (e.g. abscisic acid in *Acer saccharinum* [Beardmore and Whittle, 2005). Results of the present work are not substantive enough to rule out the use of chemical pre-treatments to alleviate the adverse effects of stresses (e.g. Uchida *et al.*, 2002; Sivritepe *et al.*, 2003, 2005) such as dehydration and freezing in the recalcitrant zygotic germplasm of tropical species. However, they do suggest that certain stress acclimation treatments, e.g. osmotic or oxidative stress pre-treatments, may pre-dispose the tissues of recalcitrant zygotic germplasm to increasing damage and/or viability loss during cryopreservation, rather than inducing some stress tolerance.

This brings us to a very interesting growing opinion in the field of cryo-conservation: the process of cryopreservation involves a number of procedural steps, each one of which, while not lethal in itself, could represent a stress; and if any one or more of these stresses is intensified, then the cumulative effect of the process of cryopreservation could lead to increased viability loss (Berjak et al., 1999b; Padayachee et al., 2009; Pammenter et al., 2010). For instance, a burst of ROS has been identified as accompanying cotyledon excision and being the causative factor in shoot tip necrosis in axes of T. dregeana (Pammenter et al., 2010). Whitaker et al. (2010), in taking these investigations further, identified two loosely-bound cell wall peroxidases that are responsible for the generation of a burst of O_2^- at every stage (including thawing) of the cryopreservation protocol. Interestingly, the ROS burst from the tropical T. dregeana axes was considerably higher than those from temperate *Castanea sativa* seeds, which may account for the lower desiccation sensitivity of C. sativa (Whitaker et al., 2010). These results suggest that ROS bursts constitute a major, and perhaps the major, factor in the difficulties experienced with dicotyledenous species that possess fleshy cotyledons. This may explain the relatively high shoot production obtained for both amaryllid species presently investigated, since it is generally not difficult to excise the entire relatively non-fleshy cotyledonary body from recalcitrant amaryllid seeds, without imposing excision damage on the axis (Pammenter et al., 2010).

8.7 The use of stress markers for cryo-protocol optimisation

The major hindrance to the wider application of recalcitrant seed germplasm cryopreservation is the unavailability of protocols that will produce reproducible results across species (irrespective of provenance). With reference to some of the pioneering and contemporary publications on the subject (e.g. Normah *et al.*, 1986; Grout, 1986; Withers, 1988; Hor *et al.*, 1990; Pence, 1990; Wesley-Smith 1992, 2001a & b, 2004; Hu *et al.*, 1994; Berjak *et al.*, 1999b; Engelmann, 1999,

2004, 2009; Mycock *et al.*, 2000; Normah and Makeen, 2008; Walters *et al.*, 2008; Pammenter *et al.* 2010), irrespective of the species concerned or treatment combination selected, the success and reproducibility of the protocol, demands the optimisation of all the manipulations involved in the preparation of the embryos/axes for cooling, the actual cooling and their subsequent thawing and *in vitro* recovery. The empirical approach adopted by many cryopreservation studies involving recalcitrant zygotic germplasm has, however, placed limitations on the intellectual and scientific conclusions drawn to date. The present work was in no way void of empiricism but its investigation of some of the physico-chemical consequences of the various procedural steps involved in the cryopreservation of *A. belladonna* and *H. montanus* embryos confirmed that the development of improved cryo-methodologies for such explants demands a more fundamental understanding of the consequences of these procedural steps on their basic biology.

Importantly, the present work allowed for an assessment of the value of a number of markers of cryo-related stress that may be used to better optimise future cryopreservation protocols for recalcitrant zygotic germplasm. In this regard, markers such as electrolyte leakage, spectrophotometric assessment of tetrazolium chloride-reduction, thiobarbituric reactive substance production, extracellular superoxide production, antioxidant status and rate of protein synthesis, were useful in interpreting the interactive effects of WC, cryoprotection and cooling rate on subsequent zygotic embryo vigour and viability. Stress markers such as electrolyte leakage have been successfully used as indicators of freezing tolerance for embryonic cell suspensions (e.g. Jitsuyama et al., 2002), while others such as tetrazolium chloride-staining have even been used to quantify the cells critical to survival (and injured cells) in simple homogenous tissue systems (reviewed by Verleysen et al., 2004). However, as shown for both species presently investigated (Chapter 3; section 3.3), cryopreserved embryos/axes contain a mixture of living, weakened and dead cells (e.g. Sussex, 1952; Pritchard and Prendergast, 1986; Wilkinson et al., 2003; Kaczmarczyk et al., 2008), which apparently compromised the accuracy with which some of the markers employed here (e.g. electrolyte leakage and tetrazolium chloride-reduction) reflected the effects of the various pre-conditioning and cooling treatments, and/or forecasted the post-thaw viability associated with these treatments. Results of the present work also suggested that while markers of oxidative stress such as lipid peroxidation levels and extracellular superoxide production can be used to differentiate between the effects of preconditioning and cooling in complex heterogeneous tissue systems such as the embryos presently investigated, they may not be as accurate as markers based on tissue metabolic competence, e.g. antioxidant status, at forecasting post-thaw viability.

Microscopical studies have been immensely valuable in providing evidence for the explanation of recalcitrant seed behaviour and responses under a variety of conditions (reviewed by Berjak and Pammenter, 2000). The ultrastructural studies carried out here were useful in substantiating one of the major outcomes of the physiological and biochemical studies described in this work: damage incurred at each stage of the cryopreservation protocol may be compounded, thus pre-disposing tissues to further damage and/or viability loss with each progressive step. Microscopical studies also allowed for the observation of living and dead cells and facilitated the identification of a number of dehydration- and cryo-induced ultrastructural changes, e.g. an increase in the degree of vacuolation and endoplasmic reticulum fragmentation (see Chapter 3; section 3.3). Such changes may underlie the changes measured in physicochemical markers of cryo-related stresses; however, the laborious and lengthy preparative procedures associated with ultrastructural studies make them inappropriate for optimising cryoprotocols for short-lived recalcitrant zygotic germplasm. Nevertheless, they may be useful for fine-tuning an already established protocol. In the present work, ultrastructural responses were not compared directly to changes in stress markers as the material used for ultrastructural studies was harvested in 2007, while that used for the stress marker and oxidative stress studies was harvested in 2008. The reasoning behind this is that recalcitrant seeds are metabolically active, at and after shedding (Berjak et al., 1989; Farnsworth, 2000; Kermode and Finch-Savage, 2002), and in some species the shedding WC, rate of development and degree of desiccationsensitivity vary considerably from one fruiting season to the next (Pritchard et al., 1999; Berjak and Pammenter, 2004; Sershen et al., 2008). Additionally, seed developmental stage, which generally varies among seed lots (unpublished, personal observation), can influence the response to cryogenic temperatures (Kioko et al., 2003). So, as a matter of caution, if markers of cryo-related stresses are to be compared among treatments for recalcitrant zygotic germplasm then these markers should ideally be measured on seeds from the same harvest.

8.8 The vigour of seedlings recovered from cryopreserved zygotic germplasm

Despite the relatively higher post-thaw viability retention associated with Gly CP + partially dried embryos in this study, these embryos showed a number of ultrastructural irregularities (e.g. irregular cell walls, areas of amorphous cytoplasm, and condensed nuclear chromatin [Chapter 3]; these may have been associated with the reduced vigour observed in recovered seedlings (see Chapters 6 and 7). It has been known for some time now that overzealous decontamination, excessive drying and even poor growth conditions may not produce such symptoms in control recalcitrant embryos/axes, but may do so in cryo-exposed ones by exacerbating freezing damage (Berjak *et al.*, 1999b; Walters *et al.*, 2008). Results of studies described in Chapters 6 and 7

suggest that while certain adverse effects of partial drying may be reversed during *ex vitro* growth (e.g. increased relative growth rate), the adverse effects of freeze-thawing may be carried through to the early *ex vitro* stage. However, there are a number of cases where cryoderived plants have been grown to maturity with no observed phenotypic difference from controls (e.g. Engelmann, 1991; Benson *et al.*, 1996; Côte *et al.*, 2000; Martínez-Montero *et al.*, 2002; Konan *et al.*, 2007; Caswell and Kartha, 2009) and the reduced vigour and drought tolerance of seedlings recovered from partially dried and cryopreserved embryos in the present work may well disappear with an extension in the period afforded to them for hardening-off under green-house conditions, and in the field. The results obtained here do, however, highlight the need to investigate the potential impacts of cryoinjury on the genome, transcriptome, proteome and metabolome of cryo-derived plants (see Harding and Benson, 1994; Harding *et al.*, 2009; Volk, 2010), which in disrupting established patterns of growth and reproduction may impact on the re-introduction of such plants into natural environments.

The *in vitro*-based manipulations involved in cryopreservation may result in genetic change (a process described as somaclonal variation [Scowcroft, 1984]), while exposing plant germplasm to the physical, chemical and physiological stresses associated with the procedural steps involved in cryopreservation may also cause cryoinjury, for which the effects upon the genome are unknown (Harding et al., 2009; Berjak et al., 2010). The restoration of species derived from cryopreserved germplasm is becoming increasingly relevant in the face of climate change (Lynch et al., 2007; Berjak et al., 2010), making the assessment of the magnitude of genomic alteration in material recovered from cryostorage fundamental to cryopreservation (Harding, 1999, 2004; Harding et al., 2009; Volk, 2010). Whilst various molecular techniques (e.g. random amplified polymorphic DNA and amplified fragment length polymorphism [Aronen et al., 1999; Helliot et al., 2002]) confirm genetic stability of plants recovered from cryopreserved explants, their results should be interpreted with caution (Peredoa et al., 2008), particularly as such analyses may cover only $\sim 0.001\%$ of the genome (reviewed by Harding, 2004). This was the motivation for Berjak et al.'s (2010) recent suggestion, "...until available techniques become genomically more widespread (Johnston et al., 2010) it would be prudent to reserve drawing definitive conclusions and mitigate against the potential risk of genetic change (Scowcroft, 1984)". Interestingly, there is a growing opinion that epigenetic factors may well give rise to genomic changes in material recovered from cryostorage (e.g. Peredoa et al., 2008; Johnston et al., 2009) and studies planned in the immediate future will look at possible DNA methylation and/or acetylation as a consequence of each procedural step in the cryopreservation of the species presently investigated.

8.9 The problems encountered in this work and their relevance to the field of nonorthodox seed cryo-conservation

The problems encountered in this work reinforce the views of Berjak *et al.* (2010), who in reviewing the status of the cryo-conservation of germplasm from non-orthodox-seeded species said that problems associated with attempts to cryopreserve such germplasm fall into two categories: (a) the practical aspects of developing appropriate protocols, often approached empirically; and (b) the intellectual problems of understanding the impact of the cryopreservation procedures on the underlying biology. Harding *et al.* (2009) also suggested that the lack of a fundamental understanding of the physico-chemical consequences of cryopreservation hampers our ability to generate generic protocols for different types of material, or comprehend the reasons for failure.

A major problem, of an intellectual nature, discussed in the Introduction to this thesis, and evidenced by the cryo-successes and failures cited throughout this work, is that explants of temperate species are far more amenable to the manipulations required for successful cryostorage. The cryopreservation of the zygotic germplasm of tropical/sub-tropical species has turned out to be much more difficult (for reviews see Walters *et al.*, 2008; Berjak *et al.*, 2010). As discussed in overviews by Berjak and Pammenter (2004) and Walters *et al.* (2008) the reasons for this are not fully understood and even now, success remains elusive in many cases (Engelmann, 2009). Even though certain treatments employed in the present work resulted in relatively high post-thaw viability retention, a number of difficulties were encountered during the experimental phase of this thesis and some of these are discussed below.

While embryo WCs associated with particular drying times remained relatively constant for non-cryoprotected material, from any given seed lot, for 2-3 weeks, this was not true when embryos were cryoprotected prior to partial dehydration (data not shown). As a consequence, the drying time required to achieve a target WC in cryoprotected embryos from a given seed lot had to be applied to those embryos within days of its optimisation, for reproducibility.

A pilot study aimed at optimising the fixation and embedding protocol for the ultrastructural studies carried out here, showed cryoprotected embryos to resist the infiltration of resin during embedding, even when the size of the specimen was dramatically decreased; evidenced by the presence of holes in ultra-thin sections. This was rectified by simply extending the incubation time in 100% resin from 12 to 18 h.

Ultrastructural studies revealed embryos within the same treatment to display one of three variations: (1) all cells lysed; (2) all cells ultrastructurally intact; or (3) a mixture of non-lysed and ultrastructurally intact cells. This is a common phenomenon in plant germplasm recovered from cryostorage (e.g. Benson and Noronha-Dutra, 1988; Wilkinson *et al.*, 2003; Kaczmarczyk

et al., 2008) and in such cases a suitably high number of specimens need to sampled for an accurate assessment of the responses of the explants to the treatment. Due to limited seed availability, just five embryos could be sampled for each treatment in ultrastructural studies carried out here. The images subsequently analysed were selected on the basis that they represented the general appearance of cells from each treatment; however, it is suggested that future studies on the ultrastructural responses of recalcitrant zygotic germplasm to cryopreservation employ significantly larger sample sizes, to ensure an accurate assessment of ultrastructural responses.

Recalcitrant seeds remain metabolically active after shedding and display a steadily changing metabolic status as they approach germination (Tompsett and Pritchard, 1993; Hong and Ellis, 1995; Berjak and Pammenter, 2004). Desiccation sensitivity generally increases as germination progresses, in relation to the increase in rate and complexity of metabolism (e.g. as shown for Avicennia marina [Farrant et al., 1988]; Landolphia kirkii [Berjak et al., 1992]; Camellia sinensis [Berjak et al., 1993]). Cryo-sensitivity of recalcitrant seeds is therefore assumed to change with developmental status (Kioko et al., 2003; Sershen et al., 2007). As a consequence in studies such as the present one, measures of stress markers and embryo viability can only be compared among different treatments when these treatments have been applied to seeds that are of the same 'storage age', which does not necessarily equate to 'developmental age'. In the present work, this had two major implications: firstly, the number of parameters that could be measured as part of any particular experiment was limited by the number of seeds available at each harvest; and secondly, the period between the optimisation of the individual procedures involved in the cryopreservation of the material and their combined application in the cryoprotocol had to be limited to a few days (usually 2-4 d). Additionally, embryo drying kinetics and the period required for post-cryo recovery (i.e. root and shoot production), differed among seed lots so it is recommended that any future studies aiming to repeat the cryopreservation protocols presented in this work, recognise and accommodate for these differences (no matter how slight they may be).

Lastly, the measurement of leaf-based photosynthetic rates for 'small-leaved' seedlings is practically difficult. The use of a portable photosynthesis measuring system fitted with a chamber specially designed for small leaves, such as the *Arabidopsis* chamber (Li-Cor, Nebraska, USA) used in this study, can aid in measuring 'real' changes in chamber carbon dioxide (CO₂) concentration; however, when CO₂ exchange rates are very low, as in stressed material, differences in CO₂ concentration are often too small to measure.

8.10 Concluding remarks

The results of the present work reinforce the notion that each successive manipulation involved in the cryopreservation of recalcitrant zygotic germplasm has the potential to inflict damage on tissues and post-thaw survival in such germplasm relies on the minimisation of structural and metabolic damage at each of the procedural steps involved in their cryopreservation (Berjak *et al.*, 1999b; Pammenter *et al.*, 2010). The results also highlight the need to design research programmes aimed not only at developing protocols for cryopreservation of plant genetic resources, but also at elucidating and understanding the fundamental basis of both successes and failures (e.g. see Harding *et al.*, 2009).

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APPENDIX A

Germination characteristics and embryo morphology of recalcitrant amaryllid seeds

Germination in amaryllid seeds is unusual in that part of the cotyledon, termed the cotyledonary body, to which the embryonic axis is attached, grows right out of the seed (Fig. A1a). Zygotic embryos (Fig. A1b) do not exhibit any visible attachment to the surrounding endosperm and in *Haemanthus montanus* (Baker) and *Amaryllis belladonna* (L.) seeds, range from c. 0.5-1.0 cm in length. The root and shoot meristems (Fig. A1c), each of which can occupy an area of between 0.5-1.0 μ m², lie within 3-4 μ m of each other and cannot be differentiated from the surrounding cotyledonary tissue with the naked eye.

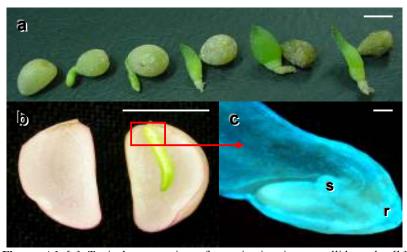


Figure A1 [a] Typical progression of germination in amaryllid seeds; [b] zygotic embryo lying within fleshy endosperm with box indicating the location of the embryonic axis (e); and [C] cross section of amaryllid embryonic axis, showing root (r) and shoot (s) meristems enveloped by cotyledonary sheath, as visualised by fluorescence microscopy. Bar = 1 cm for [A] and [B] and 1 μ m for [c].

Drying characteristics of *Haemanthus montanus* and *Amaryllis belladonna* zygotic embryos Materials and Methods

Freshly excised zygotic embryos were rapidly dried via flash drying (devised by Berjak *et al.*, 1990) for various times. In order to minimise the potential variation in drying rate as a function of embryo size, only embryos of between 4-6 mm in length were used for all the experiments described below. At each drying interval, water content (WC) of ten embryos was determined gravimetrically while ten to fifteen embryos were rehydrated, decontaminated and regenerated *in vitro* as described in Chapter 2 (section 2.2). Vigour (in terms of mean time to

germinate [MTG] and germination index [GI]) and viability were thereafter assessed as described in Chapter 2 (section 2.2). Results of these studies were used to construct curves of water content (WC) and viability vs. drying time, which informed the cryopreservation studies described in Chapter 2.

Results and Discussion

As is characteristic of recalcitrant seeds (King and Roberts, 1980; Farrant et al., 1986; Pammenter and Berjak, 1999), A. belladonna and H. montanus possessed highly hydrated embryos at shedding (c. 4.7 and 5.1 g g⁻¹, respectively), which upon rapid dehydration lost some viability at relatively high WCs (1.0-0.42 g g⁻¹; Fig. A2a, b; Table A1a, b). This was not surprising since many of the processes that lead to viability loss during dehydration in recalcitrant seeds appear to be aqueous-based and can occur at relatively high (intermediate) WCs; in the order of 1.0-0.3 g g⁻¹ (Vertucci and Farrant, 1995; Farrant et al., 1997; Walters et al., 2001). Unlike the desiccation curves of whole seeds, the pattern of which are usually characterised by a simple exponential function (e.g. Tompsett and Pritchard, 1998; Pammenter et al., 1998; Makeen et al., 2005), initial dehydration in the embryos of both species were faster than exponential; a trend observed in the embryos/axes of other recalcitrant-seeded species (e.g. Citrus suhuiensis cv. Limau langkat [Makeen et al., 2005]; and a number of amaryllids [Sershen et al., 2008]). As reported for recalcitrant embryonic axes/zygotic embryos elsewhere (e.g. Pammenter et al., 2003), WC decreased in a non-linear fashion with damage accumulating until a point at which some viability loss occurred. The fact that this loss in viability was most severe at WCs <0.34-0.29 g g⁻¹ was not surprising since recalcitrant seeds characteristically lose viability during drying at WCs ranging from 0.6-0.2 g g⁻¹ with this wide range in sensitivity being attributed to morphological, physiological, biochemical and genetic differences (Vertucci and Farrant, 1995; Berjak and Pammenter, 1997; Daws et al., 2006). These differences may even give rise to variability among embryos/axes of the same seed lot or from one seed lot to another (Berjak et al., 1996). In this study, the degree of desiccation sensitivity (assessed as the WC at which viability was first lost) differed slightly between harvests, most especially in H. montanus, but the embryos/axes of both species could nevertheless be dried to WCs between 0.34 and 0.29 g g⁻¹ while still retaining \geq 70% viability, over both harvests (Fig. A2a, b; Table A1a, b). As discussed in Chapter 2, such rapid (as opposed to slow) drving allows for the survival of the embryos/axes of several recalcitrant-seeded species to very low WCs (c. 0.20-0.44 g g⁻¹ [e.g. Normah et al., 1986; Pritchard and Prendergast, 1986; Berjak et al., 1993; Pammenter et al., 1993; reviewed by Normah and Makeen, 2008); possibly because the tissue passes through intermediate WCs so fast that damage typically associated with intermediate WCs (that can be lethal) may not have time to accumulate (Pammenter *et al.*, 1998; Walters *et al.*, 2001, 2002).

Additionally, there were three striking inter-species differences in terms of drying characteristics in the embryos presently investigated. Firstly, while root meristems were more sensitive to desiccation than shoot meristems in A. belladonna, these growing points appeared to be equally sensitive to desiccation in *H. montanus* (Table A1a, b). Differences in sensitivity between root and shoot meristems in recalcitrant zygotic germplasm, may be based on differences in desiccation tolerance per se, perhaps as a consequence of cell architecture (e.g. degree of vacuolation), differential WC, differential drying rates (and hence time of exposure to the dehydration stress), or a combination of these factors (Assy-Bah and Engelmann, 1992; Pritchard and Manger, 1998). Secondly, while slight dehydration stimulated germination rate in A. belladonna this effect was not observed in H. montanus (Table A1a, b). Partial drying can have an initial promotive effect in recalcitrant seeds but as observed in both species here (see Table A1a, b), any further drying is usually followed by a decline in GI and/or viability (Farrant et al., 1985; Tompsett and Pritchard, 1998; Eggers et al., 2007). In other species (e.g. embryos of Zizania palustris [Aldridge and Probert, 1992] and Acer pseudoplatanus seeds [Hong and Ellis, 1990]) this promotive effect has been attributed to a continuation of the earlier maturation processes which occur naturally on trees as seeds become drier (Tompsett and Pritchard, 1998). Alternatively, a decline in vigour, as observed at some of the lower embryo WCs here (Table A1a, b), is usually indicative of damage incurred and/or repair processes (Pammenter et al., 2002). Lastly, there were marked inter-species differences in drying kinetics: A. belladonna embryos could be dried to comparable WCs more than ten times faster than H. montanus embryos (Table A1a, b; Fig. A2a, b). This was unlikely a consequence of inter-species differences in embryos mass since mean fresh mass of freshly excised embryos were comparable between species (0.0080±0.0021 g for A. belladonna and 0.0071±0.0013 g for H. *montanus*). As explained in Chapter 2, desiccation damage in recalcitrant seeds appears to be a function of two interrelated parameters; i.e. the intensity and duration of dehydration (Pammenter et al., 1998; 2002; 2003; Walters et al., 2001; Liang and Sun, 2002; Pammenter et al., 2003). This suggests that at comparable WCs, H. montanus embryos may have been exposed to a greater degree of desiccation stress than those of A. belladonna.

						a
Drying time (min)	Embryo WC ¹ (g g ⁻¹)	Roots (%)	Shoots (%)	Viability ² (%)	*MTG ³	*GI ⁴
YEAR 1: 0	5.05±0.92	100	100	100	12.6	44.4
60	1.17±0.36	100	100	100	11.9	39.1
120	1.01±0.19	100	90	90	13.7	14.6
150	0.73±0.14	100	90	90	12.1	28.8
180	0.67±0.25	100	90	90	15.6	14.1
210	0.67±0.28	100	90	90	15.1	6.7
240	0.52±0.15	100	90	90	15.9	6.2
300	0.34±0.09	80	70	70	13.7	4.8
330	0.29±0.10	30	20	20	13.5	1.1
YEAR 2: 0	4.66±0.76	100	100	100		
120	0.94±0.37	100	100	100		
180	0.43±0.13	80	70	70		
240	0.33±0.13	70	70	70		
300	0.26±0.11	40	40	40		
360	0.22±0.15	30	40	30		

 Table A1 Embryo water content, drying time, vigour and viability for rapidly dried [a] A. belladonna and

 [b] H. montanus zygotic embryos.

Drying time	Embryo WC ¹	Roots	Shoots	Viability ²	*MTG ³	*GI ⁴
(min)	$(g g^{-1})$	(%)	(%)	(%)	MIG	01
YEAR 1: 0	4.67±0.57	100	100	100	9.3	69.4
15	$0.42{\pm}0.09$	100	100	100	5.3	204.1
30	0.32±0.10	80	93	80	10.0	14.2
60	0.17±0.04	20	87	20	9.3	0.7
90	0.16±0.02	13	87	20	14.0	0.4
120	0.13±0.01	7	80	13	9.0	0.3
180	0.17±0.15	7	87	7	17.0	0.2
240	0.10±0.01	0	40	7	0	0
300	0.08±0.03	0	33	0	0	0
YEAR 2: 0	5.71±1.25	100	100	100		
5	0.53±0.09	90	90	90		
15	0.29±0.05	80	90	80		
30	0.17±0.04	30	80	30		
60	0.13±0.02	0	40	0		
90	0.10±0.03	0	20	0		
120	0.10±0.02	0	20	0		b
180	0.07±0.02	0	10	0		
240	0.07±0.02	0	0	0		

¹ water content; ² mean time to germinate; ³ viability = root and shoot production; ⁴ germination index. Mean time to germinate and germination index was based on viability. Water content, % root and shoot production and viability data (after 40 d) were tested for significant differences across drying times, within years: p < 0.05 for water content data (Mann-Whitney-U test, n = 10); p < 0.05 viability and % root and shoot production data (null model chi-squared analysis, n = 10 for year 1 and n = 15 for year 2). * Replicated once; not tested for significant differences.

a

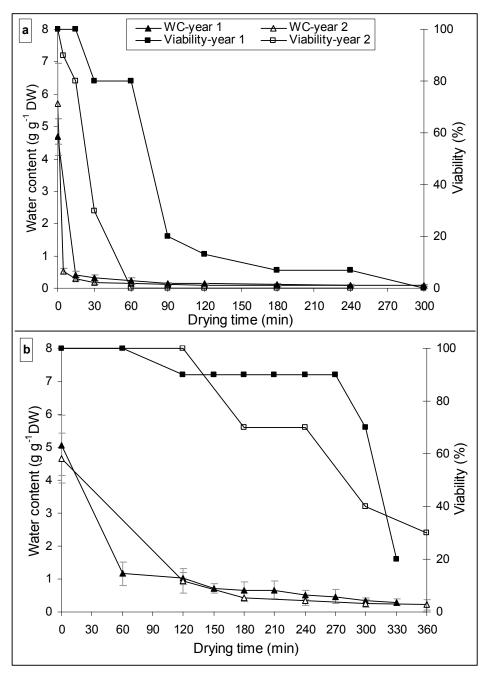


Figure A2 Water content and viability vs. drying time for [a] *A. belladonna* and [b] *H. montanus* zygotic embryos. For water content n = 10; for viability n = 15 for year 1 and n = 10 for year 2.

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APPENDIX B

Estimation of zygotic embryo sucrose concentration to check for potential interference caused by carbohydrates in the thiobarbituric acid-reactive substances assay (Heath and Packer, 1968)

Rationale

When estimating lipid peroxidation via colorimetric assays such as the thiobarbituric acidreactive substances (TBARS) assay (originally described by Heath and Packer, 1968) there is always the risk that phenolic and carbohydrate compounds can cause interference, since, like TBARS, many of them also absorb at 532 nm (Du and Bramlage, 1992). Correction for interference caused by carbohydrates (monosaccharides and disaccharides, such as sucrose and trehalose) in sugar-rich plant tissue has been suggested to guard against over-estimation of lipid peroxidation (Du and Bramlage, 1992). Those authors presented a modified TBARS assay for sugar-rich plant tissue extracts which involved, apart from measuring absorbance at 400, 532 and 600 nm instead of just at 532 and 600 nm as in the original TBARS assay described by Heath and Packer (1968), rectifying the interference of soluble sugars in samples using a standard curve for sucrose (2.5-10.0 µmol ml⁻¹). In the present study, sucrose concentration of freshly excised (fresh) and sucrose (Suc) and glycerol (Gly) cryoprotected (CP) Amaryllis belladonna (L.) and Haemanthus montanus (Baker) zygotic embryos was estimated using the colorimetric anthrone assay (after Jermyn, 1956). This was done to assess whether CP enhanced tissue sucrose concentrations relative to fresh embryos, and if so, whether these concentrations fell within the interfering range (i.e. 2.5-10.0 μ mol ml⁻¹) for sucrose (see Chapter 4; section 4.2), as suggested by Du and Bramlage (1992).

Materials and Methods

Embryo pre-treatment

After 14 d of 'hydrated' storage (see Chapter 2; section 2.2), zygotic embryos of *A*. *belladonna* and *H. montanus* were excised with the entire cotyledonary body attached and cryoprotected with either aqueous Gly or Suc as described in Chapter 2 (section 2.2). After CP, embryos were briefly rinsed with water to remove residual cryoprotectant and immediately measured for Suc concentration as described below.

Reagents

Anthrone reagent was purchased from Sigma Chemical Co. (Germany) and all other reagents were analytical grade and locally available.

Extraction of water soluble carbohydrates and estimation of sucrose concentration

Four batches of 15 embryos each (c. 100 mg) from fresh, Suc CP and Gly CP treatments, were freeze-dried, ground (using inert sand) in a pestle and mortar to pass a 0.5 mm mesh and extracted for water soluble carbohydrates (WSC) according to Trethewey and Rolston (2009), with slight modifications after Chow and Landhäusser (2004). Here, weighed samples (four samples of approximately 20 mg each, across all three treatments) were extracted three times with 1 ml 80% (v/v) aqueous ethanol by heating the samples in capped glass tubes in a 95° C water bath for 10 min. This fraction is termed the 'mobile' fraction and is composed of low molecular weight WSC, mainly sucrose and monosaccharides (Carpita et al., 1989). Extracts were then centrifuged at 13,000 g for 10 min, combined and evaporated under vacuum to remove pigments, and then re-suspended in 2 ml deionised water. Sucrose concentration was thereafter determined using a colorimetric anthrone assay (Jermyn, 1956), whereby 1 ml extracts were mixed with 25 ml of anthrone reagent (62.5% [v/v] sulphuric acid, 37.5% [v/v]ethanol, 0.00125% anthrone [v/v]), incubated at 100°C for 20 min and then read for absorbance at 620 nm. Sucrose concentration was calculated using a sucrose standard curve and expressed as mg sucrose g⁻¹ DW. Assays were carried out twice on four different extracts. For dry weight estimation, three batches of five embryos each, across all three treatments, were dried in an oven at 80°C for 48 h to determine DW.

Results and Discussion

Sucrose concentrations in fresh embryos of both species (Table B1) were much lower than concentrations reported for zygotic embryos or embryonic axes of other recalcitrant-seeded species (Steadman *et al.*, 1996). Cryoprotection did result in a marginal increase in Suc concentration relative to fresh zygotic embryos but these differences were not significant. When mg/g DW Suc concentrations were translated to μ mol ml⁻¹ concentrations, all treatments for both species exhibited mean concentrations that were <0.1 μ mol ml⁻¹ (Table B1). These data suggested that sucrose concentrations across all three treatments (for both species) were lower than the interfering range (2.5-10.0 μ mol ml⁻¹) as suggested by Du and Bramlage (1992). Also, if interference did occur in the assays carried out according to Heath and Packer (1968) in studies described in Chapter 4 (section 4.2) then the degree of interference was likely to have been comparable across CP and non-CP treatments.

Sucrose concentration in terms of mg g ⁻¹ DW							
	Fresh	Suc CP	Gly CP				
A. belladonna	1.65±0.21 ^a	1.71±0.19 ^a	1.77±0.21 ^a				
H. montanus	1.42±0.13 ^a	1.51±0.20 ^a	1.60±0.24 ^a				
Sucrose concentration in terms of µmol ml ⁻¹							
A. belladonna	0.022 ± 0.003^{a}	0.026 ± 0.003^{a}	0.025±0.003 ^a				
H. montanus	$0.023{\pm}0.002^{a}$	0.019 ± 0.002^{a}	0.025 ± 0.004^{a}				

Table B1 Sucrose concentration in terms of mg g^{-1} DW and μ mol ml⁻¹ for fresh and cryoprotected (CP) *A. belladonna* and *H. montanus* zygotic embryos.

Values represent mean±SD. Values followed by similar letters are not significantly different; p > 0.05 when tested for significant differences across embryo pre-treatments, within species (*ANOVA*, n = 4).

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APPENDIX C

Experimental design for studies on *ex vitro* vigour of *Amaryllis belladonna* (L.) seedlings recovered from fresh, partially dried and cryopreserved zygotic embryos

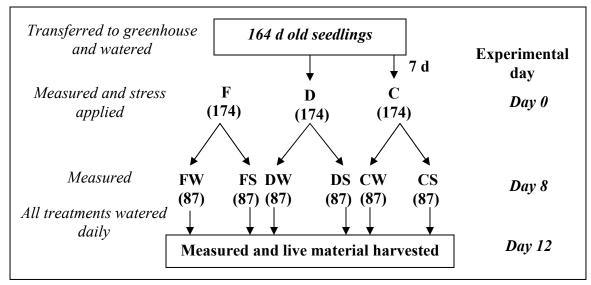


Figure C1 *Ex vitro* experimental design. Measured: CO₂-assimilation, potential photochemical efficiency, leaf water, osmotic and pressure potential measured on this day. Seedlings were generated from fresh (F), partially dried (D) and cryopreserved (C) zygotic embryos. Recovered seedlings were subjected to either 12 d of watering (W) or 8 d of water deficit followed by 3 d of re-watering (S). The number of replicates associated with each treatment combination is given within brackets.

Leaf stomatal density of *Amaryllis belladonna* seedlings recovered from fresh, partially dried and cryopreserved zygotic embryos

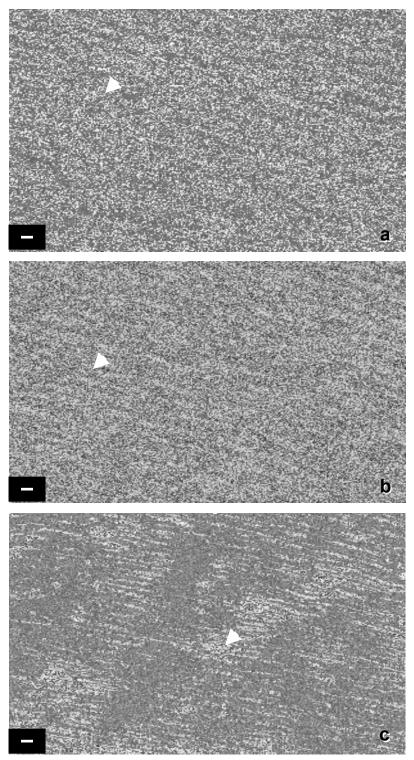


Figure C2 Scanning electron micrographs of stomata (arrow heads) on adaxial leaf surface of unstressed seedlings generated from (a) fresh; (b) dried; and (c) cryopreserved zygotic embryos. All images were captured at magnification: $200\times$, using a constant field of view (0.988 mm²). Bar = 10 μ m.