UNIVERSITY OF KWAZULU-NATAL

EFFECTS OF SOME OF THE PROCEDURAL STEPS OF CRYOPRESERVATION ON CRYO-RECALCITRANT ZYGOTIC EMBRYOS OF THREE AMARYLLID SPECIES PRODUCING DESICCATION-SENSITIVE SEEDS

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2013

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As the candidate's supervisor I have approved this dissertation for submission.

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Preface

The experimental work contained in this Master's dissertation has been carried out at the School of Life Sciences (formerly known as the School of Biological and Conservation Sciences), of the University of KwaZulu-Natal, on the Durban - Westville Campus. Supervision was provided by Professors P. Berjak and N.W. Pammenter, and Dr Sershen of the same university.

This study represents original work by the author as a partial fulfillment of the requirements for a Master's degree, and has not been submitted to any other university for a similar or relevant purpose. Where use was made of the work of others, it has been duly acknowledged in text.

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Nomali Ngobese

July 2013

Declaration

I, Nomali Ziphorah Ngobese, declare that:

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

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

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

4. This dissertation 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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Nomali Ngobese

July 2013

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I thank God for the opportunity, ability and resources granted to me to pursue this degree, which has been a dream come true.

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Gratitude is also due to all the members of the Plant Germplasm Conservation Research Group, who have become fond friends and colleagues over the years shared together.

I am also sincerely grateful to all my friends and family, who have supported me during the course of this project; and to Mr Linda Gcwensa, for sharing some of these days with me.

Dedication

This dissertation is dedicated to my late mother, Miss Nomusa Ngobese; whom I cherish dearly.

Abstract

Cryopreservation is the most promising method for the long-term conservation of germplasm of plants producing desiccation-sensitive seeds. While such seeds are generally termed recalcitrant in the context of conventional storage practices, the term 'cryo-recalcitrant' is used for germplasm which is not readily amenable to cryopreservation. Cryo procedures usually involve a sequential combination of steps which must be optimised to limit the stresses experienced by specimens, thus promoting their survival.

The present contribution reports on the effects of some of the steps involved in cryopreservation on the survival of the embryos of the amaryllids, *Ammocharis coranica*, *Brunsvigia grandiflora* and *Haemanthus albiflos*, with the ultimate aim of developing a protocol(s) for the successful cryopreservation of the germplasm of these species. The main foci of the investigations were the effects of rapid (flash) drying, the use of the cryoprotectant additives, glycerol (5 & 10%) and DMSO (0.1 & 0.25%), and employment of different cooling rates on the zygotic embryos of the selected species, which are known to be recalcitrant as well as being cryo-recalcitrant. Furthermore, this study reports on attempts at improving the rapidity of dehydration during flash drying by applying a vacuum, and also of providing cathodic protection (via highly reducing cathodic water and/or direct exposure to a static {negatively-charged} cathodic field during flash drying) to the explants at various stages in the protocol. These techniques were employed in attempts to ameliorate the adverse effects of reactive oxygen species associated with stresses imposed by the procedures during the cryopreservation process.

The embryos of *Ammocharis coranica*, *Brunsvigia grandiflora* and *Haemanthus albiflos* were initially at water contents (WCs, dry mass basis) of 3.28±0.52, 2.55±0.22, 4.48±0.92 g g^{-1}, respectively, after harvest. These embryos proved to be tolerant to moderately rapid water loss in the short term, with >60% retaining germinability at water contents ≥0.5 g g^{-1}. The results from this study confirmed that dehydration to water contents below 0.5 g g^{-1} (dry mass basis) compromised survival, and that this effect was exacerbated if the embryos were cryoprotected prior to drying. Interestingly, the rate of water loss in embryos of these species

differed, with *A. coranica* and *H. albiflos* drying at a (comparably) much slower rate than those of *B. grandiflora*. Subsequent rapid cooling yielded promising results when compared with slow cooling, as 30% of glycerol cryoprotected, rapidly cooled *A. coranica* embryos that had been flash-dried to 0.36±0.10 g g^{-1} generated normal seedlings. It was clear, however, that the effects of these procedures were exacerbated when all the steps of the cryo procedure were applied sequentially. However, the work also showed that these adverse effects may be ameliorated if each step of the cryopreservation protocol is optimised on a species-specific basis, thus promoting the chances of survival after cryopreservation and facilitating subsequent seedling establishment. This was evident in the 30% germination obtained when embryos of *A. coranica*, which had been cryoprotected with glycerol prior to flash drying before exposure to rapid cooling, while those that had not been cryoprotected or were cryoprotected with DMSO before drying did not survive. The incorporation of cathodic protection during flash drying appeared promising as it promoted the survival of 10% of *H. albiflos* embryos dehydrated to WCs between 0.37 and 0.26 g g^{-1} (whereas no survival was achieved without the inclusion of this step), and 70% of *A. coranica* embryos that were dehydrated to 0.35±0.21. In addition, the reduction of the explant size, from a whole 6 mm embryo to a 3-4 mm excised axis, promoted survival by up to 30% for *A. coranica* and *H. albiflos*, even at higher WCs. However, survival in these cases was based on observations of abnormal development, i.e. the development of roots or shoots, or calli. No surviving embryos were obtained from *B. grandiflora* after cooling, regardless of the preconditioning treatment or rate of cooling, and this was accredited to the greater degree of sensitivity of these embryos to the cryo procedures than those of the other two species. The use of cathodic water to re-hydrate explants after dehydration and of applying a vacuum during flash drying did not result in any observable benefits, and require further investigation for optimisation.

The very limited success towards establishing a cryopreservation protocol for the species investigated in this study reinforces the difficulties associated with the cryopreservation of recalcitrant germplasm, which informs the cryo-recalcitrance of some explants. However, the results obtained have helped to identify a number of intervention points that could be used to minimise the damage incurred during the various procedural steps involved in cryopreservation.

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Abbreviations and Symbols

$^1O_2$	singlet oxygen
$^{\bullet}OH$	hydroxyl radical
BAP	6-benzylaminopurine
c.	circa
C	cooling
CA	sodium cacodylate
$Ca(OCl)_2$	calcium hypochlorite
$CaCl_2.2H_2O$	calcium chloride dihydrate
CaMg	calcium magnesium solution
Cat FD	cathodic flash drying
Cat H_2O	cathodic water
$CeCl_3$	cerium chloride
CPA	cryoprotectant additive
DM	dry mass
dmb	dry mass basis
DMSO	dimethyl sulphoxide (Me_2SO)
DT	drying time
FD	flash drying/dried
FM	fresh mass
fmb	fresh mass basis
G	germination
GA	glutaraldehyde
Gly	glycerol
H_2O_2	hydrogen peroxide
HSP	heat shock protein

LEA	late embryogenic abundant proteins
LN	liquid nitrogen
MD	molecular dynamics
$MgCl_2.6H_2O$	magnesium chloride hexahydrate
MS	Murashige and Skoog
NaOCl	sodium hypochlorite
NMR	nuclear magnetic resonance
O_2	oxygen
$O_2^{\bullet-}$	superoxide anion/radical
PA	paraformaldehyde
PEG	polyethylene glycol
QDS	quarter-degree square
WC	water content
RNS	reactive nitrogen species
ROS	reactive oxygen species
SEM	scanning electron microscope
TEM	transmission electron microscope
v/v	volume per volume
VFD	vacuum flash drying
w/v	weight per volume
Ψ_w	water potential

Units of Measurement

%	percent
°C	degree Celsius
d	day(s)
g	gram(s)
g g^{-1} dmb	g H_2O per g of dry matter, dry mass basis
h	hour(s)
KV	kilovolt(s)
l	litre(s)
min	minute(s)
ml	milliliter(s)
mm	millimeter(s)
mM	millimolar
MPa	megaPascal(s)
ms	millisecond(s)
nm	nanometer(s)
s	second(s)
µ	micron(s)
µmol m^{-2} s^{-1}	micromoles per square metre per second

Chapter 1

1. Introduction

1.1. Biodiversity in South Africa

Africa is well known for its richness in plant biodiversity and South Africa is said to possess one of the highest plant species diversity world-wide (Wiersum *et al*., 2006; Berjak *et al*., 2011a). This diversity is mainly attributed to the country being characterised by contrasting biomes, which host close to 10% of the world's plant species (Berjak *et al.*, 2011a). Such diversity can contribute to a country's economy as it has implications for the nation's food security and agro-biodiversity status, and also constitutes a rich source of compounds for potential medical purposes and food/crop protection industries (Panis and Lambardi, 2005). However, the persistence of populations of many of these species is threatened by anthropogenical pressures. These include the overexploitation of natural plant resources to meet the rising demand for traditional medicine; pollution; land transformation for agricultural purposes and the loss of habitats due to urbanisation (Rao, 2004; Wiersum *et al*., 2006). Unpredictable changes in climate, natural disturbances, as well as invasion by alien species, also threaten plant biodiversity (Berjak, 2000; Rouget *et al*., 2003; Olivier *et al*., 2009).

The terrestrial ecosystems of South Africa are fragile and, according to the Red Data List (Fig. 1.1; SANBI, 2012), almost 25% of the flora is considered either threatened with extinction or of conservation concern. A substantial number of taxa are already extinct while some already fall within the "critically endangered/possibly extinct" and "endangered" categories (Berjak *et al.*, 2011a). Whilst much effort should be directed towards developing *in situ* conservation measures to prevent the continuing loss of plant biodiversity, the problem also necessitates the global development of *ex situ* conservation methods (Paunescu, 2009). Additionally, until recently, biodiversity conservation in South Africa was based primarily on law-enforcement approaches, and since these have largely been unsatisfactory, participatory methods to conservation are now being explored and/or implemented (Wiersum *et al*., 2006; van Niekerk, 2012).

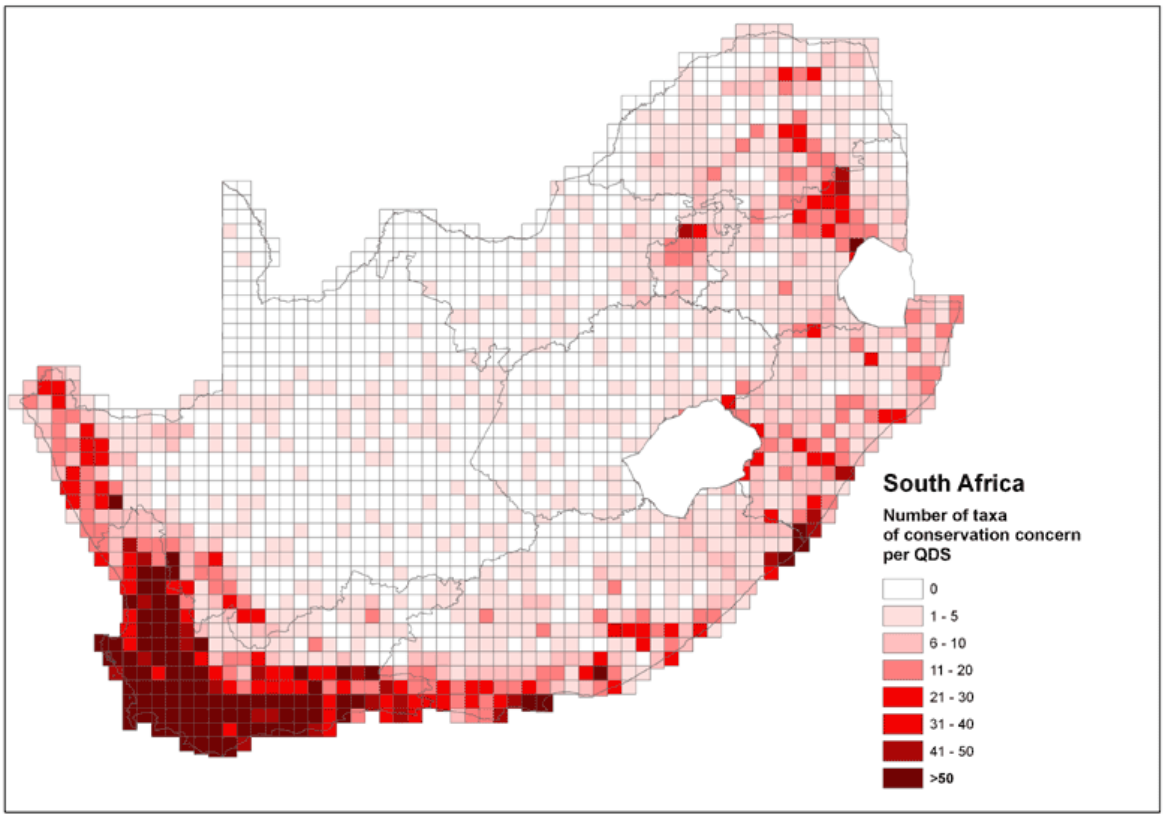


Figure 1.1: Map of South Africa showing areas of high concentrations of taxa of conservation concern per quarter-degree square (QDS, ~700 km^2; SANBI, 2012).

1.2. Available approaches for plant germplasm conservation

In situ conservation entails conserving species within their natural habitat, whereas *ex situ* conservation involves preserving and maintaining samples of living organisms outside their natural habitat (Engelmann and Engels, 2002). Various *in situ* and *ex situ* conservation methods can potentially be adopted for the conservation of plant genetic resources of species considered to be endangered (Engelmann and Engels, 2002; Berjak *et al.*, 2011a). *In situ* conservation methods are considered to be the ideal route for preserving genetic diversity, particularly because *ex situ* methods do not allow for the selection and adaptation of genotypes within their natural habitats. However, when *in situ* methods cannot be used effectively for such purposes, *ex situ* conservation then becomes a preferred alternative to extinction (Paunescu, 2009; Berjak *et al.*, 2011a). This may be because some species cannot successfully be conserved via *in situ* techniques at all or because, even when success is achieved, the resources can be maintained only for a limited period. In such cases *ex situ*

strategies are used to complement *in situ* methods, but become the only alternative for species that cannot be conserved via *in situ* methods (Thielges *et al.*, 2001; Li and Pritchard, 2009).

Ex situ conservation can be achieved using plant material in a variety of forms: whole plants, seeds, pollen, vegetative propagules, and tissue or cell cultures (Engelmann and Engels, 2002; Paunescu, 2009; Reed *et al.*, 2011). These genetic resources can be maintained *ex situ* within botanical gardens, arboreta or genebanks, whereas for *in situ* conservation plants are maintained within their natural habitats by formally protecting these spaces (e.g. as national parks and forest reserves) (Engelmann and Engels, 2002; Rao, 2004). Of the variety of *ex situ* conservation methods developed to date, cultivation of resources at botanical gardens, seed storage and *in vitro* cultivation are the most widely used (Kaviani, 2011).

1.3. Conventional conservation

The key to the maintenance of biological diversity is said to lie largely in the maintenance of tree species diversity (Thielges *et al.* (2001), and seems to be well underway as Paunescu (2009) pointed out that more than one-third of the world's flowering plants have already been cultivated at botanical gardens. This indicates the value of employing conventional *ex situ* methods for conserving global plant biodiversity. However, although cultivation of species in botanical gardens is a valuable *ex situ* conservation method for endangered species, the practice is limited by factors such as labour availability, time and space, and also presents the inconvenience of species having to overcome acclimatisation- and environment-related problems (Engelmann, 2004) before success can be claimed. *Ex situ* collections also remain exposed to natural disasters and attacks by pests and pathogens, apart from requiring considerable financial investment (Engelmann, 2004).

Among the various *ex situ* conservation methods, seed storage is widely regarded as the most convenient and popular method for conserving plant genetic diversity over extended periods (Engelmann and Engels, 2002; Paunescu, 2009). This is because seeds carry the genetic information of the parent plants and possess the ability to germinate into whole plants, yet require far less space for storage in comparison with large-scale cultivation (Li and Pritchard, 2009). Additionally, seeds are the most convenient means of transporting and distributing germplasm to farmers, breeders, scientists and other users (Engelmann and Engels, 2002). Seed storage/banking is the most convenient way of conserving biodiversity in the long-term,

but some species do not bear seeds, instead propagating vegetatively; such species are conserved as whole plants in field genebanks for the short- to medium-term (Gonzalez-Benito *et al.*, 2004) or as *in vitro* collections (Reed *et al.*, 2011).

1.3.1. The importance of seed storage

Conventional seed storage practices usually entail maintenance at low relative humidities and temperatures, and are applied successfully to seeds categorised as being 'orthodox' (i.e. desiccation tolerant [Roberts, 1973]). Such storage is generally achieved by drying seeds to low moisture/water contents (~3-7%, on a fresh mass basis [fmb]) and storing them at low relative humidity (RH) in hermetically sealed containers at low temperatures (usually at -18˚C) (Engelmann and Engels, 2002). Orthodox seeds exhibit certain characteristics (to be discussed later) that allow them to withstand the stresses imposed by these procedures and many of the world's major food crop species which produce orthodox seeds are stored this way (Engelmann and Engels, 2002). Those authors also pointed out that most of the global accessions stored at genebanks are maintained as seed, and advances in technologies over the years have devised means to store seeds of various species in this way for several decades. Unfortunately, this approach cannot be used for the conservation of species which produce seeds that are immature, sterile, or intolerant of the low moisture contents necessary for long-term maintenance via conventional seed storage practices (Engelmann, 2004; Paunescu, 2009).

1.3.1.1. Seed storage behaviour and categorisation

The initial categorisation of seeds by Roberts (1973), established the terms seed 'orthodoxy' for seeds that can tolerate extreme dehydration and survive in a dehydrated state for prolonged periods at reduced temperatures and relative humidities, and 'recalcitrance' for those that are sensitive to dehydration and cannot be stored in the same manner as orthodox types. However, seed post-harvest physiology is believed to encompass a continuum of responses to dehydration between the extremes of recalcitrance and orthodoxy (Pammenter and Berjak, 1999a; Berjak and Pammenter, 2004a, b), which also accommodates another, rather diffuse, category, i.e. those showing intermediate post-shedding/-harvest behaviour, as proposed by Ellis *et al.* (1990). Desiccation tolerant orthodox seeds will survive at low water contents with a non-significant effect on viability (Roberts, 1973). In contrast, recalcitrant seeds, which are shed at high water contents, are desiccation sensitive, becoming damaged

even upon slight drying, after which viability is soon lost (Chandel *et al*., 1995, Pammenter and Berjak, 1999a; Pritchard *et al.*, 2004). Death at high water content is particularly the case when dehydration is slow (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999a; Walters *et al.*, 2001). However, as mentioned above, the seeds of many species may not be exclusively orthodox nor recalcitrant, falling into the intermediate category of post-harvest behaviour (i.e. seeds that cannot withstand desiccation to water contents below 10-12% [fmb], and may also be chilling sensitive [Hong *et al.*, 1996; 1998]). Examples of species that produce intermediate seeds include some species of *Coffea* (Ellis *et al.*, 1990; Dussert *et al.*, 1998), neem (*Azadirachta indica*, Sacandé *et al.*, 2001) and the wild date palm (*Phoenix reclinata*; von Fintel *et al.*, 2004; Ngobese *et al.*, 2010). It should be noted though, that seeds of the majority of non-cultivated species are yet to be categorised (Hong and Ellis, 1996; Liu *et al.*, 2008).

In essence, seeds that exhibit post-harvest physiology rendering them intolerant to conventional seed-storage procedures are broadly categorised as being 'non-orthodox' (Roberts, 1973; Ellis *et al.*, 1990; Hong and Ellis, 1996). These seeds are normally characterised by high water contents at shedding and remain metabolically-active before and after being shed from the parent plant (Pammenter and Berjak, 1999a). A substantial number of species, principally of tropical and subtropical origin, such as coconut, cocoa, and many forest and fruit tree species produce non-orthodox seeds (Engelmann and Engels, 2002). Recalcitrant seeds generally lose viability relatively fast if germination does not occur shortly after shedding and, as they do not survive desiccation, they cannot be stored using conventional seed storage techniques (Chin, 1995; Berjak *et al.*, 2011a; b). Advances in biotechnology now provide some important tools for the conservation of the genetic resources of such species, e.g. *in vitro* slow/minimal growth techniques (Nassar, 2003; Sarasan *et al.*, 2006; Reed *et al.*, 2011), but this is labour-intensive and often affords only short- to medium-term germplasm conservation. In such cases, cryopreservation (i.e. storage at ultra-low temperatures usually in liquid nitrogen [LN] or in the vapour phase above LN) then becomes a promising alternative for the long-term preservation of genetic resources of such species. However, this technique requires optimisation of the procedural steps both before and after exposure of the partially hydrated germplasm to cryogenic conditions in order to achieve success. This forms the basis of the current study, which investigates the

effects of some of the procedural steps of cryopreservation on embryos of selected species known to produce recalcitrant seeds.

1.3.1.2. The significance of events during seed development in post-harvest seed behaviour

Differences in physiological responses to desiccation stress exhibited between orthodox and non-orthodox seeds are largely due to physico-chemical differences acquired – or not –during their development, before seed maturity is reached and germinative metabolism is initiated. Seed development is generally divided into three stages (Kermode and Finch-Savage, 2002; Bewley *et al.*, 2006). Initially, there is the stage of intensive cell division and differentiation, collectively called histodifferentiation, during which there is a steady increase in fresh mass (Fig. 1.2) and the formation of the endosperm and embryo. The second stage involves cell enlargement and expansion, as the major reserves, lipids, starch and storage protein, are laid down in the endosperm and within the embryo. This contributes to a substantial increase in the dry mass of the seed and a decline in the water content, as water is displaced from the cells and replaced by the insoluble polymeric reserves (Fig. 1.2).

The final stage of development in orthodox seeds is characterised by a plateau in the content of dry matter as accumulation is arrested at physiological maturity (Fig. 1.2). The most evident developmental event separating orthodox from recalcitrant seeds occurs at this point, where orthodox, but not recalcitrant, seeds undergo significant (90-95%) water loss during this phase of development - which is referred to as maturation drying (Fig. 1.2). In orthodox seeds the water loss during the third phase of development, *viz*. the maturation phase, would not be possible without the acquisition of desiccation tolerance (Kermode and Finch-Savage, 2002; Moore *et al*., 2009). During this maturation phase, recalcitrant seeds, which have not acquired desiccation tolerance, undergo only a negligible reduction in water content, if at all, (Pammenter and Berjak, 1999a). In orthodox seeds, developmental processes associated with the acquisition of desiccation tolerance begin long before shedding, and, upon maturation drying, the seeds enter a quiescent state, resuming normal metabolism upon imbibition, when (unless the seeds are dormant) environmental conditions promote the initiation of germination (Kermode and Finch-Savage, 2002). In contrast, non-orthodox seeds remain metabolically active and hydrated throughout their development and generally do not require

the availability of suitable environmental conditions before initiating germinative metabolism (Berjak *et al.*, 1989; Finch-Savage, 1996; Pritchard *et al.*, 2004).

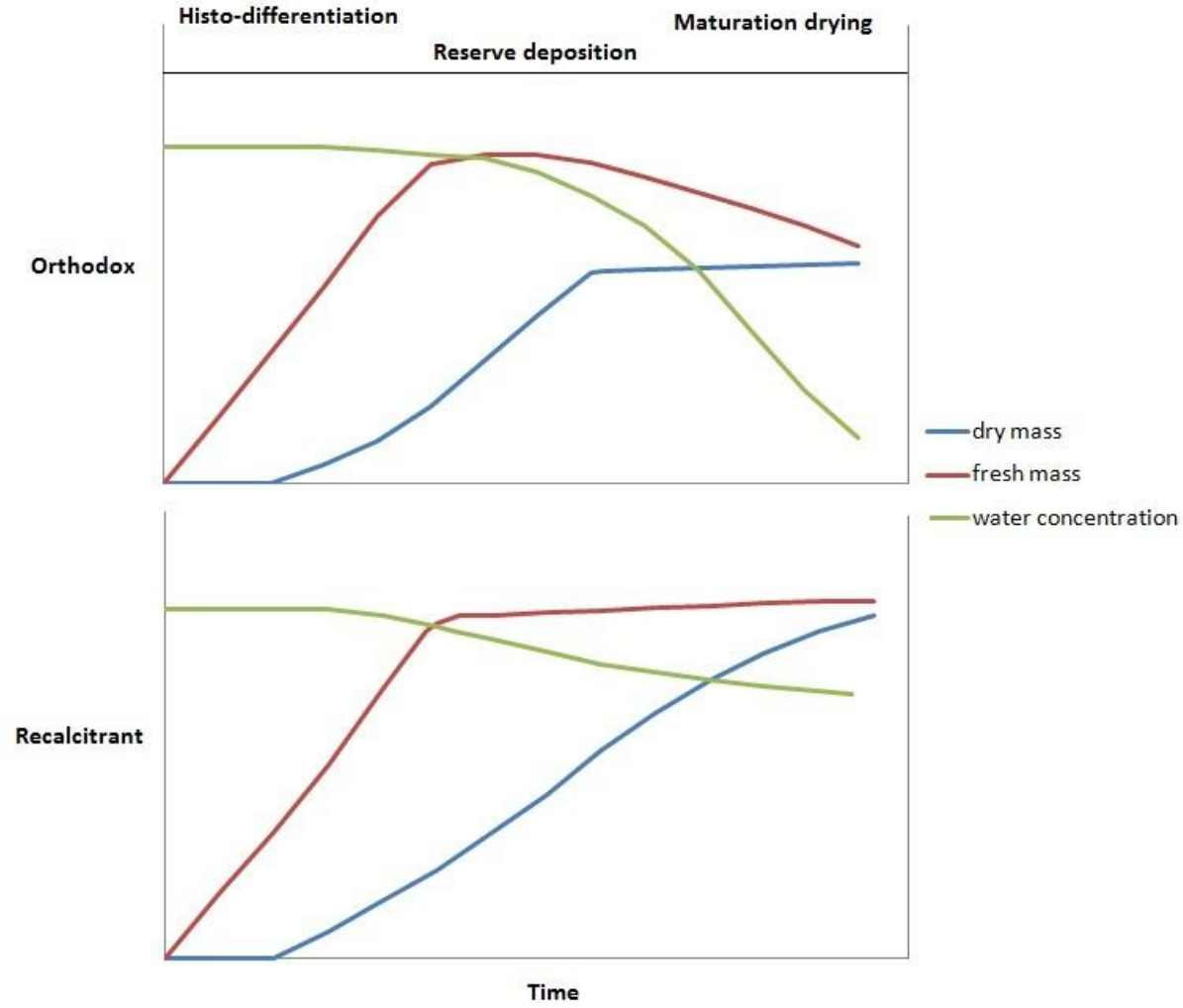


Figure 1.2: Patterns of seed development showing the changes in whole-seed fresh mass, dry mass and water content during the various stages of orthodox and recalcitrant seed development (diagram courtesy of Patricia Berjak, modified from Berjak, 2000).

1.4. Desiccation Tolerance in Seeds

Desiccation tolerance is a phenomenon which is not unusual in the plant kingdom; occurring in ferns, mosses and their spores, pollen, vegetative parts of angiosperms known as resurrection plants (Hoekstra *et al.*, 2001; Berjak, 2006), and in seeds of many higher plant species but never in gymnosperms (Illing *et al.*, 2005; Leprince and Buitink, 2007). Seed desiccation tolerance is a fundamental property of orthodox-seeded species and is considered

necessary for the completion of the life cycle of the plant (Pammenter and Berjak, 1999a). This is the feature distinguishing orthodox seeds from recalcitrant ones, and is exhibited to various degrees in intermediate seeds (Ellis *et al.*, 1990; 1991). In orthodox seeds, desiccation tolerance is completely acquired at seed maturity (Buitink *et al.*, 2003), but recalcitrant seeds are known to remain desiccation sensitive throughout their development and after they are shed from the parent plant (Vertucci and Farrant, 1995; Berjak and Pammenter, 2008). However, the recalcitrant seeds of some species are suspected to develop a certain (very limited) level of tolerance to desiccation, but not approximating to the extent of intermediate or orthodox seeds (Pammenter and Berjak, 1999b; Liang and Sun, 2000). This may explain how recalcitrant seeds of *Acer pseudoplatanus* (Hong and Ellis, 1990), *Aesculus hippocastanum* (Tompsett and Pritchard, 1993) *Quercus robur* (Finch-Savage and Blake, 1994), *Machilus thunbergii* (Lin and Chen, 1995) and *Ekebergia capensis* (Bharuth, 2011) tolerate some degree of water loss (in relation to their dry mass) during maturation.

Studies over the past two decades have suggested that several mechanisms are involved in desiccation tolerance, and that tolerance is a multifactorial phenomenon, in which each component is equally critical, as suggested by Leprince *et al.* (1993). The trait of desiccation tolerance in seeds is known to be the outcome of the interaction of a spectrum of properties (Pammenter and Berjak, 1999a) that are under genetic control, and are best described as intrinsic cell characteristics (Walters *et al*., 2005). Therefore, arguably, desiccation sensitivity is said to be a qualitative feature, dependent on the expression, or lack thereof, of the properties conferring tolerance (Kermode and Finch-Savage, 2002). However, the absence of particular features is likely to be different across recalcitrant-seeded species (Berjak and Pammenter, 2008). The stresses associated with loss of a significant proportion of water include the mechanical stress associated with turgor loss leading to the interruption of the normal metabolic functions of the cell, oxidative stress from free radical-mediated processes and the destabilisation or loss of macromolecular integrity (Vertucci and Farrant, 1995; Illing *et al.*, 2005). Those groups of authors suggest that mechanisms that confer desiccation tolerance facilitate the protection, and ultimately the repair necessary to avoid/overcome the stresses associated with desiccation. Berjak (2006) further stressed that desiccation tolerance involves not only the facility to survive extreme water loss, but also the ability to survive for prolonged periods in such a dehydrated state. In seeds, desiccation tolerance is believed to be based on the induction of several relatively complex protection mechanisms preceding early

before and during drying, with minimal reliance on the repair mechanisms of desiccation-induced damage during rehydration (Vertucci and Farrant, 1995; Illing *et al.*, 2005). These mechanisms include the synthesis and subsequent accumulation of sugars (sucrose and raffinose family oligosaccharides [RFOs]). The accumulation of such components was originally suggested to be important for stabilising membranes (Leopold and Vertucci, 1986) and more recently, to hinder the close approach of membranes to one another (Koster and Bryant, 2005; Halperin and Koster, 2006); and together with the presence of proteins and other macromolecules, are known to contribute towards vitrification of the cytoplasm (i.e. glassy state formation) during dehydration (Berjak, 2006; Buitink and Leprince, 2008). Intracellular vitrification is held to maintain subcellular integrity in the dry state (Illing *et al.*, 2005).

The desiccation sensitivity of recalcitrant seeds appears to be the outcome of either the absence, or poor expression, of these, and other, protective mechanisms (Berjak and Pammenter, 2008); findings supporting this suggestion are reviewed below.

1.4.1. Mechanisms involved in desiccation tolerance

Water is an intricate part of cells, not only as a solvent for biochemical reactions but also as a stabiliser of macromolecular structures. The hydrophilic and hydrophobic interactions that impose structure to macromolecules and organelles within cells require protection when a cell dehydrates. Membrane structure, which particularly depends on these complex interactions, is often regarded as a primary site of desiccation damage (Koster and Leopold, 1988) and therefore protective mechanisms in desiccation tolerant seeds are geared to avoiding loss of membrane integrity during dehydration rather than investing in subsequent repair mechanisms during rehydration (Oliver and Bewley, 1997; Illing *et al.*, 2005). Some of the main protection mechanisms considered important include the following (Berjak *et al.*, 2007; Berjak and Pammenter, 2008; Farrant *et al.*, 2012):

i. Intracellular physical characteristics changing to accommodate the reduction in the availability of water within the cells, particularly by the
   a) reduction of the degree of vacuolation,
   b) amount and nature of insoluble reserves accumulated, particularly sucrose and certain raffinose family/series oligosaccharides during maturation drying (Koster and

Leopold, 1988; Leprince *et al*., 1993; Obendorf, 1997; Hoekstra *et al.*, 2001; Leprince and Buitink, 2010), which contribute towards formation of a glass during dehydration (Leopold *et al*., 1994). The sugars are also thought to hinder the close approach of membranes to one another and alter the physical properties of dry membranes so that they resemble those of fully hydrated biomolecules (Koster and Bryant, 2005; Halperin and Koster, 2006).

c) reaction of the cytoskeleton (i.e. orderly dismantling of the cytoskeletal elements; Faria *et al.,* 2005), and
d) conformation of the DNA, chromatin and nuclear architecture (i.e. for the protection of the integrity of genetic information);

ii. Intracellular de-differentiation, which effectively results in the minimisation of surface areas of membranes and also of the cytoskeleton (Pammenter and Berjak, 1999a);

iii. 'Switching off' of metabolism at physiological maturity (i.e. quiescence), until conditions favouring germination are prevalent (Rogerson and Matthews, 1977; Leprince *et al.*, 2000);

iv. Presence and efficient operation of antioxidants such as ascorbate, glutathione and tocopherol, and enzymes such as peroxiredoxins, peroxidases (ascorbate peroxidase, glutathione peroxidase, thioredoxin peroxidase, catalase), glutathione reductase and superoxide dismutase *inter alia* (Pammenter and Berjak, 1999a);

v. Accumulation and roles of putatively protective (stress-associated) molecules, including the late embryogenic abundant ([LEA], Manfre *et al.*, 2006) and heat shock proteins ([HSPs], Kermode and Finch-Savage, 2002; Buitink *et al.*, 2006).

Apart from retaining subcellular integrity during drying, desiccation tolerant plant tissues also possess mechanisms to repair desiccation-induced damage during subsequent rehydration (Oliver, 2006). Evidence of the existence of such mechanisms derives mainly from studies on desiccation tolerant vegetative tissues, particularly those of resurrection plants (Illing *et al.*, 2005; Farrant *et al.*, 2012). In fact, molecular studies on these plants have revealed the

presence of genes associated with intracellular repair, e.g. those that, when expressed, result in the degradation of damaged proteins; such genes are activated upon rehydration and some have been shown to be expressed even later, during germination (Oliver, 1991). These genes code for proteins generally called the rehydrins (Bewley *et al.*, 2006). Evidentially, Cooper and Farrant (2002) showed that survival of *Craterostigma wilmsii* (a resurrection plant) was compromised when transcription and translation of some rehydrins were inhibited by distamycin A or cyclohemixide, respectively, during and after the rehydration stage of recovery from rapid dehydration. DNA repair processes are also present in seeds and contribute towards maintaining the genetic integrity of cells during the desiccation and rehydration cycles. The extent of repair that occurs depends on the presence and operation of repair mechanisms during rehydration, which, when blocked, can result in the loss of viability of the seed or plant (Cooper and Farrant, 2002).

1.4.2. Recalcitrance in relation to mechanisms conferring desiccation tolerance

Recalcitrant seeds never become quiescent, even though respiration rates are suggested to decline to some extent in a number of more tolerant, temperate recalcitrant species (Kermode and Finch-Savage, 2002); nevertheless they retain metabolic activity throughout development. In evolutionary terms, it is still not known whether the ability to develop full desiccation tolerance in recalcitrant seeds was never gained at all, or whether desiccation sensitivity is a result of the lack of full expression of desiccation tolerance (Kermode and Finch-Savage, 2002). No simple consistent relationship has emerged from the literature between seed type and total sugar content or sucrose level in the context of recalcitrant and orthodox seeds (e.g. Steadman *et al.*, 1996); however, recalcitrant seeds generally have a lower content of raffinose and stachyose (Greggains *et al.*, 2000) than orthodox seeds, which has been suggested to contribute to the lack of tolerance displayed in those of some species. Pammenter and Berjak (1999a) suggest that the inconsistencies may be because the proposed mechanisms for the involvement of sugars in desiccation tolerance operate at water contents below those at which recalcitrant seeds can survive. There is a range in the degree of reserve accumulation in recalcitrant seeds; particularly the extent of vacuolation is correlated with the amount of water loss tolerated before viability is lost (Vertucci and Farrant, 1995). Recalcitrant seeds do not undergo extensive reductions in vacuolation as do orthodox seeds during development, or de-differentiation of organelles, or the shut-down of metabolism that are necessary for desiccation tolerance (Pammenter and Berjak, 1999b). However, other

processes like those involving LEAs (generally represented by dehydrins in the earlier studies) do occur in some recalcitrant seeds, but not in an analogous manner as in orthodox seeds. For example, dehydrin proteins could not be detected in mature embryos of various recalcitrant-seeded species (undried), even though these proteins could be detected during seed development and in response to drying (Farrant *et al.*, 1996; Greggains *et al.*, 2000). Furthermore, LEA proteins and HSPs have been detected in some recalcitrant seeds (Collada *et al.*, 1997; Han *et al.*, 1997) but not to the degree of expression found in orthodox seeds. Some studies have shown that recalcitrant seeds fail to reconstitute their cytoskeletal elements following damaging degrees of dehydration (e.g. in the embryonic axes of *Quercus robur* [Mycock *et al.*, 2000] and *Trichilia dregeana* [Gumede *et al.*, 2003]) while cytoskeleton re-assembly upon rehydration occurs in orthodox seeds (e.g. in *Medicago truncatula* [Faria *et al.*, 2004]) (Berjak and Pammenter, 2008).

The protective mechanisms can be either constitutive or inducible (Leprince *et al.*, 1993; Pammenter and Berjak, 1999a); desiccation sensitivity is, therefore, considered to result from the absence or inadequate or non-expression of these mechanisms (Greggains *et al.*, 2000). Some of the protection and repair mechanisms described above will be elaborated as they become relevant later in this study. The degree of tolerance is said to differ across species depending on the variable expression of genes, but this variation may also exist within species for seeds of different provenances (e.g. *Azadirachta indica*). Interestingly, depending on the provenance of the parent trees, the seeds of *A. indica* have been variously characterised as orthodox, intermediate or recalcitrant (Berjak and Pammenter, 1997). However, the possibility does exist that intra-species variability in desiccation tolerance may be due to differences in the genetic make-up of parent plants resulting from natural selection under different environmental conditions (Bharuth, 2011). Sufficient evidence has now been gathered to show that the response to desiccation is not solely dependent on the inherent characteristics of the species or the developmental status of the seeds, but that it also depends on the conditions under which they are dried, with the rate of dehydration being a major contributor (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999a; Pammenter *et al*., 2002).

Vertucci and Farrant (1995) pointed out that at different cell-hydration-levels the water in seeds has distinctly different properties and that different chemical and metabolic processes can occur at each of these different levels. When water is removed from the tissues the

processes occurring at that specific hydration level are disrupted and may no longer occur, including protective functions; this may result in damage unless the seed has the appropriate tolerance mechanisms required for survival (Pammenter and Berjak, 1999a). Such effects are exacerbated as more water is removed. Although repair does occur in desiccation-tolerant seeds during rehydration (Cooper and Farrant, 2002; Bewley *et al.*. 2006), Oliver and Bewley (1997) emphasised that they rely more on protective mechanisms during water loss than on repair processes following rehydration for their ultimate retention of viability. The lack of some of these protective mechanisms in desiccation-sensitive seeds greatly informs their recalcitrance (Greggains *et al.*, 2000) to conventional storage practices, which then leaves cryopreservation as the next best alternative for long-term conservation.

1.5. Cryopreservation and cryo-recalcitrance

Conventional cryopreservation protocols often include the use of cryoprotective additives (CPAs), and partial physical dehydration of tissues before exposure to ultra-low cooling temperatures, usually in LN (-196°C) (Muldew *et al.*, 2004; Panis and Lambardi, 2005). These measures result in the arrest of cellular metabolic functions and should limit the damage caused by intracellular ice formation induced in partially-hydrated plant tissues during cooling and subsequent cryogenic storage (Mycock *et al.*, 1995; Sakai, 2004). At the ultra-low temperatures maintained during cryopreservation (-140 to -160°C [vapour phase over LN] or -196°C in LN), all cellular divisions and metabolic processes, and therefore biological 'ageing', are theoretically stopped indefinitely (Kartha, 1981; Engelmann, 2004). While this is mainly attributed to the fact that at such temperatures there is insufficient thermal energy for chemical reactions (McGee and Martin, 1962), Mazur (1984) further indicates that liquid water does not exist below -130°C and that the only physical states that can exist at such extremes are crystalline or glassy, as viscosity is so high ($>10^{13}$ poises). Under these conditions plant material can then be assumed to be able to be stored without alteration or modification for an unlimited period of time. However, Walters *et al.* (2009) suggest that this assumption may not be accurate, as there is evidence that even dry orthodox seeds can deteriorate during cryogenic storage as a consequence of low levels of damage accumulating over many years. Nevertheless, cryopreservation allows for the storage of large volumes of germplasm accessions (whether as orthodox seeds or explants of recalcitrant-

seeded species) in a small volume of space with minimum maintenance, whilst protecting them from contamination (Radha *et al.*, 2012).

Intact recalcitrant seeds are generally large and highly hydrated, and as such, cannot be cryostored (Berjak and Pammenter, 2008). Consequently, segments of the material – e.g. embryonic axes, monocot embryos (if sufficiently small) or vegetative buds need to be excised to obtain the smallest possible specimens for cryopreservation (see below). Such specimens are collectively called explants. Successful cryopreservation of plant tissues depends on the inherent tolerance of the material to the stresses induced by the preconditioning procedures (e.g. explant excision and partial dehydration) and exposure to cryogenic temperatures. Embryos/embryonic axes excised from recalcitrant seeds (recalcitrant germplasm) are known to be sensitive to even slight drying and often also to chilling (Berjak and Pammenter, 2004b; 2008; Pritchard *et al.*, 2004), and these properties alone may constitute a major challenge in developing a successful cryopreservation protocol for particular species – and may even render some germplasm unstorable (Radha *et al.*, 2012). There are a number of different approaches currently pursued to develop practical cryostorage protocols for such recalcitrant germplasm (e.g. Walters *et al.*, 2008; Normah *et al.*, 2011).

The term 'cryo-recalcitrance' (a contraction of 'cryostorage recalcitrance' [Benson, 2008]) is used to describe germplasm that is intractable to storage in a cryobank, i.e. displaying responses that are not conducive to cryogenic storage. This may be attributed to the intrinsic characteristics of the explant or it may be due to sub-optimal culture and cryogenic manipulations, which may be corrected through further optimisation (Withers, 1985a, b; Yoon *et al.*, 2006; Benson, 2008). The intrinsic traits may result from adaptations that would normally confer advantages in the native habitat but which become a disadvantage in facilitating the survival of germplasm in a cryobank (Benson, 2008). The defining problem of intrinsic storage recalcitrance is that the innate adaptive properties of the germplasm appear to be consistently incompatible with cryopreservation. Germplasm can also become storage-recalcitrant through physiological factors such as *in vitro* ageing when explants are derived from long-cultured stock material (Benson 2000a, b) and endophytic contamination (Benson, 2008). A wider definition of recalcitrance in the context of cryostorage proposed by Benson

(2008) is that it is the inability of germplasm to cope with the critical factors that dictate whether or not it can be stored in a viable state and retrieved fit-for-purpose from cryobanks.

The response of cells to cryopreservation – specifically, but not exclusively those of plant tissues – is intricately affected by the type and size of explant, the initial and final water contents (WCs) after dehydration, the rate of water loss to achieve dehydration, the rate of cooling and re-warming, and, if used, the type and concentration of CPA (Benson, 1999; Reed, 2001; Sakai, 2004; Panis and Lambardi, 2005). Although successful cryopreservation of germplasm derived from recalcitrant seeds has been quite limited, success has been reported for some species (e.g. embryonic axes of *Hevea brasiliensis* [Normah *et al.*, 1986] and *Camellia sinensis* [Chaudhury *et al.*, 1991; Wesley-Smith *et al.*, 1992], and the zygotic axes of *Quercus robur*, [Berjak *et al.*, 1999]. As a *caveat* though, *H. brasiliensis* seeds have recently been suggested to be of the intermediate variety [Normah *et al.*, 2011] and both *C. sinensis* and *Q. robur* are of a temperate provenance, which as a 'group', appear more amenable to cryopreservation than is material of tropical provenances [Berjak and Pammenter, 2013]). This notwithstanding, germplasm of some species/varieties seems to be distinctly cryo-recalcitrant (Benson, 2008; Reed, 2008). The lack of success, measured by the loss of viability/lack of survival, is widely accredited to the mechanical and physiological stresses caused by the various procedural steps involved in the cryopreservation of initially-hydrated plant tissues, which have a detrimental effect on the structural and biochemical integrity of cells (Lane, 2004; Sershen *et al.*, 2007). The degree of interference with metabolic processes induced by oxidative stress and disruption of intracellular integrity varies, depending on the characteristics of the cells of individual explants (Sakai, 2004).

1.6. Factors influencing cryopreservation success of germplasm of recalcitrant-seeded species

The optimisation of any cryopreservation protocol, particularly for plant germplasm representative of recalcitrant-seeded species, begins with the selection of a suitable explant. According to Keller and Senula (2010), the chosen explant is usually preconditioned via the use of CPAs or partial dehydration (induced physically) or the combination of CPA and partial dehydration by physical means, before cooling to cryogenic temperatures. Furthermore, procedures upon retrieval of explants from cryogenic storage are also of significant importance. To date, protocols have emerged as being largely species-specific

(Kaviani, 2011); this means that a protocol that may work for one species may not work for another, no matter how closely related they may be (Dussert *et al.*, 1998; Sershen, 2006; Sershen *et al*., 2007). Water content when seeds are shed, for example, is said to be intra- and inter-seasonally variable within a species, and so should be considered anew for each accession (Berjak *et al.*, 1990; Sershen *et al.*, 2008). Factors that influence the survival of germplasm after cryopreservation include the developmental status and health of the explant, the personnel and culture/treatment conditions and facilities (Kaviani, 2011) - some of these generic factors/procedures are reviewed below.

1.6.1. Explant type and size

An explant is a segment of plant tissue that can be regenerated in culture and has the capacity to develop into a new plant. The most ideal structure for germplasm conservation would be the intact seed as it contains the embryo tissue that gives rise to a genetically representative offspring of both female and male parent plants, and the nutritive reserves that will support germination and seedling establishment. The difficulties associated with cryopreserving recalcitrant seeds begin with their unsuitably large size, and the fact that they are too hydrated to facilitate the rapid dehydration and cooling rates required to minimise the physical and metabolic stresses induced by exposure to cryogenic temperatures (Berjak and Pammenter, 2008). This, then, leaves excised embryos or embryonic axes as the most suitable explant-types for cryopreservation as they contain all the necessary genetic information for development of the next generation (Bajaj, 1984; Berjak, 1989; Radhamani and Chandel, 1992; Normah *et al.*, 1994; Makeen *et al.*, 2005; Pammenter *et al.*, 2011).

In some cases though, especially in recalcitrant seeded species, the embryonic tissues are recalcitrant to cryostorage and, although this may be overcome by ameliorative treatments (Berjak *et al.*, 2011b), in such cases vegetative explants such as shoot tips may represent the best alternative. Explants are categorised depending on whether they contain undifferentiated or differentiated cells. Differentiated tissues are considered to be more genetically stable than those that are undifferentiated, as the latter are more susceptible to somaclonal variation (Ashmore, 1997; Reed, 2001). Many classical cryopreservation protocols have achieved success with undifferentiated cell suspensions and callus (Kartha and Engelmann, 1994). Fay (1992) defines an ideal explant as one extracted from a normal/healthy, true-to-type donor plant that is vigorous and disease free. Recent protocols most often use differentiated tissues

and organs for germplasm conservation, i.e. embryonic axes, shoot tips and zygotic/somatic embryos (Rao, 2004). In particular, meristems, whether those of axes or shoot apices, have the ideal characteristics (compact cells with a large nuclear:cytoplasmic ratio) for cryopreservation (Engelmann, 2011). However, undifferentiated tissues, such as callus, still remain an alternative (although the least desirable in terms of genetic diversity conservation) for cryopreservation where success is unachievable for differentiated tissues (Kaviani, 2011; Engelmann, 2011).

Studies have shown that the use of the smallest explant capable of onwards development is best for maximizing the rapid dehydration and cooling rates necessary for limiting the damage associated with the procedural steps involved in cryopreservation: this is particularly the case for desiccation-sensitive material (Muldew *et al.*, 2004; Hor *et al.*, 2005). However, the excision necessary for the acquisition of the explant of interest in most cases involves trimming away superfluous seed tissues (e.g. cotyledons). This wounding injury inflicted on the explants during their excision appears to precondition the explants to even greater damage during the subsequent procedural steps, compromising their post-cryo recovery (Naidoo *et al.*, 2011; Pammenter *et al.*, 2011). However, besides having a meristem at each pole, embryonic axes and embryos are particularly complex structures with a heterogeneous cellular composition, and require protection to ensure preservation of their structural integrity (Gonzalez-Arnao *et al.*, 2008). Severing of the seed tissues surrounding these structures also often makes them more vulnerable to stresses associated with the chemical decontamination necessary for contamination-free *in vitro* culture (Paunescu, 2009). Although rare, some success has been achieved with cryopreserving whole non-orthodox seeds, although these were probably of intermediate status (e.g. those of neem [*Azadirachta indica*; Chaudhury and Chandel, 1991] and African pepperbark [*Warburgia salutaris*; Kioko *et al.*, 1999; 2000]).

1.6.2. Explant developmental status

If seeds undergo stress before they acquire the appropriate mechanisms to withstand that stress, their intolerance is often seen in their loss of vigour and if the stress is severe or persists, the effects can result in the loss of viability (Pammenter and Berjak, 1999a). This explains why even orthodox seeds do not withstand desiccation if they are dried when immature (Dasgupta *et al*., 1982; Fischer *et al*., 1988; Hong and Ellis, 1992; Ellis *et al*., 1993). A seed known ultimately to have the capacity to tolerate desiccation will often not

germinate and may die if dried before reaching the desiccation tolerant stage of its development (Koster and Leopold, 1988; Nedeva and Nikolova, 1997). Orthodox seeds also do not survive if they are dried after germination has progressed too far, as they tend to lose desiccation tolerance as seedling establishment progresses (Buitink *et al.*, 2006) and their response to dehydration then resembles that of recalcitrant zygotic embryos (Koster and Leopold, 1988).

Goveia *et al*. (2004), when working on the recalcitrant seeds of *Trichilia dregeana*, also suggested that the developmental stage of excised recalcitrant embryonic axes plays a critical role in promoting their post-cryo survival. Those authors reported that storing the hydrated seeds of *T. dregeana* for at least 6 months before axis excision, allowed for the shoot apex to elongate such that the apical meristem was no longer in such close proximity to the excision wounds inflicted when freeing it from the cotyledonary tissue. Berjak *et al.* (2011b) reported more successful cryopreservation when working with axes from 35-d stored endospermous recalcitrant seeds of *Strychnos gerrardii* than when the material was newly-harvested. However, the *caveat* is that the seeds must be in the best condition, as, if sub-standard, axes do not respond well if excised from stored rather than fresh seeds[1]. In some cases, it has been noted that immature, but germinable, axes do not survive the cryopreservation procedures (Vertucci *et al.*, 1995), while in other cases some will survive but show lower germination totalities than mature axes (von Fintel *et al.*, 2004). Some authors have indicated that the lack of survival may be due to the physiological characteristics of the seeds (Berjak *et al.*, 2000) that change with development: these could include the inherent oligosaccharide content which potentially could contribute to the cryoprotection of cells during cryopreservation (Wesley-Smith *et al.*, 1995).

1.6.3. Water content and drying rate

The water content of explants at the time of exposure to the cryogen is critical to their survival of cryopreservation (Wesley-Smith *et al.*, 1995). This is because cryogenic temperatures are well below the freezing point of water and therefore induce rapid ice crystallisation in solution upon exposure, which could result in lethal freezing damage in

[1] Information from current experiments on *Trichilia dregeana* by Drs Sershen and Varghese, School of Life Science, University of KwaZulu-Natal (Westville Campus), Durban, South Africa.

tissues (Wesley-Smith *et al.*, 2001b). For this reason it is important to reduce the water content of hydrated plant tissues, via partial dehydration, before exposing the explants to the cryogen.

Most hydrated plant cells are sensitive to the excessive removal of water (as explained above in section 1.3.1.1), and those of recalcitrant seeds/embryos/axes are particularly so. Water plays an important role in controlling metabolism, since it is involved in most biological reactions as either a reactant or product. Among the other biological functions of water within cells, it also affects the structural attributes of macromolecules (Vertucci, 1990; Vertucci and Farrant, 1995). Water provides turgor and fills intermolecular spaces and also allows for hydrophilic and hydrophobic associations at the molecular level, controlling intermolecular distances that determine the conformation of proteins and polar lipids; water is also involved in partitioning molecules within organelles and limiting reactivity among metal ions (Ntuli and Pammenter, 2009). Water content is the most important factor affecting the ability of germplasm to survive dehydration and cooling, which are intrinsic to cryopreservation protocols (Stanwood, 1985). Further to this, the rate (Pammenter *et al.*, 1998) and temperature (Berjak *et al*., 1994; Ntuli *et al*., 1997) at which water is removed from the cells during dehydration, and also the degree to which the structure-associated (non-freezable) water fraction of the cells is impinged upon, are important considerations when optimising a cryopreservation protocol (Berjak and Pammenter, 2013).

The formation of ice in pure water becomes energetically favourable when water at atmospheric pressure is cooled to below 0°C. In order for ice formation to occur, however, a group of water molecules has to become arranged in a stable crystalline nucleus (Hobbs, 1974). Once a stable ice nucleus is formed, additional molecules of water will attach and the crystal will grow as long as the temperature remains below the equilibrium melting point. The formation of the initial crystal nucleus is stochastic (Turnbull, 1956; Angell, 2002), resulting from random motion of the liquid molecules. Nucleation becomes increasingly likely as temperature decreases because the number of molecules that have to coordinate to form a stable crystal nucleus decreases with decreasing temperature. Usually the formation of a crystal nucleus is abetted by the presence of a non-water molecule that imposes a certain amount of local order on the surrounding water molecules, thereby increasing the probability that they will form an ordered crystal (Turnbull, 1962). These types of objects are

heterogenous nucleators, and they usually cause ice to form at temperatures between -10 and -18°C (Franks, 1985). Even if no heterogeneous nucleators are present, however, a nucleating crystal will form by random action of the water molecules at about -40°C in most circumstances (Griffith and Antikainen, 1996). If the water is able to reach a temperature of about -138°C without crystal formation, then a glass phase-transition occurs and the water will remain in an amorphous solid state for as long as it is held below the glass-phase transition temperature (Chen, 2000). The ability to reach -138°C without crystal ice formation should provide the perfect cryopreservation method. Glass formation is a second-order phase transition in which the specific heat and the viscosity of the substance change significantly. The viscosity of glass is so great that inter-molecular relaxation and diffusion will not occur in ordinary laboratory time scales (Kalogeras and Lobland, 2012). In a glass, crystals do not form, even though they are energetically favourable, because the molecules are no longer free to arrange themselves into a crystal structure (Mauro *et al.*, 2009). In such cases, chemical reactions, such as those necessary for cellular degradation, become virtually impossible due to molecular immobility.

Water within cells can be differentiated into free (solution/bulk/unbound/freezable) and structure-associated (non-bulk/bound/non-freezable) water (Meryman, 1974; Cameron *et al.*, 1997; Feig, 2010). As the water content decreases in cells, the interactions between water and the solutes become stronger and the concentration of the solutes increases. If more water is removed from the system, the cytoplasm becomes sufficiently concentrated and viscous and develops properties of a glass (Leopold and Vertucci, 1986; Koster, 1991). A glass, in this respect, is the metastable state of water that resembles a solid, brittle material that retains the disorder and physical properties of a liquid (Franks, 1985; Franks *et al.*, 1991; Walters, 1998). If extensive dehydration is necessary, the water content of the cells can be reduced to levels at which all the remaining water is of the fraction that is tightly associated with macromolecular surfaces (bound/non-freezable water) and its mobility is reduced (Vertucci, 1990). If it can be achieved, this would be the ideal to obviate freezing injury during cryopreservation of desiccation-sensitive axes.

Desiccation damage occurs when water that is critical for survival is removed from cells (Walters *et al.*, 2001). Two types of cellular damage can be induced by the removal of water from plant tissues (Vertucci and Farrant, 1995; Pammenter and Berjak, 1999a; Walters *et al.*,

2001): metabolism-induced damage and desiccation damage *sensu stricto*. Desiccation damage *sensu stricto* is said to occur at low water contents (Walters *et al.*, 2001). The damage associated with the removal of water at high water contents is induced mainly by a reduction in cell volume which exerts a number of mechanical stresses on the cell contents (Levitt, 1980; Vertucci and Farrant, 1995). Desiccation damage at low water contents includes the damage that occurs when the water required to maintain the integrity of intracellular structures is removed (Pammenter *et al.*, 1991; Wolfe and Bryant, 1999; Walters *et al.*, 2001). In rapidly-dehydrated recalcitrant tea (*Camellia sinensis*) axes such damage occurred at water contents below 0.5 g g^{-1} dmb (g H_2O per g of dry matter, dry mass basis) or at Ψ_w lower than -15MPa and was correlated with the loss of viability (Walters *et al.*, 2001). Metabolism-induced damage is held to occur at intermediate water contents (Ψ_w between -5 and -15MPa [Pammenter *et al.*, 1998; Leprince *et al.*, 2000; Walters *et al.*, 2001]). The damage that occurs at intermediate water contents is related to the failure to regulate co-ordinated metabolic functions within the cells, resulting in imbalanced metabolism, leading to aqueous-based degradative processes that are probably mediated by free radicals (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999a; Walters *et al.*, 2001; 2002a). Because the natural, protective antioxidant systems may also fail at such reduced water contents, damage resulting through these processes can be highly detrimental (Finch-Savage *et al.*, 1994). Failure of the antioxidant systems on slow water loss from recalcitrant embryonic axes of *Trichilia dregeana*, has been shown to be a major factor in metabolism-linked damage (Varghese *et al.*, 2011).

Water is known to have distinctly different properties at different hydration levels in seed tissues, which translates into the different chemical and metabolic processes that can occur at these levels (Vertucci and Farrant, 1995). Pammenter and Berjak (1999a) stress that, "…no matter how rapidly desiccation-sensitive tissue is dried, there is a lower limit below which it cannot survive". Generally this limit is not lower than the water content at which only the non-freezable fraction of water remains (Pammenter *et al.*, 1993; Pritchard and Manger, 1998). This led to the suggestion that truly recalcitrant seeds, or recalcitrant-seed-derived germplasm, cannot survive the removal of structure-associated water (Pammenter and Berjak, 1999a; Berjak and Pammenter, 2013).

Desiccation rates influence the extent of water loss that recalcitrant seeds, embryos/embryonic axes can tolerate. Excised embryonic axes from non-orthodox seeds survive cryogenic storage at recovery levels commensurate with the ability to survive transiently under ambient conditions after rapid (Berjak *et al*., 1993; Pammenter and Berjak 1999a; Makeen *et al.* 2005), or ultra-rapid drying (Berjak *et al.* 1990; Vertucci *et al.* 1991; Berjak *et al.* 1999; Walters *et al.* 2001, 2002a; Pammenter *et al.*, 2002). Under slow drying conditions plant tissues remain at intermediate water contents (Ψ_w between -5 and -15MPa) for prolonged periods, permitting damage associated with unbalanced metabolism to increase. As a consequence of the time taken to dry hydrated seed tissues slowly (e.g. intact seeds dried over silica gel [Pammenter *et al.*, 1998]), each water potential range is likely to contribute to the overall assessment of the effects of drying (Walters *et al.*, 2001), and such drying rates are unlikely to be tolerated by most desiccation-sensitive material. The axes of tea (*C. sinensis*), for example, lost viability at water potentials between -3 and -15 MPa when dried slowly, but viability loss did not commence until water potentials well below -15 MPa were reached, when dried rapidly (Walters *et al.*, 2001). In light of the above, recent cryopreservation protocols for recalcitrant seed germplasm employ partial dehydration methods that facilitate as rapid removal of water from the tissues as can be achieved, so that the cells pass through the intermediate water content ranges as quickly as possible, thus reducing the damage incurred (e.g. Farrant *et al.*, 1993; Kioko *et al.*, 1998; Pritchard and Manger, 1998; Pammenter *et al.*, 1999; 2002). Flash-drying (as originally reported by Berjak *et al.*, 1990) is thus far the most efficient of these methods and involves drying naked explants in a stream of dry air. However, the actual rate of water loss is also governed by morphological and histological attributes of the explants themselves. For example, far better survival of embryos of *Amaryllis belladonna* than of *Haemanthus montanus* was achieved, which was attributed – at least partially – to the very rapid rate of water loss of the former, as opposed to the extended flash-drying period necessary for embryos of *H. montanus* to attain similarly low water contents (Sershen *et al.*, 2012a; b).

Dehydration, alone, or in combination with cryoprotection, has long been known to reduce the amount of free water available for freezing, and in that way enhance the chances of survival after cooling (Mazur, 1984; 2004). Most recent cryo-protocols include techniques such as encapsulation-dehydration, vitrification, encapsulation-vitrification or plain dehydration as strategies to reduce the water content of tissues in order to promote the

formation of glasses during cooling (Engelmann, 2011). However, as these procedures are not relevant to the current investigation, they are not detailed here.

1.6.4. Cryoprotection

Classical cryopreservation techniques include a cryoprotection step, which usually involves the treatment of tissues with a mixture of concentrated penetrating and/or non-penetrating CPAs applied at non-freezing temperatures (McGann, 1978; Engelmann, 2011). This step precedes that of partial dehydration and therefore serves as preconditioning for the drying and cooling step(s) (Kaviani, 2011; Kaczmarczyk *et al.*, 2012). The use of CPAs is aimed at aiding the vitrification of the cytoplasm during dehydration by acting colligatively with other molecules to depress the freezing point of water (Dereuddre and Kaminski, 1992; Mandumpal *et al.*, 2011). This is held to confer protection of cells during dehydration and particularly, to alleviate cooling stress by promoting the formation of a glassy state and preventing/limiting ice nucleation (crystallisation) during cooling and subsequent re-warming (Fahy *et al*., 1984; Panis and Lambardi, 2005).

Cyoprotectants are separated into two groups, *viz.* penetrative and non-penetrative, based on their ability, or not, to penetrate cell membranes, and generally CPA treatments involve mixtures of both types. Examples of penetrative CPAs are dimethyl sulphoxide (DMSO/Me_2SO) and glycerol. Non-penetrative CPAs include substances such as sugars, some sugar alcohols and high molecular weight substances (e.g. polyethylene glycol [PEG]) that are known to induce osmotic dehydration by providing a higher concentration of solutes outside the cells thereby causing movement of water from the inside of the cell to the outside. As water is removed from the cells, the ability of the cytoplasm to become viscous and develop properties of a glass is enhanced (Finkle *et al*., 1985; Leopold and Vertucci, 1986; Koster, 1991), which therefore confers protection and contributes to maintenance of intracellular integrity.

The most commonly used CPAs are DMSO, glycerol, PEG, sucrose, sorbitol and mannitol (Mathur *et al.*, 2003; Moges *et al.*, 2004; Jain, 2011; Kaviani, 2011). CPAs are usually applied in relatively high concentrations; however, overexposure of tissues may cause damage to the cells owing to CPA toxicity in some cases, or the excessive dehydration they can induce in cells (Kaczmarczyk, 2012). Upon penetrating cells, CPAs like DMSO promote

vitrification and confer protection against ice crystallisation, thus contributing to the maintenance of intracellular integrity during the cryopreservation procedures (Kaviani, 2011). Although DMSO is preferred for a broad range of explants as a consequence of its extremely rapid penetration into the cells, concerns about its cytotoxicity often favour the selection of glycerol and other additives (e.g. the amino acid additive, proline) (Withers and King, 1979). One important virtue of glycerol and DMSO as opposed to low-molecular-weight compounds is that they have a large molar volume (Meryman, 2007). The volume of 1 mole of glycerol, for example, will be 40.74 ml, whereas that of one mole of sodium chloride will be 27 ml. This means that intracellular glycerol will occupy a proportionally larger volume than the intracellular salts at any given osmolality (Meryman, 2007). Therefore, as the cell loses water to extracellular ice, the large volume of intracellular cryoprotectant will postpone cell volume reduction to a lower temperature. Additionally, DMSO is popular for its ability to scavenge reactive oxygen species (ROS), particularly hydroxyl radicals (Benson and Withers, 1987; Yu and Quinn, 1994; Fleck *et al.*, 2000; Naidoo *et al.*, 2011). CPAs are usually applied in combination, as in plant vitrification solutions (PVS1 and 2; Sakai *et al.*, 1990), but potentially damaging effects of the high concentrations of components in these solutions are often considered the barrier to successful cryopreservation (Kami *et al.*, 2008). The choice of cryoprotectant(s) is therefore critical and, as emphasised by El-Danasouri and Selman (2005), optimal CPAs are usually those that are of high permeativity (low molecular weight) and low toxicity.

1.6.5. Cooling

Cooling of biological systems, no matter how rapid, will cause some ice crystallisation (Karow, 1969), unless all remaining intracellular water is non-freezable (Vertucci, 1990). (The latter situation, however, is thought to be incompatible with viability retention of desiccation-sensitive embryos/embryonic axes [Pammenter *et al.*, 1993; Pammenter and Berjak, 1999a].) The main challenge to cells during cooling is not just their ability to tolerate storage at cryogenic temperatures, but particularly to counteract the lethality of the intermediate temperatures (from -15 to -60°C) as they are cooled down to, and warmed back from, LN temperature (Mazur, 1984). These intermediate temperatures are known to favour ice formation and growth (Moor, 1973), which is regarded as the main source of cryo-damage. Also, according to Rice (1960), this temperature range allows for photophysical reactions that can result in the formation of free radicals and breaks in macromolecules as a

direct result of hits by background ionising radiation or cosmic rays. While such damage may be negligible, direct 'hits' can produce breaks in, or cause enough DNA damage, to become deleterious after re-warming to physiological temperatures, especially since no enzymatic repair can occur at such low temperatures (Mazur, 1984). Upon cooling, once the cells have passed through the intermediate temperature range, metabolic reactions will not persist, especially at LN temperatures, at which none of the thermally driven reactions can occur (Özkavukcu and Erdemli, 2002).

Cryoprotection and partial dehydration (employed individually or in combination) are therefore necessary in virtually all cases, to promote intracellular vitrification and avoid lethal intracellular ice crystallisation at sub-zero temperatures when cryopreserving hydrated plant tissues (Mycock *et al.*, 1995). Ice crystallisation has been shown to be the predominant cause of intracellular damage during cryopreservation (Moor, 1973; Pearce, 2004; Sakai, 2004), but most contemporary cryobiologists agree that the damage acquired during cryopreservation is due to the effects of both desiccation (with its associated solution effects [Withers and King, 1979] and freezing (Kaviani, 2011). Cryoprotectants increase solute concentration inside the cell and decrease the amount of freezable water available intracellularly (Lovelock, 1953; 1954). Glycerol and DMSO are known to decrease the freezing point of water and many biological fluids by colligative action; glycerol decreases it to ~-46 ºC and DMSO decreases it to ~-73 ºC (Meryman *et al*., 1977). DMSO also protects the fluidity of membranes (Gurtovenko and Anwar, 2007a). Particularly, sugars (trehalose, sucrose) if present intracellularly, and glycerol as a CPA, protect against damage due to excessive water loss (Jochem and Korber, 1987).

Successful cryopreservation protocols entail optimisation of cooling rates in conjunction with explant-tissue hydration level, to eliminate – or at least minimise – nucleation of potentially lethal intracellular ice crystals. These factors are important determinants of cryopreservation success. Conventional cryopreservation protocols have mainly utilised slow (equilibrium) cooling rates (i.e. 0.5 to 2.0°C min^{-1} to ~-40°C [Kartha, 1985]), which favour extracellular ice nucleation and growth (Mazur, 1990) but allow for dehydration during cooling (Karow, 1969); however, the latter can, itself, be detrimental (Pritchard *et al.*, 1995). Mazur (1963; 2004) showed that lethal intracellular freezing could be avoided if cooling was slow enough to permit sufficient water to leave the cell during progressive freezing of the extracellular

fluid (also known as equilibrium cooling). He also indicated that the rate of cooling differs between cells of differing size and water permeability, stating that a typical cooling rate around 1°C min^{-1} is appropriate for many mammalian cells after treatment with cryoprotectants such as glycerol or DMSO, but this rate is not a universal optimum. However, it needs to be pointed out that these observations and recommendations come from work on cell suspensions (generally of animal origin), and not complex tissues such as embryos/axes excised from recalcitrant seeds.

Cooling rates that successfully minimise lethal crystallisation in plant cells still need to be empirically determined (Sershen *et al.*, 2007), but in contrast to the arguments for equilibrium cooling, recent cryopreservation protocols for plant germplasm favour faster cooling rates (i.e. rates greater than 10 to hundreds of °C s^{-1}, Walters *et al.*, 2002b; Wesley-Smith *et al.*, 1992; 2004a; b) that restrict the growth of intracellular ice crystals to non-lethal dimensions (Engelmann, 2004). Luyet *et al*. (1962) were the first to employ rapid (non-equilibrium) cooling rates (hundreds of °C min^{-1}), and showed that intracellular ice formation could be restricted to below lethal levels. Faster cooling rates are also known to promote supercooling of the cell interior and to limit the extent of cellular dehydration incurred (Franks, 1985). As the cooling rates increase, ice crystals become more numerous but very small (Carrington *et al.*, 1996), and may well be uniformly distributed intra- and extracellularly. The small intracellular crystals do have the potential to cause damage due to disruption to the cellular ultrastructure and can lead to cellular death (Karow, 1969); however, their impact on post-cryopreservation survival may be related to their localisation (Wesley-Smith, 2003). Based on their work on recalcitrant embryonic axes, Wesley-Smith and colleagues (2004a;b), also explain that at sufficiently low water contents (if achievable) the high intracellular viscosity slows ice crystallisation, making survival independent of cooling rate. They further highlight that at higher water contents, the reduced viscosity requires faster cooling to prevent ice crystal damage. The ability to cool such explants rapidly with increasing hydration therefore needs to be in balance with an increasing limitation to dissipate heat fast enough to prevent severe damage.

It is imperative that cryostored explants be rapidly re-hydrated. As with rapid cooling, this minimises the time during which explants pass through the temperature range which promotes ice crystallisation. Rapid rates of rehydration have been shown to result in higher

survival and normal onwards development of axes, compared with slow rehydration rates (Wesley-Smith *et al.*, 2004a).

1.7. The problem of oxidative stress during cryopreservation

The unregulated production of reactive oxygen species (ROS) has been implicated as one of the major detrimental stresses leading to the loss of viability of cryopreserved tissues (Roach *et al.*, 2008; Varghese and Naithani, 2009; Whitaker *et al.*, 2010; Pammenter *et al.*, 2011). ROS are normally unavoidably produced as a consequence of aerobic metabolism, and include radicals such as the superoxide anion/radical ($O_2^{\bullet-}$) and the hydroxyl radical ($^{\bullet}OH$), and some non-radical species, e.g. hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Sharma *et al.*, 2012). These are formed by the inevitable leakage of electrons to O_2 from the electron transport chain activities of chloroplasts and mitochondria, and plasma membranes or as a by-product of various metabolic pathways occurring in different compartments of a cell (Foyer and Harbinson, 1994; Del Rio *et al.*, 2006; Blokhina and Fagerstedt, 2010). It has, however, recently emerged that ROS are essential components in cell signal transduction pathways that control key metabolic processes such as growth, development, abiotic and biotic stress responses and programmed cell death (Laloi *et al.*, 2004; Bhattacharjee, 2005; Pitzschke *et al*., 2006) and the ROS-mediated signaling is controlled by a delicate balance between production and scavenging (Halliwell, 2006). However, detrimental imbalances occur under conditions of stress (see below). Since plants are sessile organisms and cannot escape from adverse environmental conditions, their adaptations include an elaborate system to control cellular ROS concentrations (Mittler *et al.*, 2011). In addition, plants have evolved a way to utilise lower concentrations of ROS as signalling molecules for a number of regulated processes during plant growth and development, e.g. cell elongation (Foreman *et al.*, 2003) and differentiation (Tsukagoshi *et al.*, 2010). However, the production of ROS can become unregulated due to disruption of cellular homeostasis and this translates to oxidative stress. Such ROS production has been implicated in salinity stress (Wi et al., 2006), drought stress (Price *et al*., 1989), metal toxicity (Sharma and Dubey, 2007; Mishra *et al*., 2011), UV-B radiation (Han *et al*., 2009) and also as a consequence of chilling (Hu *et al*., 2008).

In healthy aerobic organisms, production of ROS is counter-balanced by antioxidant defence systems (Halliwell, 2006). According to that author, the balance is not perfect, such that ROS-mediated damage occurs continuously and damaged molecular components have to be

repaired or replaced, e.g. those of DNA and proteins. Oxidative stress refers to the situation of substantial imbalance between the production of ROS/reactive nitrogen species (RNS) and antioxidant defences (Halliwell, 2006). This can better be explained as the disturbance in the pro-oxidant–antioxidant balance in favour of the pro-oxidants, leading to the potential for damage (Sies, 1991). The ROS can cause serious oxidative damage to membrane lipids, proteins and nucleic acids if not regulated. In some studies on recalcitrant seeds (e.g. those of *Castanea sativa*, *Trichilia dregeana*, *Aesculus hippocastanum* and *Quercus* spp.), oxidative damage has been shown to contribute significantly to the loss of viability during and after drying (Finch-Savage *et al.*, 1994; 1996; Cheng and Song, 2008; Roach, 2009; Whitaker *et al*., 2010). This is primarily attributed to the increased production of ROS in conjunction with a decline in cellular antioxidant activity, which has also been shown to occur during dehydration of germinating orthodox seeds and is associated with the loss of desiccation tolerance (Leprince *et al.*, 1994; 1999). Recently, however, Varghese *et al.* (2011) showed that ROS-mediated damage in slowly-dried, recalcitrant seeds of *T. dregeana* compared with those that were flash-dried was primarily the outcome of failure of the antioxidant system rather than an increase in the ROS produced.

1.7.1. The use of cathodic protection to quench unregulated ROS

Cathodic protection (CP) is used in industry to minimise oxidative damage, e.g. the corrosion of metals in a variety of large structures, including bridges and pipelines (Srinivasan *et al.*, 1996). Cathodic protection is based on the principles of electrode kinetics, with the first reported practical demonstration generally being credited to Sir Humphry Davy in 1824 (Kean and Davies, 1981). Davy found that he could preserve copper in seawater by the attachment of small quantities of iron, zinc or tin. His discovery was based on the fact that metal that has been extracted from its primary ore (metal oxides) has a natural tendency to revert to that state under the action of oxygen and water (Davy, 1824). This phenomenon is called corrosion and the most common example is the rusting of steel. Corrosion is an electro-chemical process that involves the passage of electrical currents on a micro or macro scale. The change from the metallic to the combined form occurs by an ‘anodic’ reaction which produces free electrons, which pass within the metal to another site on the metal surface (the cathode), where they are consumed by the cathodic reaction (Davy, 1824; Kean and Davies, 1981).

Provision of cathodic protection to plant tissues in an analogous way was first investigated by Pammenter *et al*. (1974) who showed that placing *Zea mays* seeds on the cathode of a static electric field extended their viability when maintained under accelerated ageing conditions. In that study, it was hypothesised that such effect was afforded by cathodic protection, which reduced free radicals in the seed tissues by providing a source of electrons (Pammenter *et al.*, 1974; Berjak, 1978). In a recent major breakthrough, Berjak *et al.* (2011b) extended the principle of cathodic protection with the use of the cathodic fraction of a dilute electrolysed salt solution ('cathodic water'), which they used in place of distilled water as the solvent during cryo-procedures. Such electrolysed reduced water has been suggested to have the ability to scavenge reactive oxygen species and protect DNA from oxidative damage (Shirahata *et al*., 1997; Hanaoka, 2001). This beneficial effect was conjectured to have enabled seedling development from cryopreserved embryonic axes of *Strychnos gerrardii*, where none could be produced in all previous attempts (Berjak *et al*., 2011b).

1.7.2. Assessing ROS production resulting from the cryopreservation process

Unregulated ROS production has been found to result from each procedure of the cryopreservation process – and upon retrieval from cryogenic storage – with the effects being exacerbated when all procedures are applied sequentially, as is required for cryopreservation (Whitaker *et al.*, 2010; Berjak *et al.*, 2011b; Sershen *et al.*, 2012c). Among the ROS, the hydroxyl radical is the most damaging in biological tissues, as it will react with virtually all intracellular structures including DNA (Benson and Bremner, 2004; Halliwell, 2006). However, most studies that aim to assess the oxidative status of plant tissues resulting from exogenous stress, assess extracellular superoxide ($O_2^{\bullet -}$) production and lipid peroxidation as popular 'markers' (e.g. Misra and Fridovich, 1972; Grzegorz, 1997; Varghese and Naithani, 2009; Whitaker *et al.*, 2010). Oxidative stress has been identified as a major component of chilling and cryo-injury in plant tissues (Tapell, 1966; Benson, 1990; Fleck *et al.*, 2003; Whitaker *et al.*, 2010; Berjak *et al.*, 2011b), and assessments of freezing injury of cryopreserved germplasm generally consider two key factors, dehydration (osmotic and evaporative) and ice formation (Benson, 2008), as both can have effects on free-radical chemistry (Benson and Bremner, 2004). Excision-related injury of axes from recalcitrant seeds also, is correlated with a ROS burst with the potential for lethal damage of the shoot apical

meristem. This has been counteracted for different species by the provision of DMSO with and without ascorbic acid (Naidoo *et al.*, 2011) and cathodic water (Berjak *et al.*, 2011b).

Hydrogen peroxide is said to be particularly detrimental intracellularly (Riley, 1994) and can also be used as a marker of changes in the oxidative status of plant tissues (Oracz *et al.*, 2007). The particular hazard posed by non-dismutated H_2O_2 is its potential involvement in Fenton chemistry, by which hydroxyl radicals are generated (Hendry, 1993; Benson and Bremner, 2004). Hydrogen peroxide formation is the result of a two-step reduction of molecular oxygen (the first step leading to the superoxide radical) and has a relatively long life in comparison with other ROS. The long half-life (1 ms) of H_2O_2 and its small size allow it to traverse cellular membranes and migrate to different compartments, which facilitate its signalling functions (Bienert *et al.*, 2006). As a result, it is now well established that H_2O_2 is a regulator of a multitude of physiological processes, including resistance acquisition, cell wall strengthening, senescence, phytoalexin production, photosynthesis, stomatal opening and the cell cycle (Petrov and Van Breusegem, 2002). The multi-functionality on the one hand, and the danger presented by elevated concentrations on the other, require the very strict control of H_2O_2 concentration in plant cells.

Active production of H_2O_2 is known to occur mostly apoplastically and is required for triggering the 'oxidative burst' that is a part of the hypersensitive response to pathogens, but is also a prerequisite for normal growth, development and cell death (Miller *et al.*, 2010). The main source of this H_2O_2 is a class of cell membrane NADPH-dependent oxidases, e.g. respiratory burst oxidase homologues (Rho), which are regulated by a unique class of Rho-like proteins called ROPs (Rho-related GTPases from plants) (Agrawal *et al.*, 2003), as well as cell wall-associated peroxidases (Bolwell *et al.*, 2002). However, other sources of H_2O_2 are known in different plant cell compartments, but these are the result of increased metabolism (e.g. photorespiration and fatty acid oxidation in peroxisomes and glyoxisomes, as well as over-energisation of the electron transport chains in chloroplasts and mitochondria) (Petrov and Van Breusegem, 2002). Particularly the mitochondrion is known to be the main intracellular source of oxidants (Turrens, 2003). In most cases, H_2O_2 is formed after reduction of superoxide radicals catalysed by superoxide dismutase. Simultaneously, a network of antioxidants is constantly 'on the alert' for rising H_2O_2 concentrations and provides effective scavenging (Apel and Hirt, 2004; Gechev *et al.*, 2006; Miller *et al.*, 2010). The biological

effect of H_2O_2 is mostly dependent on its concentration, but also on the site of production, the developmental stage of the plant and previous exposures to different kinds of stress. Generally, at low concentrations it is known to act as a signalling molecule, while at higher concentrations it provokes the onset of cell death (Gechev and Hille, 2005).

1.8. The present study

This study aimed to investigate the effects of each of the procedural steps of cryopreservation on the viability of zygotic embryos excised from the recalcitrant seeds of three members of Amaryllidaceae, *viz. Ammocharis coranica* (Ker Gawl.) Herb., *Brunsvigia grandiflora* Lindli. and *Haemanthus albiflos* Jacq. The *ex situ* germplasm conservation of these, and numerous other indigenous species, is presently a priority as many indigenous South African species which are important for traditional medicine are under threat (Mander *et al.*, 2007; Nair *et al.*, 2011). A number of the Amaryllidaceae, in particular, are unsustainably harvested for medicinal purposes throughout the country (Bastida *et al.*, 2011) and therefore represent a conservation priority. The amaryllids are monocotyledonous, bulbous geophytes, and the three species selected for investigation in this study are generally found growing in the coastal areas of South Africa (see Fig. 1.3, for distribution maps). Even though, according to the national Red Data List (SANBI, 2012), the species selected for this study are currently listed as those of 'low concern', they may soon be endangered as their bulbs are targeted for the types of alkaloids they contain (Hutchings et al., 1996; Bastida et al., 2011). Since harvesting the bulb destroys the entire plant this has compromised the continuing existence of some amaryllid species, with a number of them being listed as either 'endangered' or 'vulnerable' in South Africa (SANBI, 2012). This is worrying as some 19 of the 59 amaryllid genera (Snijman, 2000) occurring in South Africa are endemic. *Ammocharis coranica* (commonly known as the Karoo Lily) is used for treating mentally ill patients (Koorbanally *et al.*, 2000). The bulb of *A. coranica* is particularly used to make an antidote against witchcraft (Pooley, 1998) and can also be used as a substitute hallucinogen in place of its close relative, *Boophane distica*. *Brunsvigia grandiflora* is used to soothe and heal wounds after circumcision and as a treatment for renal and liver ailments; it can also be used for chronic coughs, as can *H. albiflos* (Pooley, 1998).

The conservation status of a number of amaryllid species is further threatened by the fact that a great number produce recalcitrant seeds that cannot be stored hydrated for longer than a

matter of weeks (von Fintel, 2006; Sershen *et al.*, 2008). Furthermore, previous attempts by von Fintel (2006) to cryostore embryos of the species selected for the present study were all unsuccessful, suggesting perhaps the elements of cryo-recalcitrance. However, the lack of success could also have originated from a spectrum of other factors (including seed developmental status and experimental parameters). This provided the motivation for the study at hand, which sought to develop successful cryopreservation protocols for the seed-derived germplasm of three amaryllid species, *A. coranica*, *B. grandiflora* and *H. albiflos*. Apart from the fact that these species produce typically recalcitrant seeds (von Fintel, 2006), these propagules were considered to be ideal for the investigation of the phenomenon of possible cryo-recalcitrance. Despite the lack of success in cryopreserving the embryos, the studies by von Fintel (2006) revealed valuable information about the shedding water content and desiccation sensitivity; these are reviewed in Table 1.1 below.

Table 1.1: Review of results obtained from investigations conducted on the zygotic embryos of the three amaryllid species by von Fintel (2006).

Species	[1]SWC (g g^{-1})	WC (g g^{-1}) yielding 50% viability	[2]WC after FD	[3]Viability (%) after FD	[4]Viability (%) after FD+C
Ammocharis coranica	3.98±0.28*	1.8	0.68±0.36*	50**	0**
Brunsvigia grandiflora	2.84±0.46*	0.7	0.84±0.34*	60**	0**
Haemanthus albiflos	4.27±0.46*	0.9	0.73±0.20*	43**	0**

1. SWC = shedding water content
2. WC after FD = water content after flash drying for cryopreservation
3. Viability after FD = viability after flash drying
4. Viability after FD+C = viability after flash drying and cooling (cooling in LN /nitrogen slush/"Mr Frosty")

*Values represent mean±standard deviation (n=5)
**Values represent mean (n=15)

Those results showed that the embryos of all three species were highly desiccation sensitive, which is characteristic of recalcitrant seeds (Pammenter *et al.*, 1998; Walters *et al.*, 2001; Berjak and Pammenter, 2008), as 50% lost viability at relatively high water contents (i.e. higher than 0.5 g g^{-1}) after flash drying. The findings of von Fintel (2006) also showed that these embryos displayed a greater degree of sensitivity to flash drying if subjected to dehydration when germination had been initiated (characterised by embryo protrusion from

the seed) than if investigated before this occurred. The embryos also seemed to be sensitive to cooling, as none of them produced normal seedlings after that step, regardless of flash drying before being cooled either directly in LN, nitrogen slush or at a controlled rate using "Mr Frosty". (These cooling techniques are explained in **Chapter 2** of this study.) Further attempts to precondition explants for vitrification, by subjecting them to the 5 and 10% solutions of either DMSO, glycerol, sucrose, polyvinylpyrrolidone (PVP) or dextran, and the plant vitrification solution 2 (PVS2; Sakai *et al.*, 1991), before cooling did not afford any beneficial effects (von Fintel, 2006). The lack of success in cryopreserving the embryos of all three species could have been due to cooling being attempted at WCs deemed unsuitably high for cryo-exposure of plant germplasm. Such water contents are known to allow for intracellular ice formation at sub-zero temperatures (Sakai, 2000; 2004).

The current study proceeded to: 1. investigate the effect of lowering the water content of the embryos to levels which would reduce/avoid the risk of ice crystallisation during cooling; 2. reduce the damage incurred during dehydration by investigating the effects of applying cathodic protection during flash drying, in order to promote viability retention at this stage and possibly even during cooling. This study further reports on: 3. attempts at lowering the concentration of cryoprotectants used, particularly DMSO, from the high concentrations that are suspected to be toxic to explants, and using a mixture of glycerol and DMSO as a cryoprotectant for the embryos of the selected species.

A study similar to that of von Fintel (2006) was undertaken by Sershen (2006) on fifteen amaryllid species. That author achieved post-cryo survival for a number of the species and showed that, even though most members of this family are characterised by seed recalcitrance, the embryos can withstand flash drying to WCs below 0.5 g g^{-1}. Interestingly, that study also showed that flash drying of axes to below 0.2 g g^{-1} was always accompanied by severe viability loss, but that the WC at which desiccation sensitivity was manifested was not only species-specific but differed within different harvests of seeds of individual species as well. As is expected of desiccation-sensitive embryos, that study confirmed that of the explants flash-dried to WCs amenable for cooling (which seemed to broadly be within the range of 0.2-0.4 g g^{-1}), none survived for longer than a few hours if not frozen. The experiments of Sershen (2006) indicated that there could be commonalities in drying rates and the optimum developmental stage at which the embryonic axes of seeds from this family

display the least desiccation-sensitivity. That study clearly showed that compared with slower cooling, immersion of these explants in nitrogen slush (-210˚C; achieving rapid cooling at hundreds of ˚C min^{-1}) was best. It was also found that in cases where the embryos were amenable to cryoprotection using 5 and 10% solutions of glycerol in series, they could survive being flash-dried to WCs below 0.2 g g^{-1} and >40% remained viable even after cooling.

The results obtained by von Fintel (2006), Sershen (2006) and Sershen *et al.* (2007) therefore informed the approach taken in this study which sought to adapt and improve on protocols previously developed for amaryllid seed germplasm particularly for the three species selected here in view of their apparent cryo-recalcitrance. Whilst certain procedural steps such as cryoprotection, cooling and warming were largely similar to protocols for other amaryllid species, the effects of novel interventions such as cathodic protection on post-cryo survival of the zygotic germplasm of the three species was also investigated. This was done towards investigating some of the potential effects of oxidative metabolism on post-cryo survival of the embryos of the selected amaryllid species, given the current emphasis on its detrimental effects during the various procedural steps involved in cryopreservation (Sershen *et al.*, 2012c).

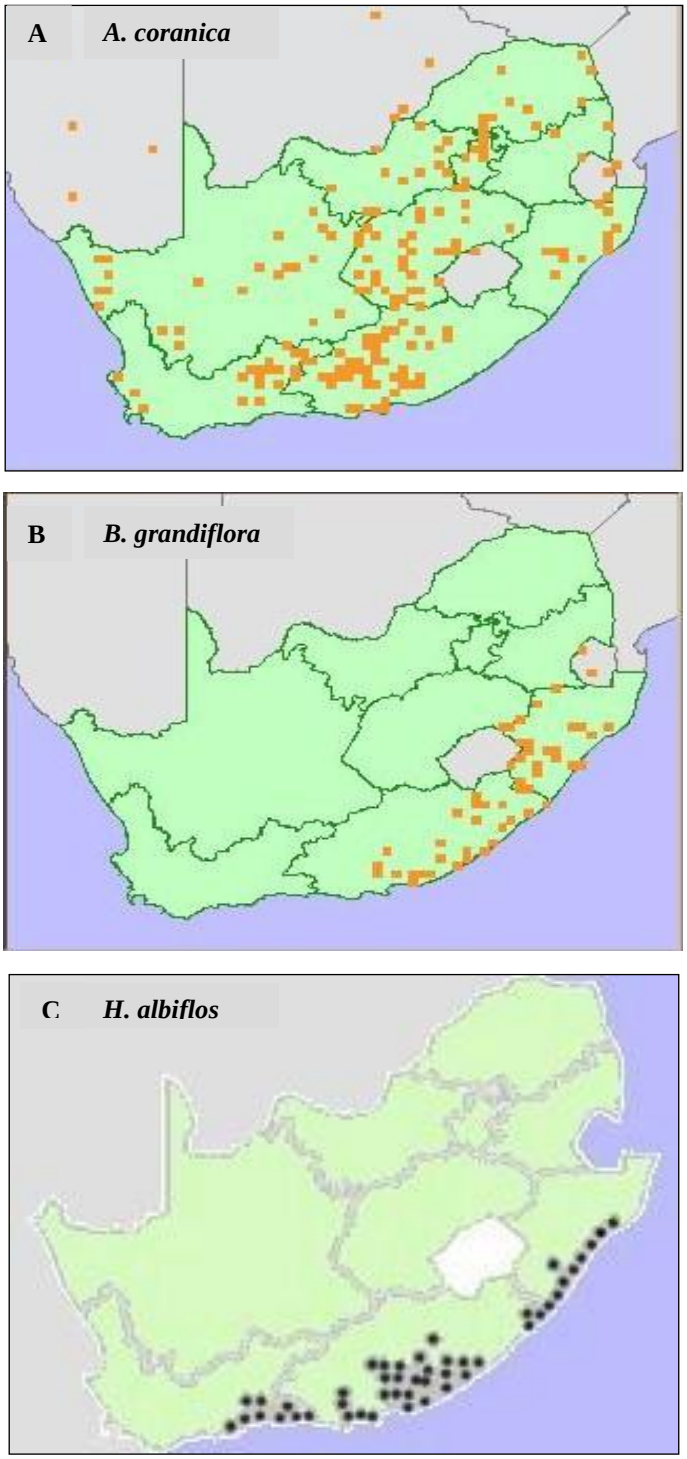


Figure 1.3: Geographical distribution of the amaryllid species investigated in this study for South Africa. A= distribution of *A. coranica*, B= distribution of *B. grandiflora*, and C= distribution of *H. albiflos* (from Snijman and Victor, 2004).

Chapter 2

2. Materials and Methods

2.1. Seed collection and storage

The ripe fruits of *Ammocharis coranica*, *Brunsvigia grandiflora* and *Haemanthus albiflos* (Fig. 2.1) used for this study were harvested from the Eastern Cape Province of South Africa at the sites indicated in Figure 2.2. The *A. coranica* fruits were collected from Rietvlei (S32º 30' 04.8" E26º 49' 12.5"), *B. grandiflora* from Rockford Bridge (S32º 28' 46.8" E26º 54' 36.0") and *H. albiflos* from Dune Forest (S32º 43' 10.2" E28º 22' 15.6"). After collection, the fruit coverings were removed and the seeds extracted, which were then couriered over-night by road to the University of KwaZulu-Natal, in Durban. Upon arrival the seeds were immediately processed by manual cleaning and removal of remaining fruit-tissue, and stored under hydrated short-term conditions at 6°C. This was effected by dusting the seeds with a benomyl-based fungicide, Benlate (active ingredient: benomyl/benzimidazole; Dupont, USA), prior to being placed in hydrated storage. Seeds were then arranged in a monolayer on a plastic grid suspended approximately 200 mm above sterile, moistened paper towel that lined the base of individual 5l buckets which were sealed to avoid or minimise dehydration during storage. The lids were lined with a layer of dry paper towel before sealing the buckets, to prevent condensate dripping down on the seeds during storage. In order to decontaminate them, the buckets and grids used had been pre-soaked for 30 min in a dilute (less than 1%) sodium hypochlorite (NaOCl; Reckitt Benckiser, South Africa) solution and allowed to air-dry over-night prior to seed storage. The seeds were left for at least seven days to mature in storage at 6°C in a constant temperature room prior to the initiation of experimentation.

Figure 2.1: Showing the fruits of *A. coranica*, *B. grandiflora* and *H. albiflos* (seeds in upper row), respectively.

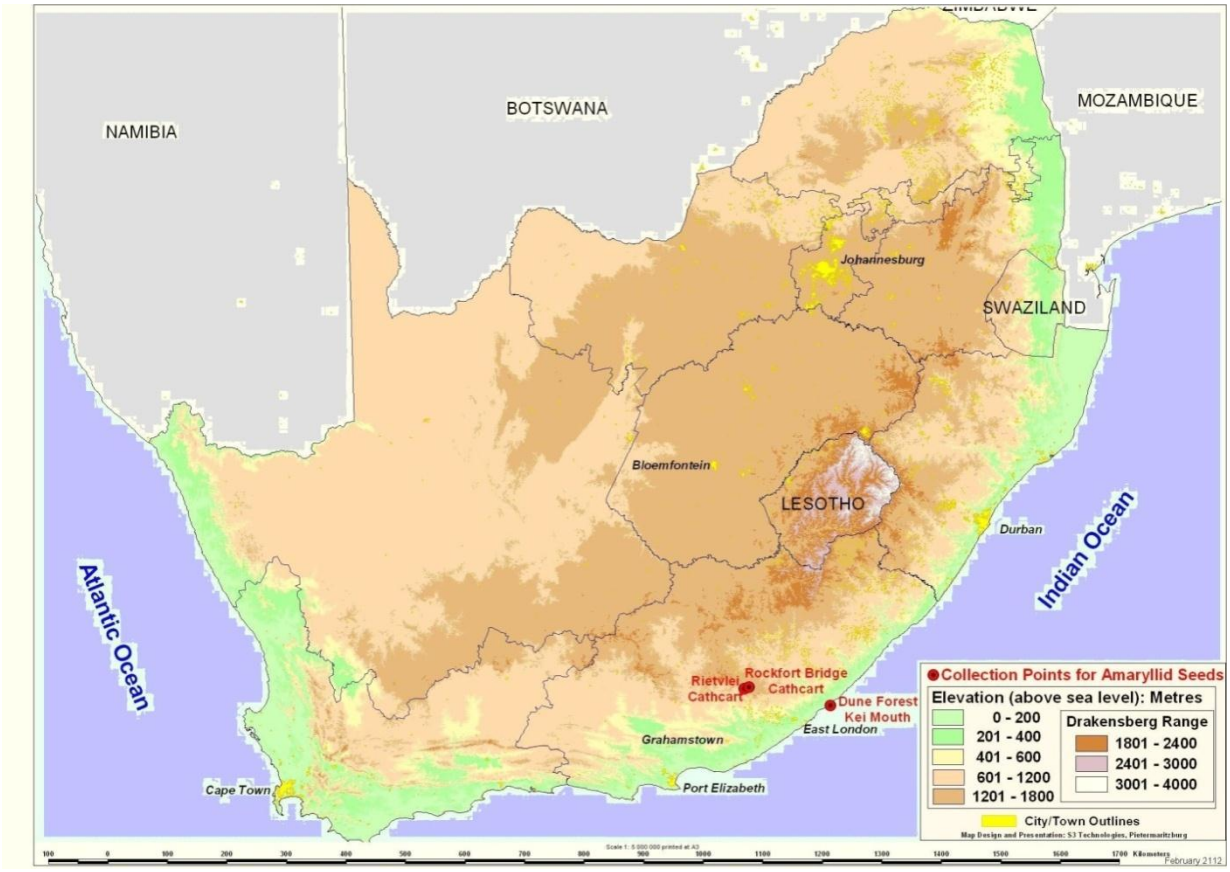


Figure 2.2: Map of South Africa (from S3 Technologies, Pietermaritzburg [2009]), showing the collection points of fruits of the species selected for this study.

2.2. Embryo excision, cryoprotection and dehydration

The first embryo dehydration trials were initiated at seed maturity, after the seven-day-storage period. Maturity was gauged by the full development of the zygotic embryos within the seeds; in all cases these were excised before protrusion of the embryo (which occurs by elongation of the cotyledonary body) to the outside of the seeds and is indicative of the start of germination (see Figs 2.3 & 2.4). The use of germinating/germinated seeds was avoided as germinating embryos of other genera and species of the same family are known to become more desiccation sensitive as germination proceeds and they are also suspected to become more cryo-intolerant (Sershen, 2006; von Fintel, 2006). This has also been seen in other recalcitrant-seeded species, e.g. the axes of *Landolphia kirkii*, (Berjak *et al.*, 1992).

Figure 2.3: Showing the developmental (maturation) stages of *A. coranica* seeds. Stages of protrusion of the cotyledonary body are shown from the seed in the centre towards the right. Arrow indicating the stage used in this study.

All the other seeds were retrieved and used within a month of hydrated storage, which was observed to be the longest seed-storage period preceding embryo protrusion. Before dehydration was attempted the embryos were exposed to selected cryoprotectant (CPA) solutions: these were glycerol (Merck, South Africa; 5 & 10% v/v solutions), dimethyl sulphoxide (DMSO; SIGMA-ALDRICH, USA; 0.1 & 0.25% v/v solutions) and a combination of glycerol and DMSO (5 & 0.1 % solutions, and 10 & 0.25% solutions, respectively). Whilst there are a number of cryoprotectants that can be used for the cryopreservation of plant material (Fuller, 2004), the choice of the penetrative cryoprotectants, glycerol and DMSO, in this study was based on their beneficial effects for other recalcitrant seed material (Sershen, 2006 [glycerol] and Naidoo *et al*., 2011 [DMSO]). The embryos were immersed into the CPA solutions contained in 90 mm diameter Petri dishes, first to the CPA solution of lower concentration for 1 h then transferred to that of higher concentration for a further hour, so as to minimise osmotic shock and allow ample time for CPA uptake by the cells (Muldew *et al.*, 2004; Benson, 2008).

The dehydration trials were initiated on the zygotic embryos having no macroscopic signs of injury after excision. The embryos were accumulated within closed 90 mm diameter Petri dishes on filter paper moistened with deionised water.

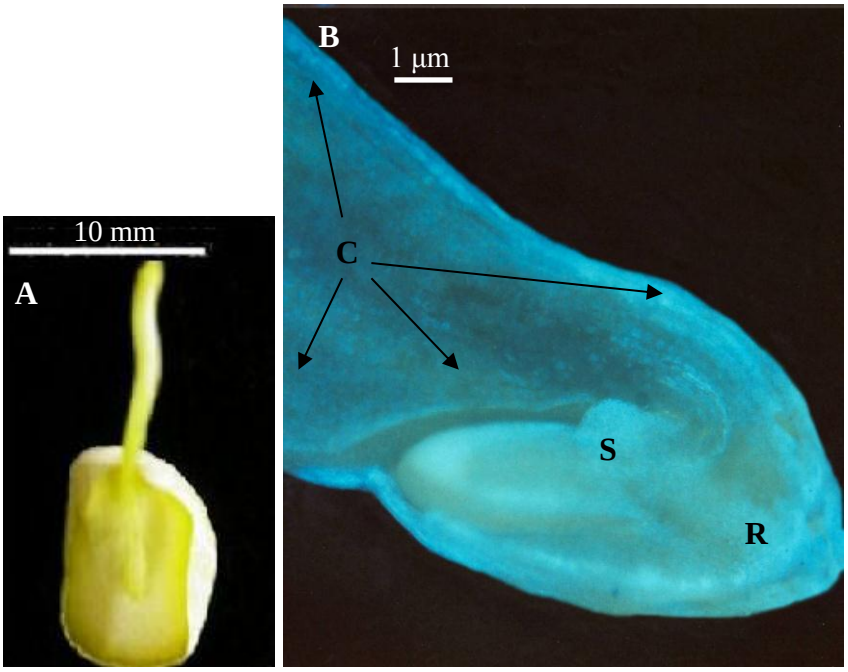


Figure 2.4: Showing (**A**) the protrusion of the embryo (cotyledonary body) from the *A. coranica* seed, and (**B**) (picture from Sershen [2006], with permission): the location of the embryonic axis at the tip of the protruded cotyledonary body. Axis for which root meristem (R) and shoot meristem (S), enveloped by the cotyledonary sheath (C), are illustrated.

The whole cotyledonary body (i.e. zygotic embryo) was selected as a suitable explant in this study, as this includes the embryonic axis from which the seedling naturally develops (Fig. 2.4). Each Petri dish was opened only briefly when an embryo was being introduced, to guard against dehydration of the explants. The accumulated embryos were then separated into batches that were rapidly dehydrated (flash-dried [FD]; Pammenter *et al.*, 2002). The embryos were evenly spaced on the grid of the flash dryer (as originally devised by Berjak *et al.*, 1989; see modified apparatus shown in Fig. 2.5), where they were subjected to a current of silica-gel-dried air from below, generated by a CPU fan. Embryos were sampled at various intervals to generate a survival (germination) vs water content curve, thus giving measures of dehydration rate and the corresponding effect on viability. Ten embryos were cultured (as described below) for viability assessment and a further 10 used for water content determination following each flash drying interval. Water content (WC) was determined by weighing individual embryos before and after oven-drying over activated silica gel for 48 h at 80°C, and quantitatively expressed on a dry mass basis (dmb) as g H_2O per g dry matter (g g^{-1}).

2.3. Cathodic protection during dehydration

For application in this study, cathodic protection was applied by attaching a cathode to the metal mesh on which the embryos were placed during flash drying, so as to provide an electron source to the tissues which could theoretically reduce ROS (Fig. 2.5). The technique, first applied to desiccation-sensitive embryos in the present study, is termed cathodic flash drying (Cat FD).

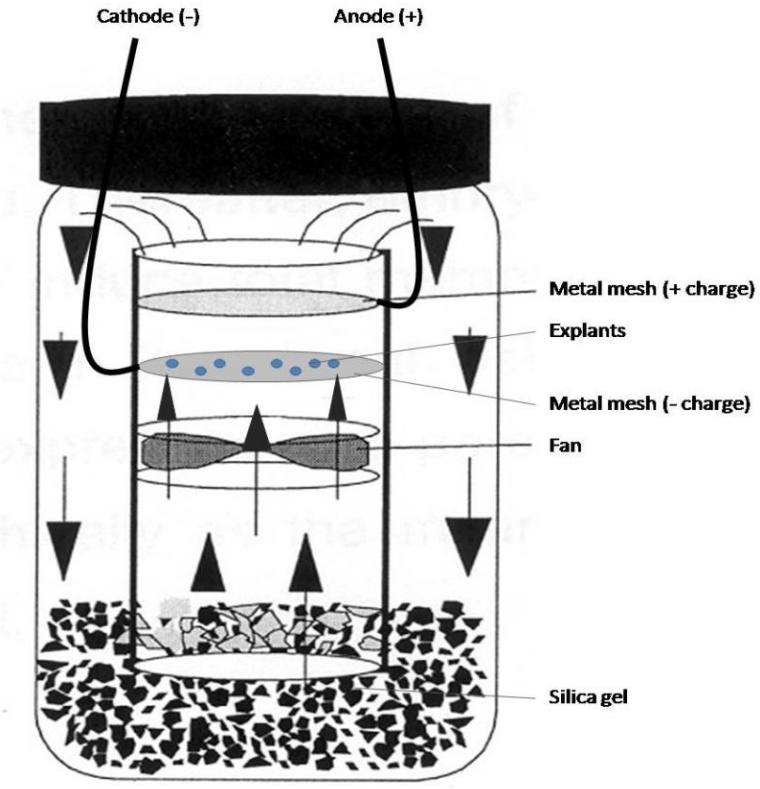


Figure 2.5: A schematic diagram of a flash dryer that has been modified to incorporate cathodic protection during flash drying by providing a negative (-ve) electric charge on the grid upon which explants are dried (modified from Wesley-Smith *et al.*, 2001a and Pammenter *et al.*, 2002).

The production of reactive oxygen species (ROS) has been shown to accompany the various steps required for cryopreservation of embryonic axes from recalcitrant seeds (Berjak *et al.*, 2011b). Therefore, in the present study attempts were made to limit ROS production accompanying the stress of dehydration of the amaryllid embryos by providing cathodic protection (Pammenter *et al.*, 1974) during the flash drying procedure.

Further attempts at employing cathodic protection were made in order to improve viability retention of the embryos after flash drying, by exploring the use of cathodic water (Cat H_2O) for rehydration after flash drying, rather than using the normal standard CaMg solution (1:1 aqueous solution of 0.5 μM $CaCl_2.2H_2O$ and 0.5 mM $MgCl_2.6H_2O$ [Mycock, 1999]). Cathodic water was produced by submerging a cathode at a charge of 60 V in 200 ml of a solution of CaMg for 60 min. Each newly-made batch of cathodic water was used within 60 min, to ensure efficacy.

In addition, another modification was made to the normal flash dryer by applying a slight vacuum in an attempt to accelerate the dehydration of explants in order to shorten the duration of flash drying required to reach WC ranges amenable for cooling. This was the expectation of altering membrane permeability slightly (Feng *et al.*, 2004) in favour of promoting water loss, without inflicting further detrimental injuries on the cells.

2.4. Rehydration and recovery

For viability assessment, the variously dehydrated embryos were rapidly re-hydrated by direct immersion in a CaMg solution (as described above) for 30 min at ambient temperature (*c*. 25˚C). Ten re-hydrated embryos were set to germinate immediately following rehydration after each interval of flash drying by culturing under aseptic conditions. The embryos were decontaminated with 1% (w/v) calcium hypochlorite ($Ca(OCl)_2$; Merck, Germany) for 3 min followed by three rinses with sterile deionised water, after which the surface water was removed by blotting embryos on sterile filter paper. These embryos were then plated for onwards development (five embryos per 65 mm diameter Petri dish) on a full-strength MS-based medium (Murashige and Skoog, 1962) containing 3% sucrose (w/v), and solidified with 8% agar (Agar Bacteriological; Merck, South Africa). The Petri dishes were then placed in a dark cupboard to minimise the risk of photo-oxidative-induced free radical damage (Touchell and Walters, 2000) until embryo growth was observed, after which they were transferred to a 16 h light/8 h dark photoperiod in a growth room at 25±1°C, under medium light intensity (52 μmol m^{-2} s^{-1}) for further development. The embryos, assessed after at least three full weeks in the growth room, were considered to have germinated upon observation of both root and shoot development. These results were used to gauge the degree of tolerance to induced dehydration which was inferred by the lowest water content commensurate with

substantial embryo viability retention. In this study satisfactory survival was considered to have resulted when germination of 60% or more embryos occurred.

2.5. Cooling

For the cooling experiments, samples (n=10) of the partially dehydrated embryos were taken immediately after flash drying, as was a control sample of undried embryos (n=10), to assess the effects of slow or rapid cooling.

Slow cooling was achieved by placing embryos in 2 ml polypropylene cryovials (Greiner™; 10 embryos per cryovial), which were then placed in a "Mr Frosty" apparatus (Nalgene, USA). The outer reservoir of "Mr Frosty" was filled with isopropanol, and the apparatus then placed in a -70°C freezer for 95 min, which achieves cooling at the rate of 1°C min^{-1} down to -70°C thus allowing for equilibrium cooling (Pearce, 2004; Wesley-Smith *et al.*, 2004a). After the allocated time, the slowly cooled samples were rapidly withdrawn from the freezer and transferred into liquid nitrogen (LN) by immediately slotting the cryovials into aluminium cryocanes and plunging these canes into LN in Dewar cryovats for cryogenic storage.

Rapid cooling was achieved by exposing explants to nitrogen slush (LN sub-cooled to -210°C under vacuum [Echlin, 1992]) immediately after drying. Nitrogen slush was prepared in a 100 mm deep × 50 mm diameter polystyrene container and used to tumble-mix 10 partially-dehydrated embryos at a time. This facilitates cooling at rates of hundreds of °C s^{-1} (Wesley-Smith *et al.*, 2004b), achieving non-equilibrium cooling. After exposure to nitrogen slush, the rapidly cooled samples were immediately transferred into LN as described above for slow-cooled embryos.

2.6. Thawing and assessment of survival

After cryogenic storage over at least one night, the embryos were rapidly retrieved from the cryogen and immediately thawed for 2 min in a CaMg solution that had been pre-warmed to, and maintained at, 40°C. These embryos were then immersed in a fresh CaMg solution at ambient temperature for 30 min for rehydration in the dark, and thereafter immediately cultured for survival assessment as described above.

2.7. Localisation of hydrogen peroxide (H_2O_2) as a representative of ROS, using transmission electron microscopy (TEM)

This part of the investigation focused on examining embryo cells of *A. coranica* using transmission electron microscopy to localise the distribution of H_2O_2, as a representative indicator of ROS and oxidation stress (protocol adapted from Bestwick *et al.*, 1997 and Oracz *et al.*, 2007). The embryos were subjected to each of the procedural steps of cryopreservation (as detailed above) and thereafter exposed to cerium chloride ($CeCl_3$) which reacts with H_2O_2 to produce electron-dense, insoluble precipitates of cerium perhydroxides, $Ce(OH)_2OOH$ and $Ce(OH)_3OOH$, that can be detected visually as an indication of the presence of H_2O_2 (Bestwick *et al.*, 1997). The zygotic embryos (5-6 mm in length) were taken immediately after treatment and incubated in freshly prepared 50 mM Mops (SIGMA-ALDRICH, USA; [3-(N-morpholino) propanesulfonic acid]) buffer, pH 7.2, containing 5 mM $CeCl_3$ (SIGMA-ALDRICH, USA) for 1 h. The samples were then processed by fixing in a mixture of 1.25% (v/v) glutaraldehyde (GA) and 1.25% (v/v) paraformaldehyde (PA) in a 50 mM sodium cacodylate (CA) buffer, pH 7.2, for at least 4 h (but not longer than 48 h) at 6 ˚C. After fixation, samples were washed in Pipes buffer (SIGMA-ALDRICH, USA; 10 min, three times) and postfixed in 2% (v/v) osmium tetroxide in Pipes buffer for 2 h. Samples were again washed in Pipes buffer (three times, 10 min each) and then dehydrated in a graded acetone series (50, 70, 80, and 90% [v/v] acetone [Platinum Line(AR)], 10 min each, followed by three changes of 100% acetone each of 20 min duration). Dehydrated samples were progressively immersed in Epon-Araldite at 3:1 acetone:resin, 15 min; 2:1 acetone: resin, 12 h; 1:1 acetone:resin, 15 min; 1:2 acetone:resin, 15 min; 1:3 acetone:resin, 15 min; fresh undiluted resin, 24 h, followed by a further change of fresh resin for 4 h. The embryos were then halved, with the halves containing the axes being transferred to fresh resin and polymerized at 60˚C for 48 h. Ultrathin sections (100 nm) of root meristem cells were cut with a Reichert (Milton Keynes, UK) Ultracut E ultramicrotome using glass knives and mounted on uncoated copper grids (200 mesh). Sections were then examined using a Hitachi H7000 Jeol JEM 1010 (JEOL, Japan) transmission electron microscope at an accelerating voltage of 95 KV. The ultrastructure of root meristem cells from each of three embryos for all treatments, including the untreated control, was assessed and images that represented the general appearance of cells from each treatment were captured using a digital camera (MegaView 3 Software System) for subsequent analysis.

2.8. Data analysis

Where applicable, data analyses were done using the statistical software programme SPSS Inc. (USA) Version 21 for Windows. Analysis of variance (ANOVA) was used to test for differences in embryo water content (WC) within and across harvests carried out over two fruiting seasons. The assumptions of normality and equality of variance were tested using a '1-sample Kolmogorov Smirnov Test' and a 'Lavene's Test of equality of Error Variances', respectively. Where WC data did not meet the requirements of the standard parametric ANOVA a parametric ANOVA was performed on ranked WC data (as an alternative method for a non-parametric ANOVA). In addition, a Tukey's *post hoc* test was performed to make pair-wise comparisons of the effects of the independent factors. The differences were determined using the probability cut-off of 95% ($p \leq 0.05$).

Chapter 3

3. Results and Discussion

This chapter describes the viability responses to cryoprotection, partial drying and subsequent cooling of the zygotic embryos of the amaryllid species investigated, and reports on various studies conducted to understand the individual and cumulative effects of these procedural steps of cryopreservation on post-cryo survival. As described in **Chapter 2**, this study investigated the effect of glycerol and DMSO as cryoprotectants, the effect of rapid (flash) drying, and subsequent rapid or slow cooling on the viability of zygotic embryos of the three selected species. These results are presented and discussed based on the step (*viz*. cryoprotection, drying and cooling) to which they pertain. Results are generally presented for all three species in order to draw comparisons across species; however, where this was not possible as a consequence of low seed numbers in some seasons, results are presented for individual species. Where experiments/treatments could be repeated over two seasons results are presented for both seasons (labelled year 1 and 2 from here on).

3.1. Response to cryoprotection

The post-harvest water content (WC) of the zygotic embryos of *A. coranica*, *B. grandiflora* and *H. albiflos* was 3.28±0.52, 2.55±0.22, and 4.48±0.92 g g^{-1}, respectively (Fig. 3.1). Such WCs are typical of recalcitrant seeds which are always shed in the hydrated condition (Pammenter and Berjak, 1999b; Sershen *et al*., 2007). The results showed that the embryos were germinable after harvest (100% across all species) and germinated after 4-5 d in culture. The cryoprotectants had variable effects on the WC of the zygotic embryos depending on whether they were used alone or in combination but these changes in WC had no detrimental effect on the viability of the embryos in relation to the control. However, there may have been some underlying effects that were not severe enough to impact on viability (Sershen *et al.*, 2007; 2012a). von Fintel (2006), for example, reported that exposure of embryos of some amaryllids to cryoprotectants led to stunted growth and abnormal development, and Sershen *et al.* (2007) also affirmed that cryoprotection results in reduced germinant size in embryos of amaryllids, when growth was assessed over a comparable period of time.

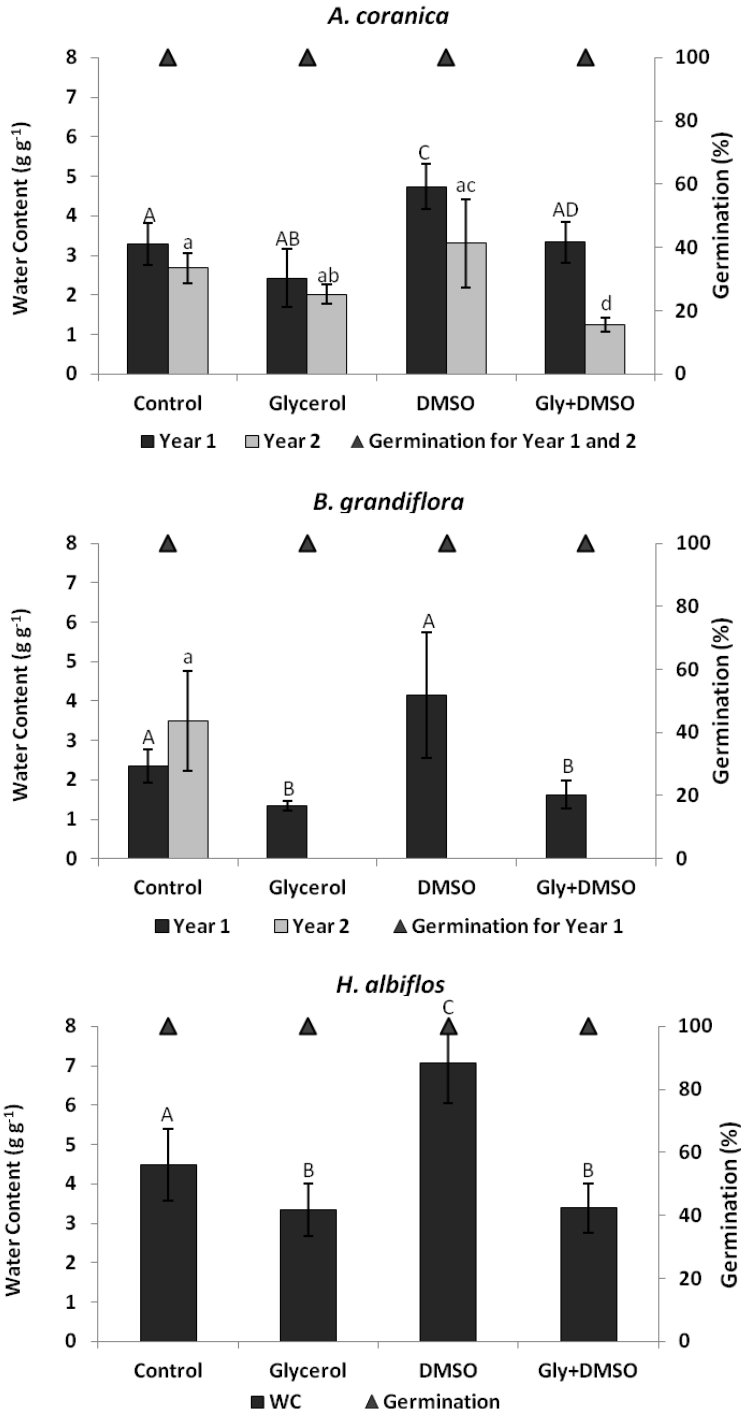


Figure 3.1: Water content of zygotic embryos excised from seeds of *A. coranica* (2 seasons), *B. grandiflora* (1 season) and *H. albiflos* (1 season), before (control) and after cryoprotection. Glycerol/Gly= glycerol (5 & 10% v/v solutions) and DMSO= dimethyl sulphoxide. (0.1 & 0.25% v/v solutions), applied ambient temperature (*c.* 25˚C). Columns represent the mean±SD (n=10). Columns labelled with different letters are significantly different ($p<0.05$; ANOVA).

Glycerol had a considerably dehydrating effect on the zygotic embryos of all three species, but this change in WC was not significant for *A. coranica* (p=0.100 in year 1, p=0.090 in year 2) embryos. The reduction in WC after exposure to glycerol was probably due to the combination of the diffusion of glycerol into cells, which ultimately increased the DM (Volk and Walters, 2006), and osmotic dehydration of the tissues, as discussed below (Sershen, 2006; Sershen *et al*., 2010). Glycerol is a chemical to which the cell membrane is permeable and it has been reported to be highly penetrative (Gao *et al.*, 1995). It is known to interact by hydrogen bonding with water and to permeate across the plasma membrane/plasmalemma of many different cell types, with minimal toxicity at comparable concentrations to those used in this study (Fuller, 2004). When a cell is placed in a solution that is hyper-osmotic with respect to a permeating cryoprotectant but isotonic with respect to the impermeable salts, it first shrinks because of the osmotic efflux of intracellular water, but thereafter increases in volume as the cryoprotectant permeates and as water concomitantly re-enters the cell (Gao *et al.*, 1995). Furthermore, on the basis of cryo-SEM observations, glycerol appears to accumulate in the apoplastic space outside embryonic cells in meristematic tissue (Sershen[1], unpublished data) and in this way could bring about osmotic dehydration of the cells. In addition to this, Sershen *et al*. (2012a) suggested the occurrence of an endocytotic uptake of glycerol based on observing plasmalemma vesiculations by glycerol-cryoprotected embryos of *A. belladonna*. These effects could then explain the 32% decrease in the WC of these embryos that were cryoprotected with glycerol compared to those not cryoprotected. The concentrations of glycerol used in this study were within the range used in the other studies mentioned and those generally used in most cryopreservation protocols (e.g. Sakai *et al.*, 1991; Nishizawa *et al.*, 1993; Pennycooke and Towill, 2000; Turner *et al.*, 2001; Chenshu *et al.*, 2003; Towill *et al.*, 2006), with which the present results were comparable.

When the embryos were cryoprotected with DMSO, there was a substantial increase in the WC in those of all three species when compared with the control (significant for *A.coranica* [year 1; p=0.000] and *H. albiflos* [p=0.000]). This increase is suggested to have been due to the osmotic diffusion of water into the cells from the aqueous DMSO solution in response to the effect of DMSO on the plasma membrane. DMSO is widely known as a membrane

[1] Information from current experiments on *Trichilia dregeana* by Drs Sershen and Varghese, School of Life Science, University of KwaZulu-Natal (Westville Campus), Durban, South Africa.

'puncher' (Anchordoguy *et al.,* 1992; Gurtovenko and Anwar, 2007a) and in this way could have facilitated the inward movement of water molecules. Studies using molecular dynamics (MD) simulations with single-component lipid bilayers have shown that DMSO replaces water in the inner region of the lipid head group (Tristam-Nagle *et al.,* 1998) and causes an increase of area per lipid and a decrease of membrane thickness (Yu and Quinn, 2000). Gurtovenko and Anwar (2007a; b) further showed a DMSO-mediated enhancement of permeability through the formation of water pores, both in atomistic and coarse-grained (Notman *et al.,* 2006) MD simulations. DMSO is said to have an effect on the phospholipid membranes or biomembranes of cells that is strongly dependent on the concentration used and the temperature, because these factors affect its physical properties in water. Yamashita *et al*. (2000) showed that a DMSO molecule strongly interacts with two water molecules at around 20˚C by hydrogen bonding at low concentrations; thereby, DMSO has an effect of rigidifying the water structure. However, at higher concentrations, DMSO tends to break the water structure (Vaisman and Berkowitz, 1992). At higher temperatures, the hydrogen bonding between DMSO and water molecules breaks and, consequently, DMSO has a more hydrophobic character and easily associates with the phospholipids of membranes due to hydrophobic association (Laggner *et al.,* 1987). This property of DMSO in aqueous solution is one of the reasons why it can be used as a cellular cryoprotectant at low temperatures while it is toxic for cells at higher temperatures (Anchordoguy *et al.,* 1992). In the present study, aside from the possibility of water influx into the cells from the dilute aqueous CPA solution used, embryos were exposed to DMSO at *c*. 25˚C, which could have resulted in the water structure becoming more rigid and hence less easily removed.

Understanding of the molecular basis for the actions of DMSO on cell membranes is still limited, especially for plant cells, mainly due to limitations of current experimental techniques. However, studies by de Ménorval *et al.* (2012) and He *et al*. (2012) showed that DMSO promotes plasma membrane permeability to water in living cells of animal cell lines, and that the permeability increases with the increase in DMSO concentration. These effects are suggested, at least partly, to explain the ultimate increase in embryo WC, especially since the concentrations of DMSO solutions (*viz* 0.1 & 0.25% DMSO) used in this study were relatively low compared with those commonly used in other plant cryopreservation protocols (Häggman *et al.,* 1998; Benson, 1999; Ford *et al.,* 2000a; Moges *et al.,* 2004; Volk *et al.,* 2006). The penetration of DMSO molecules into the cells would have probably (negligibly)

increased the dry mass of the cell, but an enhanced influx of water would have ultimately led to an increase in WC (Anchordoguy *et al.*, 1991; Gao *et al.*, 1995).

The benefit of using low DMSO concentrations may be related to the suggestion that they tend to favour higher viability retention during and after cooling (Fahy *et al*., 2004). However, other authors (Berejnov *et al.*, 2006; Volk and Walters, 2006) argue that low concentrations of cryoprotective agents (CPAs) may lower the propensity for glass formation and that cryopreservation methods require very high concentrations of CPAs in order to achieve vitrification. One of the reasons that informed the selection of the low concentrations of DMSO used in this study was the indication that these could promote embryo viability retention upon cryogen exposure, as discussed by Fahy *et al.* (2004).

The embryos that were exposed to the combination of glycerol and DMSO (5% glycerol plus 0.1% DMSO followed by 10% glycerol plus 0.25% DMSO) showed a significant decline in WC relative to the control, except for those of *A.coranica* in year 1 ($p=0.518$). This was probably due to an over-riding dehydrating effect of glycerol (at relatively high concentrations, as explained above) over the hydrating effect of DMSO on the cells, particularly because DMSO was used at such low concentrations relative to the concentrations of glycerol.

The inter-species differences in changes of WC with exposure to cryoprotectants described above cannot be easily explained as various factors influence the resulting WC when embryos are treated with any specific solution; however, differences in the rate of penetration of specific cryoprotectants are most often the consequence of differing tissue characteristics (Ferrando and Spiess, 2001). Cryoprotectants themselves also differ in terms of the rate at which they penetrate tissues; DMSO is known to penetrate cell membranes much more rapidly than glycerol (Kartha, 1985; Meryman, 2007). Using a mixture of different CPAs can also have variable effects, and can be advantageous (Fahy *et al*., 1986) affording the potential to reduce the toxicity of any single additive, limiting the impact of extreme drying and helping to stabilise the glasses formed (Benson, 2008). In this regard, most vitrification solutions applied to plant tissues use a mixture of penetrating and non-penetrating (or a slow penetrating) cryoprotectants, as is often recommended (Fahy *et al.*, 1984). In the present study, the two cryoprotectants used are both considered to be penetrative – although, as

discussed above – glycerol remaining in the apoplast could be considered to be non-penetrative. The combination of glycerol with DMSO was chosen based on their beneficial effects for other recalcitrant seed material (Sershen, 2006 [glycerol] and Naidoo *et al*., 2011 [DMSO] and to test if they could offer a beneficial interactive effect on the embryos of the selected species. When penetrative cryoprotectants are used in combination, as in glycerol and DMSO in this study, the relative molecular weight and penetrative capacity of each cryoprotectant determines the impact of cryoprotection on the dry mass and the water status of the embryos. However, DMSO has been suggested to enhance the uptake of cryoprotectants like glycerol when used in combination (Fuller, 2004), but is considered to be more toxic to plant cells than glycerol when they are used at equimolar concentrations (Kartha and Engelmann, 1994).

The use of high concentration mixtures of the cryoprotectants (i.e. DMSO and sucrose) has been reported by several authors, but with highly variable success (Uragami *et al.*, 1989; Al-Bahrany and Al-Khayri, 2012). The combination of CPAs to produce one mixture has been a common venture in cryopreservation protocols as an aim to exploit the beneficial effects that may be offered by one CPA on the mode of action of the other and in order to improve the efficacy of their effect on the cells (Finkle and Ulrich, 1979; Volk *et al.*, 2006). The synergy may be as a result of a direct interaction of the molecules of the combined chemicals, or may result from an indirect interaction through the one having a specific effect on the cells, which will then allow the other to work more efficiently. One such example is that of ethylene glycol and DMSO when used in combination with glycerol (Volk *et al.*, 2006), and propanediol used with DMSO (Boutron and Kaufmann, 1979; Szurek and Eroglu, 2011). In the former case, both ethylene glycol and DMSO were found to be permeable to the shoot tips of mint (Steponkus *et al.*, 1992; Volk and Walters, 2006) and to exhibit surfactant effects on membrane permeability, in turn, therefore, increasing the diffusion of glycerol into cells (Finkle *et al.*, 1985). Such properties would need to be determined for the concentrations of glycerol and DMSO used in this study as well, in order to ascertain the mode of action.

Since a reduction in the WC of the embryos was observed after exposure to the 5% and 10% glycerol solution (for an hour at each concentration), it was considered worthwhile to investigate whether a comparable change would occur with exposure to lower concentrations. The reasoning behind this was that the use of lower concentrations of glycerol could prove to

be beneficial by limiting any dehydration damage incurred (which might have resulted when embryos were sequentially exposed to 5 and 10% glycerol) prior to physical partial dehydration (flash drying). It was also of practical importance to investigate whether the change in WC observed after cryoprotection with 5 and then 10% glycerol solutions occurred at both concentrations or largely as a consequence of the initial exposure to the cryoprotectant (i.e. in the 5% solution). Results of these studies are reported on below.

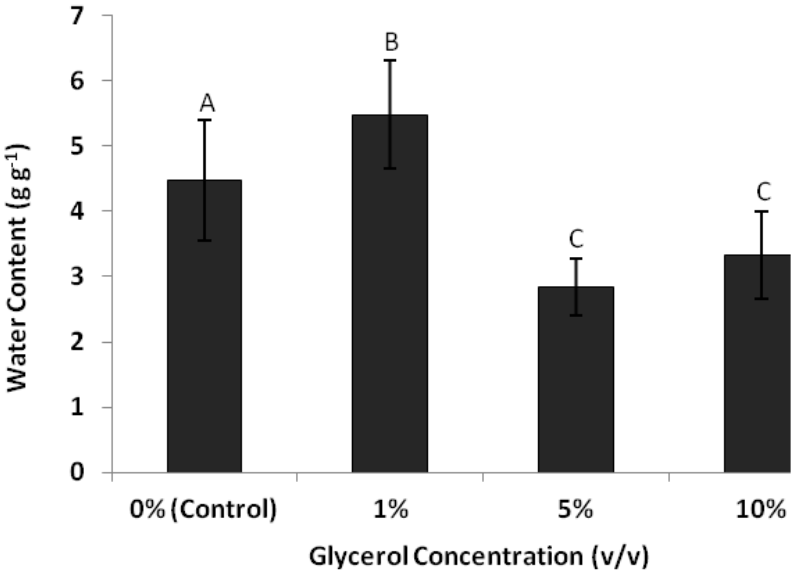


Figure 3.2: Water content of zygotic embryos of *H. albiflos* before and after exposure to different concentrations of glycerol for 1 h at *c*. 25°C. Columns represent the mean±SD (n= 10). Columns labelled with different letters are significantly different ($p<0.05$; ANOVA).

The results show that there was a significant ($p=0.023$) increase in embryo WC relative to the control (non-cryoprotected embryos) after exposure to a 1% glycerol (Fig. 3.2). However, there was a significant decrease in WC after exposure to higher glycerol concentrations, of 5 ($p=0.000$) and 10% ($p=0.007$), being more marked in the former. This indicates that lower concentrations (i.e. 1%) of glycerol do not induce osmotic dehydration, and it appears as though the chemical dehydration observed across all three species in this study was mainly the consequence of exposure to the initial cryoprotectant concentration (i.e. 5% glycerol). This experiment could be done on the embryos of *H. albiflos* only and could not be done on those of the other two species because of the shortage of seed material.

3.2. Response to dehydration

3.2.1. Rapid dehydration of explants not exposed to cryoprotectants

The initial (post-harvest) WC of the zygotic embryos of *A. coranica* and *B. grandiflora* (*c.* 2.68-3.28 and 2.55-3.50 g g^{-1}, respectively) and germinability (100%) were high over consecutive seasons (Fig. 3.1). Such high WCs are typical of recalcitrant-seeded amaryllid embryos as shown for other species (von Fintel, 2006; Sershen *et al*., 2008). The similarity in the WC of the embryos of the seeds collected over these two successive seasons could have been a consequence of the comparable temperatures prevalent during the period leading up to seeding experienced by the parent plants over the two years (see **Appendix**). The total amount of rainfall did, however, differ over the two sampling seasons for these species (139.4 mm in year 1 and 326.6 mm in year 2 for *A. coranica*, and 182.6 mm in year 1 and 297.6 mm in year 2 for *B. grandiflora*). While rainfall is known to be among the major contributing factors to the quality of seeds produced (Fenner, 1991; Fougereux *et al.*, 1997; Gutterman, 2000, Olivares *et al.*, 2009), the temperatures experienced by the parent plant during seed development also play an important role in determining seed quality (Sanhewe *et al.*, 1996; Daws *et al.*, 2004; Contreras *et al.*, 2009). Seed development and shedding for both these species coincided with the rainy season (summer), which supports the suggestion of Pritchard *et al*. (2004) and Daws *et al.* (2005) that species with desiccation-sensitive seeds may minimise the risk of seed desiccation within dry environments by timing seed shed to the period of maximum rainfall.

Inter-seasonal comparisons of embryo post-harvest WC could not be done for *H. albiflos* over two sampling seasons as all investigations were done on seeds of one collection, for which the recorded rainfall was 577 mm during seed development. The WC of the embryos was 4.48±0.92 g g^{-1} when assessed in the laboratory. Although there was a difference in the rainfall during seed development of *A. coranica* and *B. grandiflora* over the two sampling seasons, this was not considered as a major factor as the Eastern Cape, where the parent plants are found, is typified by summer rainfalls that seldom exceed 500 mm (Benhin, 2006).

Recalcitrant seeds are known to germinate rapidly when sown fresh (Mng'omba *et al.*, 2007), and, across species, those of the Amaryllidaceae will commence germination shortly after shedding, with no requirement for an extraneous water supply (personal observations). The zygotic embryos of all three species displayed 100% germination after the excision step as

reported for other members of Amaryllidaceae (Sershen *et al.*, 2008), attesting to the lack of ROS-associated excision injury to the shoot apical meristem as demonstrated for *Amaryllis belladonna* by Pammenter *et al.* (2011). Consideration of the response of the embryos of both *A. coranica* and *H. albiflos* to flash drying (Fig. 3.3) shows that there was a significant ($p<0.05$) sharp decline in WC (to <1 g g^{-1} but >0.5 g g^{-1}) within the first 60 min, without any change in viability. This was followed by a gradual (largely non-significant, $p>0.05$) decline in water content to ~0.5 g g^{-1}, after 120 min of flash drying for both species with a 10% loss in viability. In contrast, the initial sharp decline in WC to <1 g g^{-1} (but >0.5 g g^{-1}) of embryos of *B, grandiflora* occurred within the first 15 min of flash drying, also without any loss of viability. The embryos of this species reached WCs <0.5 g g^{-1} after just 30 min of flash drying, by which time 30% had lost viability. The embryos of *B. grandiflora* dried faster than those of *A. coranica* and *H. albiflos*, which is in agreement with differences in drying rates of embryos across species of other amaryllids, as reported by Sershen (2006). For all three species, further extended periods of dehydration resulted in relatively small (non-significant; $p>0.05$) changes in WC, which were accompanied by a severe loss in viability in all cases. At WCs ≤0.2 g g^{-1} ≥50% of the embryos had lost viability.

The initial decline in WC to <1 g g^{-1} (but >0.5 g g^{-1}) in the embryos of these species would probably have been due to the loss of apoplastic water and solution water from the unbound fraction in the cells, and consequently its reduced availability (Berjak *et al.*, 1990; Pammenter *et al.*, 1991), which was suggested to have been the case for embryos of 15 other amaryllid species (Sershen, 2006; Sershen *et al.*, 2008). The rapidity of the initial decline in water content is in agreement with the time-related minimisation of any effects of unbalanced metabolism, and consequently a limitation of metabolism-linked desiccation damage (Pammenter *et al.*, 1998; Walters *et al.*, 2001). This is in accordance with the fact that no viability loss occurred to that point, considering that recalcitrant seed germplasm of a number of species has been recorded to tolerate the loss of a proportion of intracellular solution water (e.g. axes of *Ekebergia capensis* [Pammenter *et al.*, 1998]; *Aquilaria agallocha* [Kundu and Kachari, 2000], *Artocarpus heterphyllus* [Wesley-Smith *et al.*, 2001a], and *Telfairia occidentalis* [Ajayi *et al.*, 2006]). The reduction of water availability is considered to decrease the rate of solute diffusion to metabolic sites where critical enzymatic activity occurs (Walters *et al.*, 2001), water being the solvent and medium in which diffusion of solutes and biochemical reactions take place in plant cells (Copeland and McDonald, 2001).

However, the initial rapid loss of water was followed by a decline in viability when further drying was effected to *c*. 0.5 g g^{-1}. The decrease in embryo viability at such relatively high WCs is common in recalcitrant seeds (Liang and Sun, 2000) and, considering the extended time necessary for dehydration to *c*. 0.5 g g^{-1}, is considered to have been the result of metabolism-linked damage (Pammenter *et al.*, 1998; Berjak and Pammenter, 2001; Walters *et al.*, 2001).

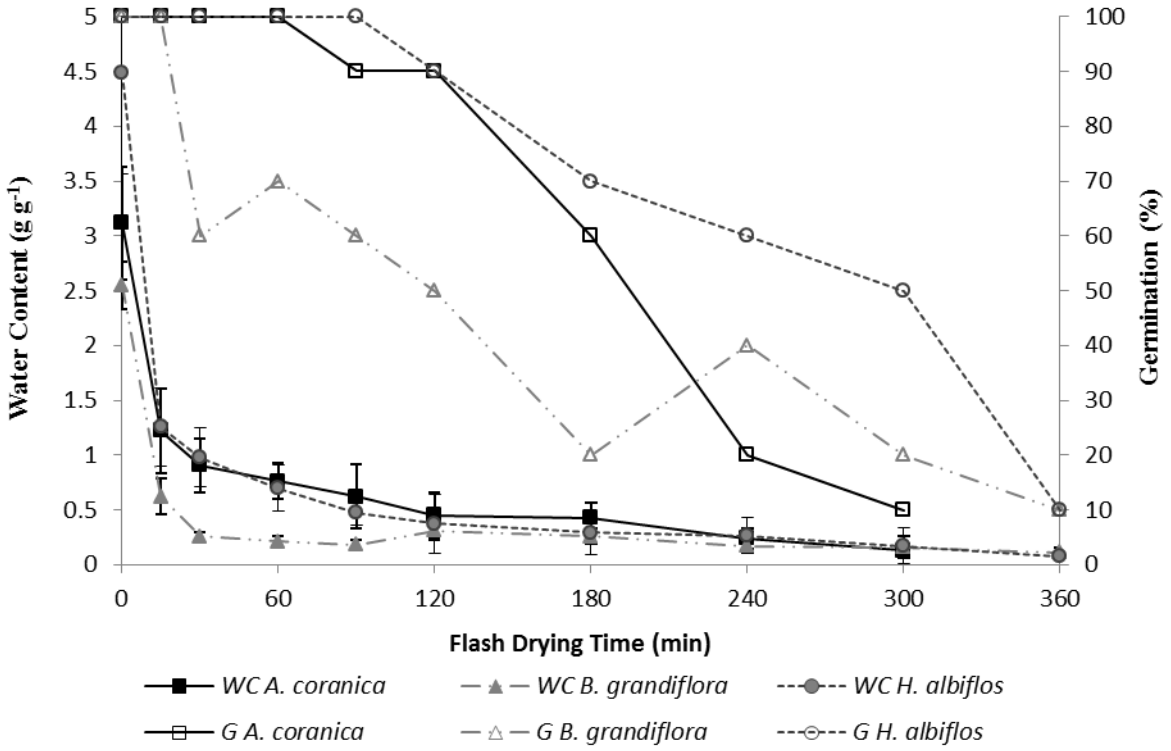


Figure 3.3: Change in viability (G= germination) with water content (WC) for excised zygotic embryos of *A. coranica*, *B. grandiflora* and *H. albiflos* after flash drying for various time intervals. Error bars represent the mean±SD (n= 10).

Fig. 3.3 further shows that continuing dehydration to WC considerably below 0.5 (≤0.2) g g^{-1} was associated with an extreme decline in viability. This viability loss is suggested to be due to the purturbation or actual removal of structure associated/bound water within cells, which is known to cause desiccation damage *sensu stricto* (Berjak and Pammenter, 2001; Walters *et al.*, 2001). The amount of structure associated water has been reported to be ≤0.28 g g^{-1} in embryos of some recalcitrant-seeded species (e.g. *L. kirkii*, Pammenter *et al.*, 1991) and removal of such water has been shown to be associated with severe damage, as was also

suggested to be the case in 10 other species of amaryllids (Sershen *et al*., 2007). Crowe *et al.* (1990) suggested that the removal of bound water may result in profound changes in the physical properties of biomolecules, particularly the phospholipids and proteins in cells.

Structure-associated water is essential to maintain cellular integrity in non-orthodox seed tissues (Pammenter *et al.*, 1993), and its loss can interfere with organisation, including causing membrane structural changes and protein conformation disturbances (Hoekstra *et al*., 2001). Such stress is known to exert mechanical strain that is associated with turgor loss (Illing *et al*., 2005), oxidative damage due to free-radical-mediated processes and destabilisation of macromolecular integrity (Dussert *et al*., 2006; Harding *et al*., 2009). During dehydration from ~0.5 g g^{-1} to the level of structure-associated water, metabolic damage would continue to occur, but actual loss of the structure-associated water is taken as an indication of desiccation-linked damage *sensu stricto* (Pammenter *et al*., 1991; Vertucci and Farrant, 1995). What is assumed to have been the outcome of desiccation damage *sensu stricto* was reported for silver maple when the (desiccation sensitive) seeds were dehydrated below 32% of their original water content, the threshold level below which only non-freezable water remained (Becwar *et al.*, 1983).

The embryos of *B. grandiflora* dried the fastest across the species, reaching WCs below 0.5 g g^{-1} after just 30 min of flash drying, whilst those of *H. albiflos* and *A. coranica* required at least 120 min to reach comparable WCs. Such differences in the rate of embryo dehydration have been reported for other members of this family (Sershen *et al.*, 2008), with *Amaryllis belladonna* requiring only 30 min of flash drying to reach water contents <0.4 (from 4.67) g g^{-1} (Sershen *et al.*, 2011) and *Haemanthus montanus* requiring 300 min to reach 0.34 (from 5.05) g g^{-1} (Sershen *et al.*, 2012b). The observations were reported to be associated with a greater degree of intracellular damage (as detected via ultrastructural studies) in embryo tissues of the slower drying species (*H. montanus*) compared with the faster drying *A. belladonna*, and were related to the prolonged period of dehydration required (Sershen *et al*., 2012b). This leads to the suggestion that decline in viability of *A. coranica* and *H. albiflos* embryos might not have been due to the stress brought on by water loss alone, but may also have resulted from the prolonged duration of dehydration necessary to attain WCs within the range considered to be suitable for cryopreservation (i.e. 0.2-0.4 g g^{-1} of amaryllid embryos (Sershen *et al*., 2012b). The detrimental effects of slowly drying recalcitrant tissues are well

documented in literature (Pammenter *et al.*, 1991; Pammenter and Berjak, 1999a; b; Liang and Sun, 2000; Walters *et al.*, 2001; Wesley-Smith *et al.*, 2001b). Slow drying rates extend the period of time that the tissue spends at intermediate WCs while incurring cumulative metabolism-linked damage (Pammenter *et al*., 1998, Pammenter and Berjak, 1999; Walters *et al*., 2001; 2002a). While excessive water extraction alone from cells will lead to desiccation damage (Crowe *et al.*, 1989; 1992; Hoekstra *et al.*, 1992; Varghese and Naithani, 2002), as it is essential to lower water contents appropriately for cryopreservation, it is imperative that the length of dehydration treatment to achieve optimal water contents is controlled and optimised (Pammenter *et al.*, 1998; 1999). There have been suggestions that drying rate and the damage incurred during drying can influence post-cryo survival (Walters *et al.*, 2008) and therefore the studies described below were initiated to investigate this phase of the cryopreservation protocol in greater depth.

The objective of the partial dehydration studies was to optimise drying times for WCs below 0.5 g g^{-1}, and ideally between 0.2 and 0.4 g g^{-1}, for subsequent cryopreservation trials. This range was selected because such WCs have been shown to be suitable for the cryopreservation of recalcitrant seed germplasm (e.g. embryos of *Zizania palustris* [Touchell and Walters, 2000], *Quercus suber* and *Q. ilex* [Gonzalez-Benito *et al*., 2002], some members of Amaryllidaceae [Sershen *et al*., 2007], and a range of other species [Normah and Makeen, 2008]). While survival can be achieved at WCs below the selected range, the choice was based on the fact that such WCs represent the balance between the avoidance of ice crystal damage, metabolism-linked damage and desiccation damage *sensu stricto*. Non-orthodox tissues tend to undergo desiccation damage at WCs lower than 0.45 g g^{-1} (Vertucci and Farrant, 1995) which culminates in substantial viability losses below 0.25 g g^{-1} (Pammenter *et al*., 1993). To revisit the results reported earlier, it took no less than 180 min to flash dry *A. coranica* embryos to WCs within the selected range, with 40-80% viability loss (Fig. 3.3). However, *H. albiflos* embryos could be dried to WCs in this range in at least 120 min with only a 10-40% viability loss (Fig. 3.3). In contrast, *B. grandiflora* embryos could be dried to WCs that were within the target range in just 30 min, while incurring a 30-40% viability loss (Fig. 3.3). Based on suggestions that longer, as opposed to shorter, drying times predispose recalcitrant zygotic embryos to higher levels of post-cryo viability loss (Sershen *et al*., 2012b) it was hypothesised that *B. grandiflora* zygotic embryos might display the best post-cryo survival.

From the results discussed above it was evident the embryos of the selected amaryllid species were losing viability at relatively high WCs (Figs. 3.3). However, it must be remembered that in recalcitrant amaryllid seeds the embryonic axis (containing the root and the shoot meristems) is enclosed within a single cylindrical cotyledonary body that protrudes out of the seed prior to radicle protrusion (see Fig. 2.3, **Chapter 2**). As the axis cannot be distinguished from the surrounding cotyledonary tissue by the naked eye the approach developed to flash drying amaryllid embryos for cryopreservation entails drying whole zygotic embryos or the embryonic axis with a portion of cotyledonary body attached (Sershen, 2006; von Fintel, 2006). As in this study, the WC determinations are also based on the average WC across the embryo (bulk embryo WC) rather than meristem WCs. This raised the possibility that the WC of the meristems, the parts of the embryo that are vital for onwards growth and development, could actually be at a WC dissimilar to the bulk embryo WC at any particular drying time. This could influence the survival obtained after cooling, thus the hypothesis presented above was tested by assessing the WC of the different parts of *H. albiflos* embryos as follows (see Figs. 2.3 and 2.4, showing the morphology of *A. coranica* seeds as the embryos are relatively similar. The WC assessments of the embryo components were not conducted on *A. coranica* and *B. grandiflora* embryos because of limited seed numbers):

a) Whole zygotic embryos flash-dried (Embryo - considered as a control) for WC assessment;
b) Embryos that were flash-dried whole after which the axes and cotyledonary portions separated from each other for WC assessment; these are referred to as Axes DBC (dried before cutting; 3-4 mm segments) and Coty DBC (2-3 mm segments - just a portion of the cotyledon remaining after separation from the axis from the whole embryo), and
c) Axes (3-4 mm segments) that were separated from the cotyledonary portions before being flash-dried for WC assessment, referred to as Axes CBD (cut before drying).

The results showed that initial bulk embryo WC was substantially higher than the WC of the 3-4 mm axes and the 2-3 mm cotyledonary tissue (Coty DBC), when assessments were done immediately after excision from mature seeds (Fig. 3.4), even though such differences were largely non-significant ($p=0.156$). The results also showed that the axes and the cotyledonary tissues were at similar WCs (Fig. 3.4) when assessments were done after excision. All embryo components (both axes and cotyledonary segments) followed the same trends of

water loss as whole embryos when flash-dried as independent entities – i.e. a significant ($p<0.05$) loss within the first 60 min of drying, followed by a more gradual (non-significant; $p>0.05$) decline in WC. However, the cotyledonary tissues (Coty DBC) dried more rapidly than the axes (Axes DBC), when both were dried when constituting whole embryos, ultimately leaving the axes at a higher WC than the cotyledons. This then indicated that the bulk water loss detected when the zygotic embryos were flash-dried was mainly due to the loss from the cotyledons, leaving the actual axes at relatively higher WCs. Interestingly, excised axes (Axes CBD) dried at a comparable rate to the whole zygotic embryos, indicating that excised axes dry considerably faster as independent explants as opposed to being dried with the entire cotyledonary body intact.

Uneven water distribution of different tissues in recalcitrant/non-orthodox seeds has been reported for some species (e.g. *Aesculus. hippocastanum* [Tompsett and Pritchard, 1993] and *Araucaria hunsteinii* [Pritchard *et al.,* 1995]), with axes generally being at higher WCs, and more desiccation sensitive, than the cotyledons (e.g. *Quercus robur* [Finch-Savage, 1992]; *Machilus thunbergii* [Lin and Chen, 1995]; *Theobroma cacao* [Li and Sun, 1999], *Dovyalis caffra* [Erdey and Berjak, 2004]; *Ekebergia capensis* [Erdey *et al.,* 2004]; and *Warburgia salutaris* [Kioko *et al.,* 2004]. Extrapolating from those reported observations on differing WCs of axes and cotyledons, meristematic tissues may have higher WCs than other parts of the axes after rapid dehydration, mirroring the situation before drying which could reflect uneven water distribution as a natural phenomenon. Whether or not this is so requires to be established, but the results described above are indicative of the phenomenon of uneven drying in different tissue components described for flash-dried recalcitrant embryos by other authors (Pammenter *et al*., 1998; Wesley-Smith *et al*., 2001b).

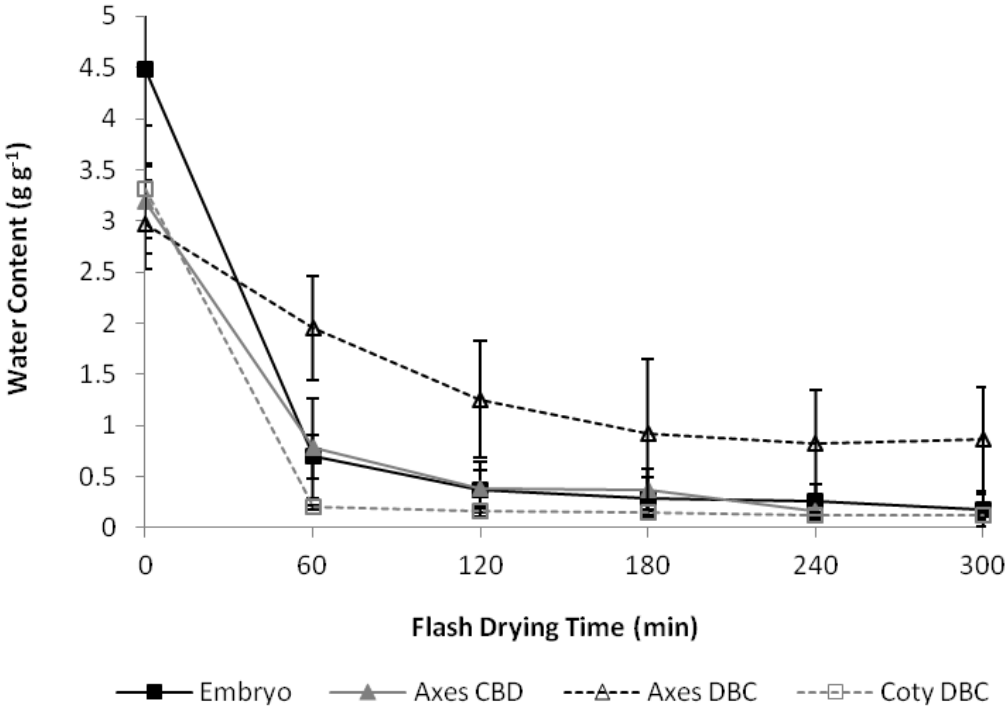


Figure 3.4: Water content of the different components of the zygotic embryos of *H. albiflos* (refer to Fig. 2.4 of Chapter 2) before and after flash drying. Embryo= whole zygotic embryos, CBD= cut before drying, DBC= dried before cutting, Axes= 3-4 mm segments of the zygotic embryos containing the shoot and root meristems. Error bars represent the mean±SD (n= 10).

3.2.2. Rapid dehydration coupled with cryoprotection

When partial dehydration was preceded by cryoprotection the embryos displayed similar trends in terms of WC and viability as those displayed by non-cryoprotected embryos (Fig. 3.5). A sharp (significant; $p<0.01$) decline in WC was observed within the first 60 min of flash drying, followed by a more gradual (non-significant; $p>0.05$) decline which was accompanied by a decrease in viability. However, the decline in viability of cryoprotected *A. coranica* and *H. albiflos* embryos was more extreme than for non-cryoprotected embryos at comparable WCs (compare Fig. 3.5 with Fig. 3.3). For example, of the *A. coranica* embryos, 80% germination was obtained after flash drying for 120 min (to reach 0.52±0.21) without prior cryoprotection, whereas of those that had been exposed to cryoprotection with either glycerol, DMSO, or a combination only 70 and 60% germinated, respectively. Also of the *H. albiflos* embryos, 90% germinated after flash drying for 180 min (to reach 0.29±0.20) without prior cryoprotection, whereas of those that had been exposed to cryoprotection with either

glycerol, DMSO, or a combination only 0, 60 and 30% germinated, respectively. This is suggested to be due to cytotoxicity of the cryoprotectant molecules (Fahy, 1986; Arakawa *et al.*, 1990; Steponkus *et al.*, 1992; Gao *et al.*, 1995). Dehydration has been reported to increase the concentration of cryoprotectant molecules within the cells (Reed, 1995; Al-Ababneh *et al.*, 2002), thus imposing an osmotic stress. Additionally, when cells with a high intracellular concentration of cryoprotectants are exposed to a less concentrated medium during rehydration (e.g. the CaMg rehydration solution used in this study), they then might swell due to an osmotic influx of extracellular water. Therefore, the loss of viability displayed by partially dehydrated cryoprotected embryos in this study coud have been the result of the combination of osmotic and mechanical stress. However, the possibility that cryoprotection intensified viability loss by simply extending the period spent at intermediate WCs cannot be ignored.

It was also noteworthy that the degree to which cryoprotection compromised post-drying viability differed across cryoprotectants. *Ammocharis coranica* embryos cryoprotected with DMSO showed a greater degree of sensitivity to flash drying than glycerol-cryoprotected embryos (Fig. 3.5). Embryos of *H. albiflos*, on the other hand, appeared to be more sensitive to flash drying after cryoprotection with glycerol as opposed to DMSO. While exposure to glycerol prior to flash drying had only minor effects on embryos of a spectrum of amaryllid species, in the case of *Brunsvigia gregaria*, viability dropped to 60% compared with 90% when embryos were similarly dehydrated without any cryoprotection (Sershen *et al.*, 2007). Such differing species-based responses to glycerol cannot be explained without considerable in-depth knowledge about the physiology and state of development of the embryos. The effect of cryoprotectants in the embryos of the two amaryllids investigated in this study seemed to be species related, even though some studies have shown DMSO to be more toxic than glycerol (Kartha and Engelmann, 1994; Gurtovenko and Anwar, 2007a). Although relatively low concentrations of DMSO were used, the concentration within the cells would have increased as a consequence of water loss during flash drying. For *A. coranica*, the detrimentally toxic effect of DMSO that is known to occur at higher concentrations (Withers and King, 1979; Kartha and Engelmann, 1994; Fuller, 2004) is suggested to have led to a decrease in viability. The effects of cryoprotection on post-drying embryo viability were not investigated for *B. grandiflora* as a consequence of a shortage of viable seeds.

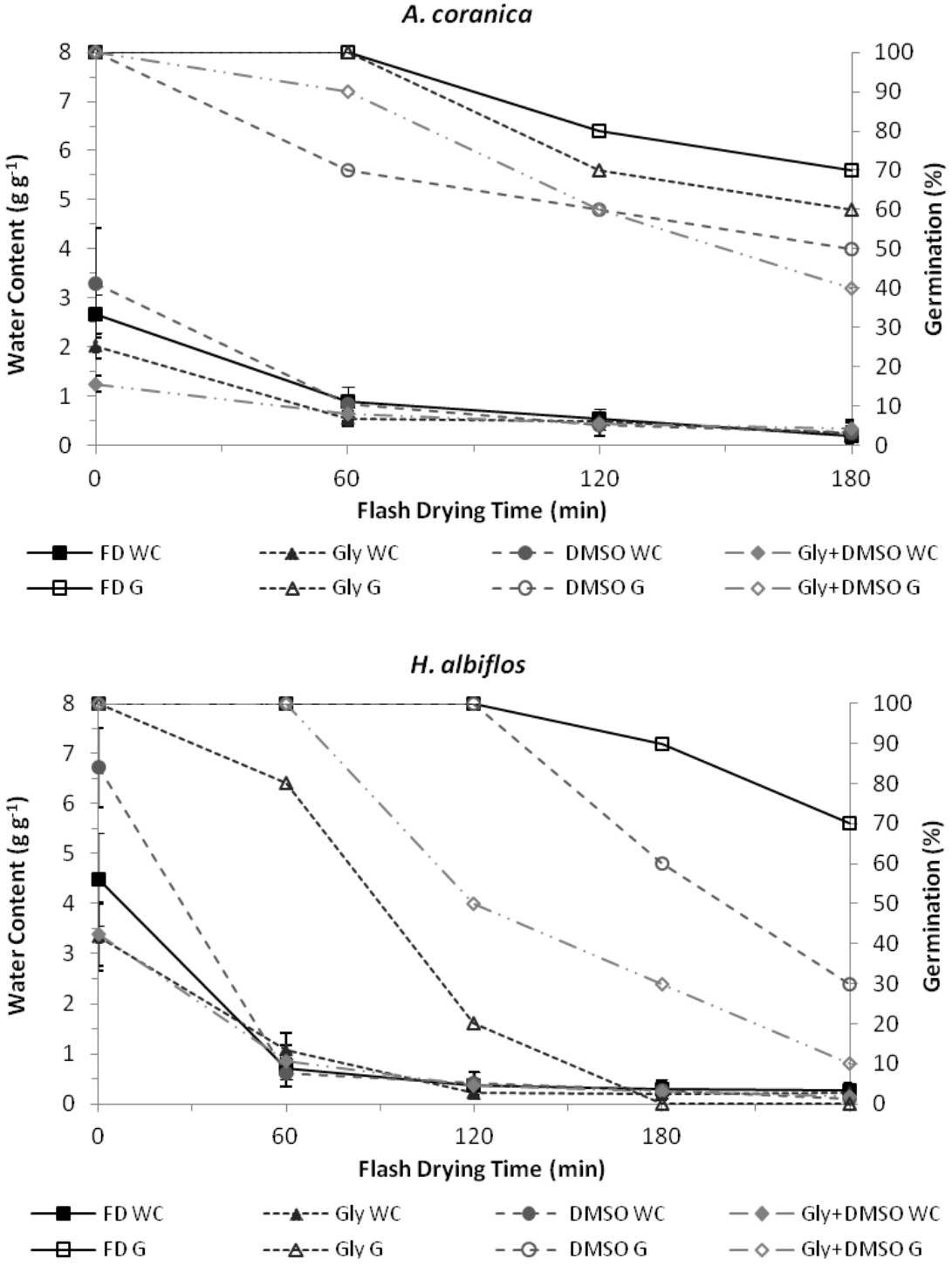


Figure 3.5: Effects of cryoprotection in combination with flash drying on water content (WC; n=10) and germination (G; n=10) of *A. coranica* (year 1) and *H. albiflos* embryos. Curves are labelled according to treatment: FD= flash-dried embryos, Gly= glycerol-cryoprotected embryos, DMSO= DMSO-cryoprotected embryos, Gly+DMSO= embryos cryoprotected with both glycerol and DMSO.

The germination results reported thus far are based on data collected after at least 4 weeks of *in vitro* recovery. When embryos were observed later during *in vitro* recovery it was evident that flash drying altered growth patterns relative to undried (fresh) embryos and this was exacerbated when embryos were cryoprotected before flash drying. Normal germination was considered to be the development of both a root and a shoot during recovery of the explants, indicative of seedling establishment. However, abnormal development was also observed to result from variously treated embryos; this was scored after observation of the development of either shoot alone, root alone, or callus (see Fig. 3.7) which were considered to be a sign of survival (but not germination). Fig. 3.6 shows that fresh embryos of *A. coranica* showed 100% germination, but this was compromised after flash drying as some of the flash-dried embryos then displayed abnormal development (Fig. 3.6A). Figs. 3.6B, C and D further show that cryoprotection before drying predisposed embryos such that production of normal seedlings was further compromised. After 120 min of flash drying, the lowest percentage production of normal seedlings occurred after cryoprotection with glycerol, while, after dehydration for 180 min seedling development was compromised the most for embryos exposed to the mixture of glycerol and DMSO. In essence, flash drying compromised the capacity of some embryos to produce and establish seedlings and this effect was exacerbated by cryoprotection prior to flash drying. Such abnormalities in growth have also been reported for *A. belladonna* zygotic embryos (Sershen *et al*., 2008), where partial dehydration (by flash drying) reduced the number of embryos that produced seedlings, as well as the subsequent *in vitro* biomass of resulting seedlings. In addition to this, a number of studies (e.g. Wesley-Smith *et al*., 2001b; Sershen *et al*., 2007; Steinmacher *et al*., 2007) have reported the development of abnormal phenotypes in seedlings recovered from partially dehydrated recalcitrant axes. The callusing of those embryos that did not form either a root or a shoot was considered an indication that flash drying had a detrimental effect on their overall germinative capacity.

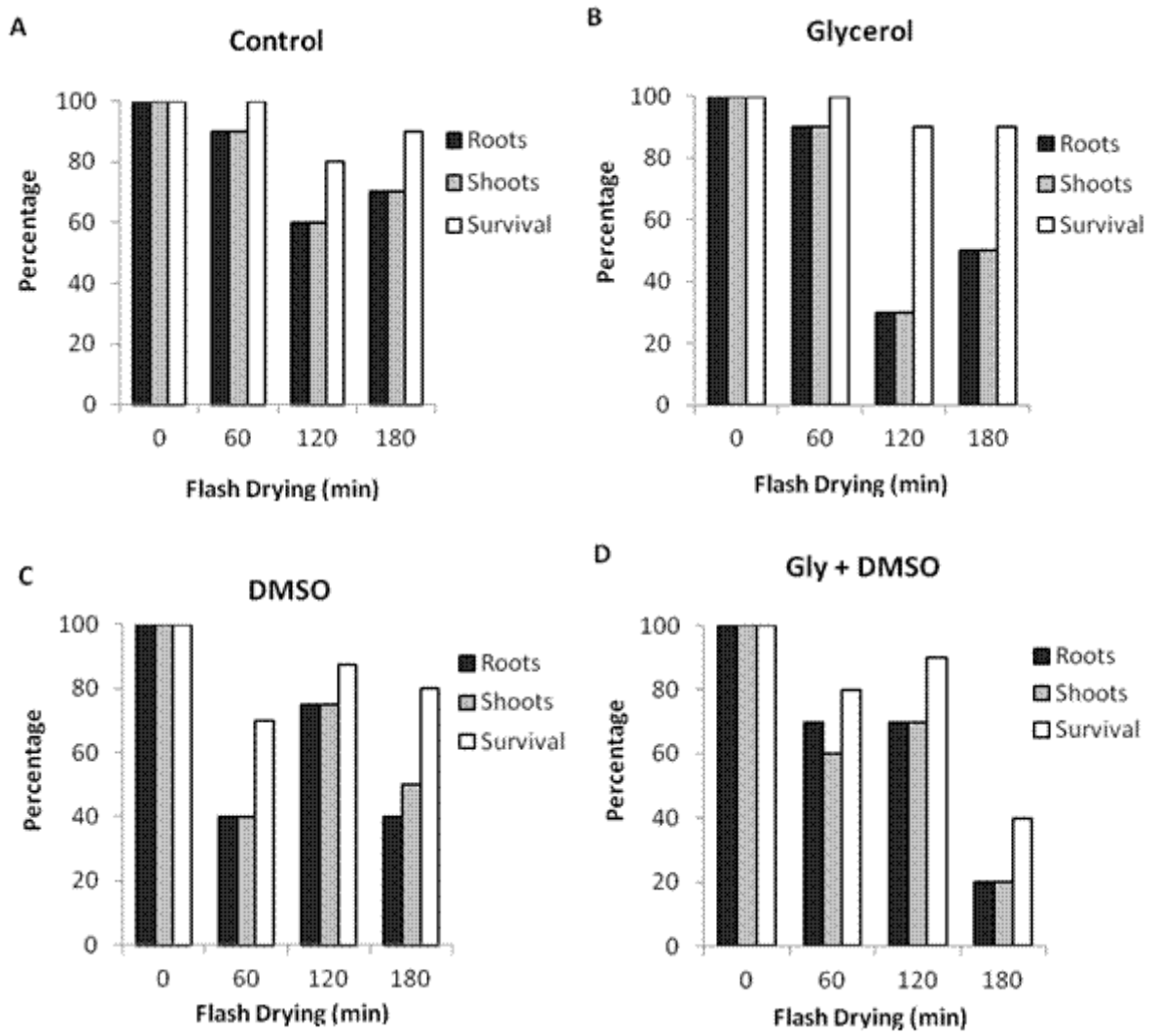


Figure 3.6: Viability of *A. coranica* embryos (n=10) after 6 weeks *in vitro* in response to flash drying before and after cryoprotection; (**A**) whole zygotic embryos without cryoprotection, (**B**) whole zygotic embryos cryoprotected with glycerol; (**C**) whole zygotic embryos cryoprotected with DMSO; (**D**) whole zygotic embryos cryoprotected with a mixture of glycerol and DMSO.

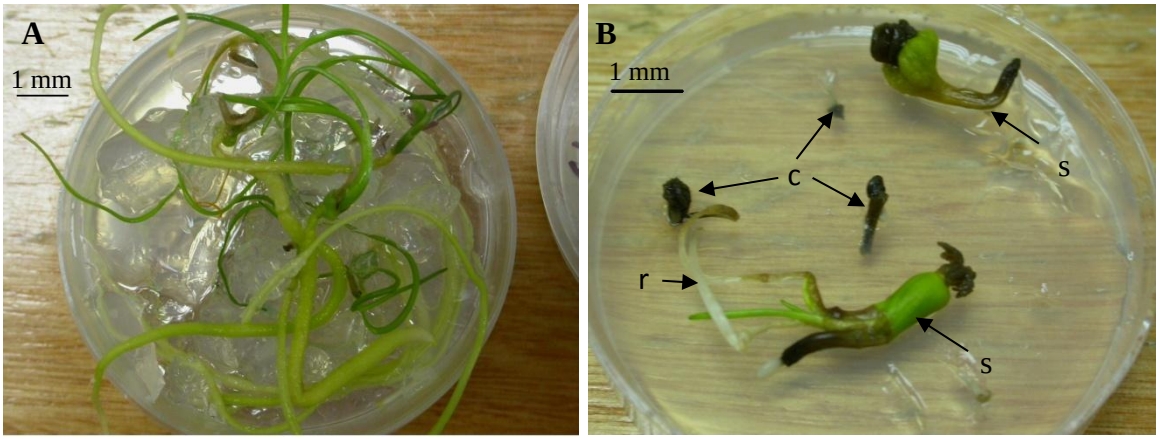


Figure 3.7: Zygotic embryos of *A. coranica* after a 1 month of *in vitro* recovery. (**A**) Showing normal development of untreated embryos (n=5), control; (**B**) showing abnormal development [s=shoot, r=root, c=callus] after flash drying (for 120 min to 0.45±0.21 g g^{-1}).

3.3. Attempts to improve survival after partial dehydration

3.3.1. Cathodic protection

If a successful cryopreservation protocol is to be established for any species, the stress incurred by explants during the preconditioning steps (such as flash drying and/or cryoprotection) must be minimised in order to promote post-cryo survival (Sershen *et al.*, 2007). Given the compromised ability for seedling production by some embryos after flash drying, it was evident that the damage incurred should be minimised in order to ensure better post-cryo survival. For this reason, the effect of incorporating cathodic protection during flash drying was investigated. As described (**Chapter 2**), cathodic protection was applied by attaching a cathode to the metal mesh on which the embryos were placed in the flash dryer (Fig. 2.5). Cathodic protection, based on the principles of electrode kinetics, is often used in industry to minimise oxidative damage to a variety of large structures (Srinivasan *et al.*, 1996). Cathodic protection applied to seeds was originally reported to have a beneficial effect on maize caryopses exposed to age-accelerating conditions (Pammenter *et al.*, 1974). In the present study cathodic protection was applied in an attempt to limit production or effects of reactive oxygen species (ROS) consequent upon deranged metabolism accompanying the stress of dehydration of the embryos.

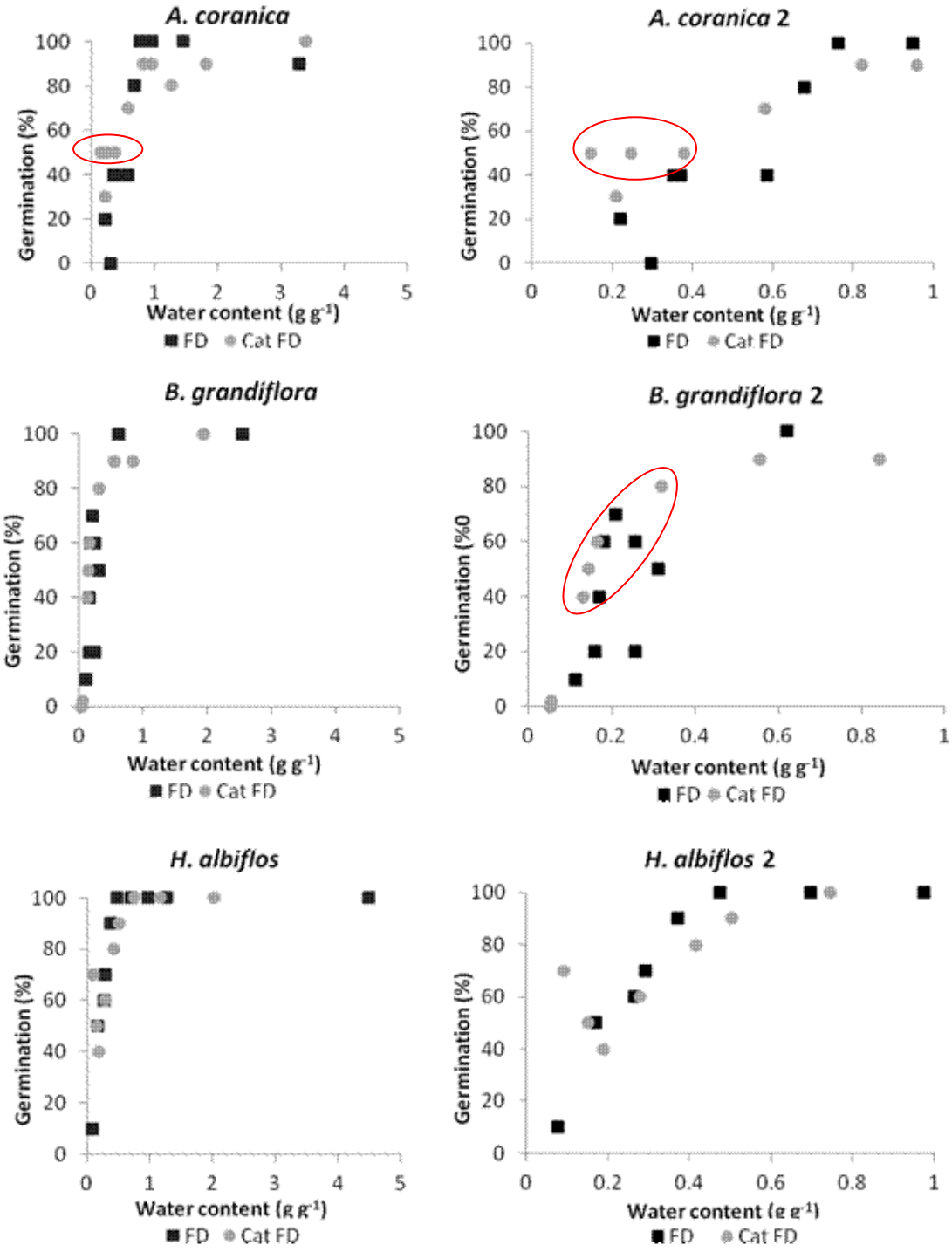


Figure 3.8: Comparison of the effect of cathodic flash drying (Cat FD) and conventional flash drying (FD) on the viability of zygotic embryos (n=10) of the selected species. Left column shows the effects of drying on germinability at the water contents tested; Right column indicates the effects on germinability at water contents below 1.0 g g^{-1}.

The results showed that there were no significant differences ($p>0.05$) in viability at comparable WCs when a comparison was made between the embryos that had been exposed to cathodic and conventional flash drying (Fig. 3.8). However, it was noted that cathodic protection promoted viability retention of the embryos of *A. coranica* at WCs in the range of 0.15 g g^{-1} to 0.40 g g^{-1}. Most importantly, these WCs were within the range (indicated by encircled datum points in Fig. 3.8) suggested for cryopreservation of recalcitrant zygotic embryos. When graphically depicted, this beneficial effect was not as apparent for embryos of the other two species; however, survival of embryos of *B. grandiflora* after cathodic flash drying was considerably enhanced (Table 3.1).

Table 3.1: Comparison of the effect of cathodic flash drying (Cat FD) and conventional flash drying (FD) on the water content (WC) and viability of zygotic embryos (n=10) of the selected species. %G= percentage of embryos that germinated.

A. coranica				***B. grandiflora***				***H. albiflos***			
FD WC	**%G**	**Cat FD WC**	**%G**	**FD WC**	**%G**	**Cat FD WC**	**%G**	**FD WC**	**%G**	**Cat FD WC**	**%G**
0.22±0.11	20	0.21±0.13	50	0.11±0.04	10	0.13±0.07	40	0.08±0.02	10	0.09±0.03	70
0.30±0.17	0	0.25±0.12	30	0.17±0.07	40	0.17±0.08	60	0.17±0.16	50	0.19±0.18	40
0.37±0.26	40	0.38±0.26	50	0.31±0.08	50	0.32±0.11	80	0.26±0.16	60	0.28±0.21	60

No statistical analysis was conducted on the %G data as the values reflected in this table were from a single trial

Further to the studies presented above, investigations were also implemented to test the effects of cathodic protection during rehydration. Cathodic protection here was afforded by using the cathodic fraction of a dilute electrolysed salt solution ('cathodic water'; Berjak *et al*., 2011b) in place of the CaMg solution to re-hydrate explants after flash drying. No improvement, in terms of viability retention, of the embryos within the targeted WC ranges was achieved by rehydration with cathodic water, as the germination totalities were not substantially higher than for those re-hydrated with the CaMg solution (data shown for *H. albiflos* in Fig. 3.9). This investigation was not conducted on embryos of *A. coranica* and *B. grandiflora* because of limitations in seed numbers.

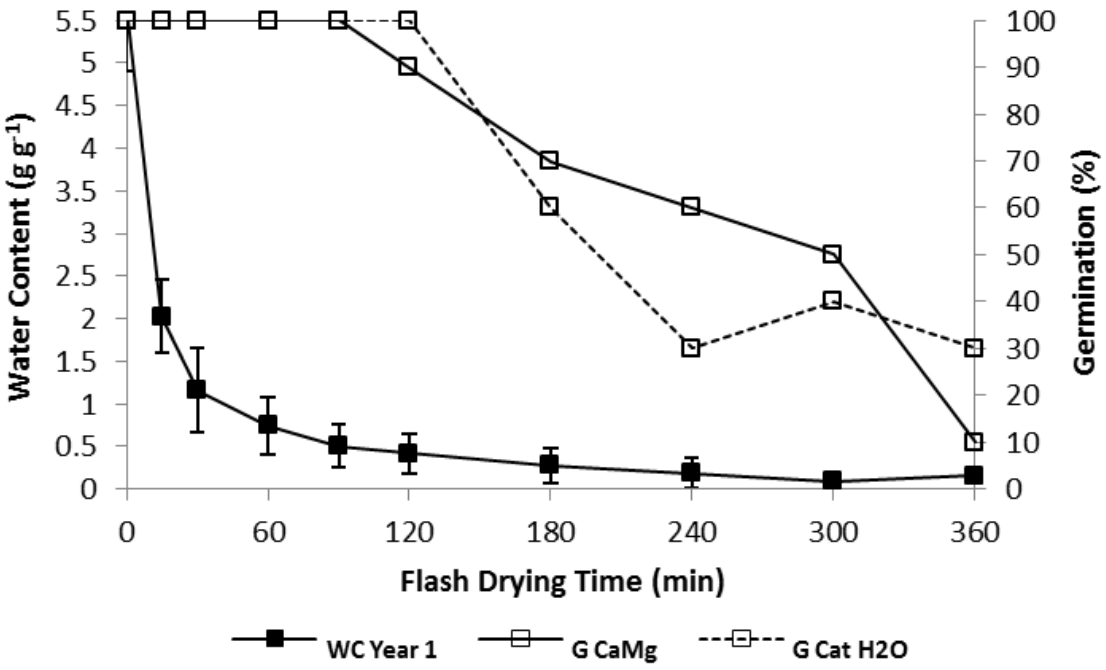


Figure 3.9: Comparison of germination (G) percentages of embryos (n=10) of *H. albiflos* that were recovered after flash drying and re-hydrated with either a calcium magnesium solution (CaMg) or with cathodic water (Cat H_2O). Error bars represent the mean±SD (n= 10).

3.3.2. Vacuum-supplemented flash drying

Vacuum-supplemented drying was effected by applying a light vacuum to the explants during flash drying. Since dehydration damage is a consequence of both drying intensity (Varghese and Naithani, 2002) and duration (Walters *et al*., 2002a), this technique was explored in an attempt to enhance the rate of dehydration of explants in order to shorten the duration of flash drying required to reach WC ranges amenable for cooling (i.e. 0.2-0.4 g g^{-1}). The results show that applying a vacuum during flash drying had no significant effect ($p>0.05$) on the rate of water loss in the embryos of *A. coranica* when compared with conventional flash drying (Fig. 3.10). For instance, vacuum and conventionally flash-dried embryos required at least 90 min to reach WCs within 0.2-0.4 g g^{-1}. However, as was the case for the embryos that had undergone cathodic protection, after vacuum-supplemented flash drying slightly higher germination percentages of *A. coranica* embryos were obtained at certain WCs within this range.

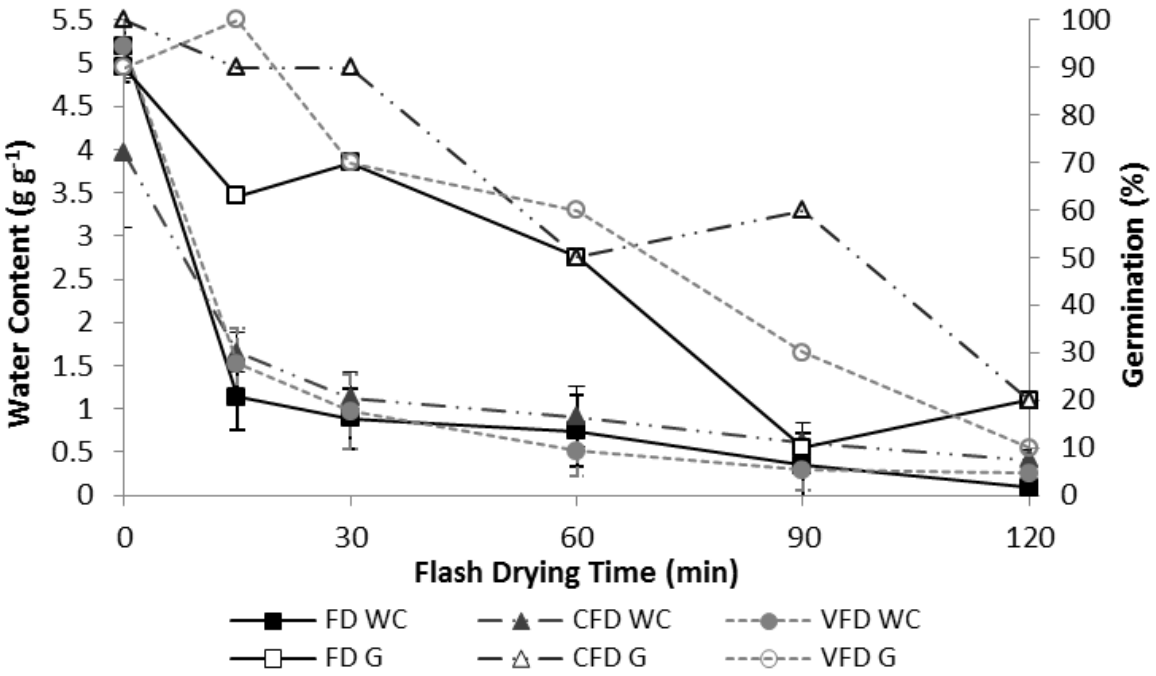


Figure 3.10: Comparison of the effects of cathodic flash drying (Cat FD) and vacuum-supplemented flash drying (VFD) to unmodified flash drying (FD) on the water content and viability (G= germination) of the zygotic embryos of *A. coranica*. Error bars represent the mean±SD (n= 10).

With reference to the results discussed above, while cathodic flash drying appeared to confer some benefit in promoting germination, this was not the case when embryos were re-hydrated with cathodic water, nor when vacuum-supplemented flash drying was carried out. Hence, cathodic protection was applied when partially flash drying the zygotic embryos used in subsequent cryopreservation studies.

3.4. Response to exposure to cryogenic temperatures

Previous studies on amaryllid embryos and zygotic embryos/axes of a number of other recalcitrant-seeded species have shown rapid, non-equilibrium, cooling rates to be far superior to slow cooling rates at promoting post-cryo survival (Sershen *et al.*, 2007; 2012a); therefore, rapid cooling rates were employed for all the cryopreservation studies described below. Pilot studies on all three species investigated here also confirmed that slow cooling rates (1°C min^{-1} down to -70°C, followed by immersion into LN [at -196°C]) were unsuitable for the material being investigated: no survival was achieved for slowly cooled embryos of

any of the species, irrespective of the WC or cryoprotectant used. The loss of viability of all the explants can mainly be attributed to the fact that slow cooling allows ample time for explants to undergo further dehydration (Karow, 1969; Mazur, 1990) – i.e. to WCs below those induced by flash drying – resulting in extreme desiccation beyond the limits that can be tolerated by such recalcitrant germplasm (Wesley-Smith *et al*., 2001a; Fuller, 2004). Extreme dehydration stress is suspected to far surpass that exerted by mechanical damage alone, and is suggested to result in intracellular perturbation due to the removal of structure-associated water. In addition, other reasons for the lack of survival of slow cooling might have been exposure of the cells to highly concentrated intra- and extra-cellular cryoprotectant solutions, and mechanical interactions between tissues and extracellular ice (Mazur, 1977). For these reasons all the results described from here onwards pertain to embryos cooled rapidly by exposure to nitrogen slush (hundreds of ˚C min^{-1}; Wesley-Smith *et al.*, 2004b). The preconditioning (cryoprotection and drying) treatments selected for these studies were also limited to those shown to be least detrimental in earlier studies, namely glycerol and DMSO cryoprotection, and flash drying (with and without cathodic protection) to WCs in the range of 0.2-0.4 g g^{-1}. Additionally, all cryopreservation studies involving cryoprotected and cathodically protected explants were performed mainly on whole zygotic embryos. Futhermore, cooling trials were conducted on excised axes with a piece of cotyledonary body attached, as carried out by Sershen *et al*. (2007); however, these experiments were limited to axes that had only undergone (unmodified) flash drying due to abating seed numbers. Reducing the explant size offered the advantage of presenting a larger surface area to volume ratio of the sample which could facilitate faster drying and cooling rates (Muldew *et al.*, 2004; Hor *et al.*, 2005; Naidoo *et al.*, 2011) and ultimately promote normal development.This was attempted for *A. coranica* and *H. albiflos*, but cryopreservation studies on excised axes could not be done for *B. grandiflora* because of limited seed numbers. Cathodic protection during flash drying could not be tested on embryos that had been cryoprotected, also, because of limited seed numbers.

When the viability data for cryopreserved embryos was assessed across species it was evident that cooling to cryogenic temperatures (and/or subsequent warming) was deleterious, irrespective of the WC prior to cooling or cryoprotectant used (Tables 3.2-3.4). Rapid cooling, unlike slow cooling, did, however, allow for 30% germination by glycerol cryoprotected *A. coranica* embryos and varying levels of callus-based embryo survival in the

case of both *A. coranica* and *H. albiflos*. In contrast, no *B. grandiflora* embryos survived after cryopreservation, regardless of the preconditioning treatment applied. In *A. coranica* embryos, callus-based survival ranging from 10 to 30%, was obtained for axes and whole embryos and was highest (70%) in embryos that had undergone cathodic flash drying down to WCs of 0.35±0.21 g g^{-1}. The only treatment that resulted in *A. coranica* zygotic embryo germination (i.e. formation of roots and shoots with no intermediate callus stage) involved cryoprotection with glycerol and subsequent (unmodified) flash drying to 0.36±0.10 g g^{-1} before rapid cooling. Other studies have also shown glycerol cryoprotection followed by dehydration to WCs in this range to be suitable for the successful cryopreservation of recalcitrant amaryllid zygotic embryos (Sershen *et al.*, 2011; 2012a).

The callus-based survival obtained for 3-4 mm axes of *A. coranica* was recorded for axes that were cooled at WCs of 3.02±0.31, 0.79±0.62 and 0.60±0.33 g g^{-1}, which were considerably higher than the WCs range (0.2-0.4 g g^{-1}) suggested to be generally suitable for the cryopreservation of recalcitrant seed germplasm (Normah and Makeen, 2008; Sershen *et al*., 2012b). The fact that axes at such high WCs showed survival, albeit callus-based, was intriguing (since vitrification during cooling was unlikely to have occurred) and is thought to be the result of reducing the explant size from the 6 mm embryo to a 3-4 mm segment. A reduction of explant size is held to allow for more homogenous drying across the specimen, and, in the present case, the removal of most of the cotyledonary tissue would have resulted in a more homogenous size, cellular composition and physiological state of the explants, as discussed by Engelmann (2004). Even though reduction of explant size allows for a faster dissipation of heat, promoting relatively faster cooling rates (Wesley-Smith *et al*., 2004a; b) than of whole embryos, water contents in the range *c*. 0.6-3.0 were most likely to have resulted in ice crystal formation within meristematic cells. As organised meristems are essential for normal growth, this may explain the rather abnormal development after cryogen exposure. Nevertheless, the fact that callusing occurred is evidence that not all the axis/meristematic cells were lethally damaged. Cryoprotection was not attempted on axes of *A. coranica* prior to cryogen exposure because of the limitation of seeds, so it is presently not known whether the effects on subsequently-cooled axes might have been beneficial.

It was also noteworthy that DMSO cryoprotection of embryos of *A. coranica* prior to cooling did not result in either germination or callus-based survival. Though DMSO was applied at

relatively low concentrations here, after flash drying for 60 and 180 min, exposure of embryos of *A. coranica* to DMSO was associated with considerable loss of viability, although root and shoot production seemed able to be sustained in more embryos flash-dried for 120 min (Fig. 3.6). Considering the survival data for 120-min flash-dried embryos to be somewhat anomalous (perhaps because of small sample sizes), there seems no doubt that the *A. coranica* DMSO-exposed embryos responded adversely to dehydration, and that the deleterious effects were lethally exacerbated by exposure to cryogenic temperatures. The toxic effects of DMSO in plant tissues have been reported in other studies (e.g. Kartha, 1985; Rudolph and Crowe, 1986), which went further in demonstrating that the alteration of a specific enzyme (fructose diphosphatase, FDPase) was the cause of impaired glycolysis after treatment with DMSO which led to viability loss. Exceptions do exist though; Kim and Oh (2009) for example, showed 10% DMSO to be more effective than 20% glycerol at cryoprotecting embryogenic cell suspension cultures. In the present study, some 30-50% of *A. coranica* embryos produced a root and a shoot after cryoprotection with glycerol followed by flash drying (Fig. 3.6). However, it appears that there were no further adverse effects upon exposure to cryogenic temperatures, as when glycerol-cryoprotected *A. coranica* embryos were dried to WCs between 0.2 and 0.4 g g^{-1} 30% exhibited normal growth (Table 3.2). It was encouraging to note that 70% of embryos which were cathodically protected during flash drying (for 180 min) showed survival as callus. It is thus conjectured that glycerol-cryoprotected embryos may exhibit far better germination (than 30%), if flash-dried to *c*. 0.3 g g^{-1} with the application of cathodic protection. The 70% survival of those non-cryoprotected embryos, although manifested by callusing, may be accredited to the ROS quenching properties by the static negative field applied to the explants during flash drying (Pammenter *et al.*, 1974). This aspect demands further investigation which unfortunately could not be presently undertaken due to the seasonality and limitation of seed numbers.

Table 3.2: The effects of rapid cooling to cryogenic temperatures on the germinability of variously-treated zygotic embryos of *A. coranica*.

Species	Explant	CPA	DT (min)	WC ($g\ g^{-1}$)	G (%)
A. coranica	Axes	-	0	3.02±0.31	20**
	Axes	-	180	0.79±0.62	10**
	Axes	-	240	0.60±0.33	30**
	Embryos	-	0	3.09±0.34	30**
	Embryos	-	120	0.80±0.32	0
	Embryos	-	120*	0.58±0.45	0
	Embryos	-	180	0.65±0.27	0
	Embryos	-	180*	0.35±0.21	70**
	Embryos	-	240	0.35±0.22	0
	Embryos	-	240*	0.25±0.12	0
	Embryos	Glycerol	0	2.87±0.49	0
	Embryos	Glycerol	120	0.53±0.26	0
	Embryos	Glycerol	180	0.36±0.10	30
	Embryos	Glycerol	240	0.27±0.09	0
	Embryos	DMSO	0	4.30±0.44	0
	Embryos	DMSO	120	0.36±0.10	0
	Embryos	DMSO	180	0.32±0.35	0
	Embryos	DMSO	240	0.12±0.25	0

CPA=cryoprotectant; DT=drying time; WC=water content; G= germination, n=10 for germinability and WC.* Embryos exposed to cathodic flash drying; ** Embryos/axes callused rather than directly developing shoots and roots on recovery

For *B. grandiflora* cooling was tested only on zygotic embryos, with the results showing that none of the treatments promoted survival after cryopreservation (Table 3.3). This was initially surprising, as the embryos of this species displayed the most rapid dehydration rate (see Fig. 3.3). However, when the drying curves for the three species were compared, it was evident that *B. grandiflora* was the most sensitive to dehydration, displaying only 20% viability at WC 0.26 g g^{-1} whilst ≥50% of those of *A. coranica* and *H. albiflos* retained viability at similar WCs (Fig. 3.3). It was also disappointing that the potential survival after flash drying with cathodic protection (Fig. 3.8; Table 3.1) was not realised after cryogen exposure. However, the low WC – 0.19±0.03 after 120 min of cathodic flash drying – is likely to have been a factor precipitating viability loss by desiccation damage *sensu stricto* – not initially, but when aggravated by cryogenic cooling. Also, as embryo viabilities after the combination of cryoprotection and flash drying (in the absence of cooling) could not be accommodated due to lack of material, there is a possibility that cryoprotection could have exacerbated the damage incurred during flash drying, predisposing the embryos to even greater damage upon exposure to cryogenic temperatures.

Table 3.3: The effects of rapid cooling to cryogenic temperatures on the germinability of variously-treated zygotic embryos of *B. grandiflora*.

Species	Explant	CPA	DT (min)	WC (g g^{-1})	G (%)
B. grandiflora	Embryos	-	0	1.95±0.35	0
	Embryos	-	60	0.32±0.11	0
	Embryos	-	60*	0.38±0.12	0
	Embryos	-	120	0.14±0.15	0
	Embryos	-	120*	0.19±0.03	0
	Embryos	Glycerol	0	0.37±0.11	0
	Embryos	Glycerol	60	0.30±0.08	0
	Embryos	Glycerol	120	0.21±0.06	0
	Embryos	DMSO	0	2.74±0.48	0
	Embryos	DMSO	60	0.23±0.17	0
	Embryos	DMSO	120	0.16±0.09	0

CPA=cryoprotectant; DT=drying time; WC=water content; G= germination, n=10 for germinability and WC.* Embryos exposed to cathodic flash drying.

When *H. albiflos* embryos and axes were subjected to cryopreservation very low levels – 10% – of callus-based survival were observed across a few treatments, mainly those involving non-cryoprotected embryos dried to WCs less than 0.4 g g^{-1} (Table 3.4). It must be noted that though these low levels of survival are reported for relatively low mean WCs (some <0.25 g g^{-1}), drying was not even across different embryos (as evidenced by the large standard deviations [Table 3.4]). At this point it was already obvious that the response of the explants to the cryopreservation procedures is species-specific, and *H. albiflos* embryos particularly, dried relatively more slowly than those of *B. grandiflora* even though they dried at comparable rates to those of *A. coranica* (Fig. 3.3). This alone probably led to a degree of damage incurred during slow drying (Sershen *et al*., 2011), preconditioning them to an even greater damage after cooling (Sershen *et al*., 2012b). In addition, it was shown that cryoprotection exacerbated the detrimental effects of drying (Fig. 3.5) as also shown for *Citrus aurantium* by Al-Ababneh *et al.* (2002), and that this had preconditioned the embryos for even further damage during and after cooling.

Table 3.4: The effects of rapid cooling to cryogenic temperatures on the germinability of variously-treated zygotic embryos of *H. albiflos*.

Species	Explant	CPA	DT (min)	WC ($g\ g^{-1}$)	G (%)
H. albiflos	Axes	-	0	3.19±0.35	0
	Axes	-	60	0.78±0.49	0
	Axes	-	120	0.39±0.18	0
	Axes	-	180	0.37±0.21	0
	Axes	-	240	0.17±0.09	10**
	Embryos	-	0	4.48±0.92	0
	Embryos	-	60	0.70±0.21	0
	Embryos	-	120	0.37±0.27	10**
	Embryos	-	180	0.29±0.20	10**
	Embryos	-	240	0.26±0.16	10**
	Embryos	Glycerol	0	3.33±0.67	0
	Embryos	Glycerol	60	1.08±0.35	0
	Embryos	Glycerol	120	0.21±0.06	0
	Embryos	Glycerol	180	0.20±0.05	0
	Embryos	Glycerol	240	0.21±0.07	0
	Embryos	DMSO	0	6.71±0.80	0
	Embryos	DMSO	60	0.62±0.28	0
	Embryos	DMSO	120	0.41±0.23	0
	Embryos	DMSO	180	0.27±0.19	0
	Embryos	DMSO	240	0.11±0.28	10**

CPA=cryoprotectant; DT=drying time; WC=water content; G= germination, n=10 for germinability and WC. ** Embryos/axes callused rather than directly developing shoots and roots after recovery

None of the embryos, of the species used in this study that were of WCs higher than the range selected as amenable for cooling (0.2-0.4 g g^{-1}) survived the cooling step. When recalcitrant axes/embryos at high water contents are cooled rapidly below 0°C, the intracellular solution becomes supercooled, which predisposes tissues to the possibility of lethal intracellular ice formation (Vertucci *et al*., 1991; Pence, 1992; Wesley-Smith *et al*., 1992). These theoretical explanations substantiate some of the difficulties in attaining successful cryopreservation of recalcitrant germplasm, as was shown by Sun (1999) for the embryonic axes of *Quercus rubra*. High tissue WCs equate to lower intracellular viscosity, which requires ultra-rapid cooling rates in order to prevent/limit ice crystal damage in cells (Wesley-Smith *et al*., 2004a). However, the ability to cool rapidly at high water contents is limited by the ability of the tissue to dissipate heat fast enough to prevent severe damage (Wesley-Smith *et al*., 2004a). For pure water, glass forms at -138 °C (Turnbull, 1956; Chen, 2000). Glass-phase water cannot ordinarily be formed because ice crystallises at considerably higher temperatures, but vitrification of pure water can be achieved by very rapid cooling (106 °C sec^{-1}) so that ice crystals do not have time to form. However, cooling rates as high as this are achievable for only up to about 10 µ in depth if the water surface is instantaneously exposed to LN temperatures (Johari *et al*., 1996). The most successful technique for the vitrification of pure water has been supersonic deposition of droplets onto cryogenically cooled surfaces (Johari *et al.*, 1996). Cooling rates as high as this have only rarely been achieved for cells (Goetz and Goetz, 1938) and there does not seem to be any way to achieve similar rates for tissues or whole organs. The best compromise towards achieving really rapid cooling rates is the use of the smallest possible explants for cryopreservation. This was factored-in in this study, as the reduction of the size of explants from the *c*. 6 mm zygotic embryos to using 3-4 mm excised axes (see Fig 3.4 and Tables 3.2-3.4) was explored.

The mechanism by which cryoprotectant solutions protect cells from damage – even in the case of vitrification solutions – is still poorly understood (Fuller, 2004; Volk and Walters, 2006). As the constituents are often applied in high concentrations, they may function as elaborate desiccants and decrease the amount of water that is available to form lethal ice crystals (Sakai *et al.*, 1991). Alternatively, they may stabilise cell structures during desiccation and cooling, promoting glassy states in relatively dry biological systems (Crowe *et al.*, 1998; Bryant *et al.*, 2001). Furthermore, vitrification agents may structure the water remaining in cells so that it is less likely to freeze (Wolfe *et al.*, 2002). Cryoprotectant

solutions, even in some instances those containing glycerol, are known to be toxic to cells, specifically during cooling, and prolonged exposure exacerbates the problem (Wang *et al.*, 2007). A number of studies using thermal analyses (DSC) of plant materials demonstrate a strong relationship between the presence of freezing or melting transitions of water and damage at subfreezing temperatures (Dereuddre and Kaminski, 1992; Vertucci and Stushnoff, 1992; Wesley-Smith *et al.*, 1992; Benson *et al.*, 1996; Martinez *et al.*, 2000; Kim *et al.*, 2005). Evidence of first order water transitions (i.e., freezing and melting) have been observed in seed tissues (Vertucci, 1989a; b; Vertucci *et al.*, 1991; Wesley-Smith *et al.* 1992; Farrant and Walters, 1998), pollen (Buitink *et al.*, 1996), winter-hardy buds (Vertucci *et al.*, 1991), naked shoot tips of olive (Martinez *et al.*, 2000), and shoot tips encapsulated in calcium alginate beads (Benson *et al.*, 1996; Dumet *et al.*, 2000; Sherlock *et al.*, 2005) at WCs >0.25-0.4 g g^{-1}. Plant organs that survive LN temperatures are usually at similar, or even lower, WCs (Dereuddre and Kaminski, 1992; Wesley-Smith *et al.*, 1992), and the window of acceptable WCs can be widened by increasing cooling rate to over 100 °C s^{-1} (Wesley-Smith *et al.*, 1992; 2001b). This then is suggested to be the main cause leading to the lack of survival after cryopreservation in the embryos/axes of the selected species that were at WCs above this range in the present study.

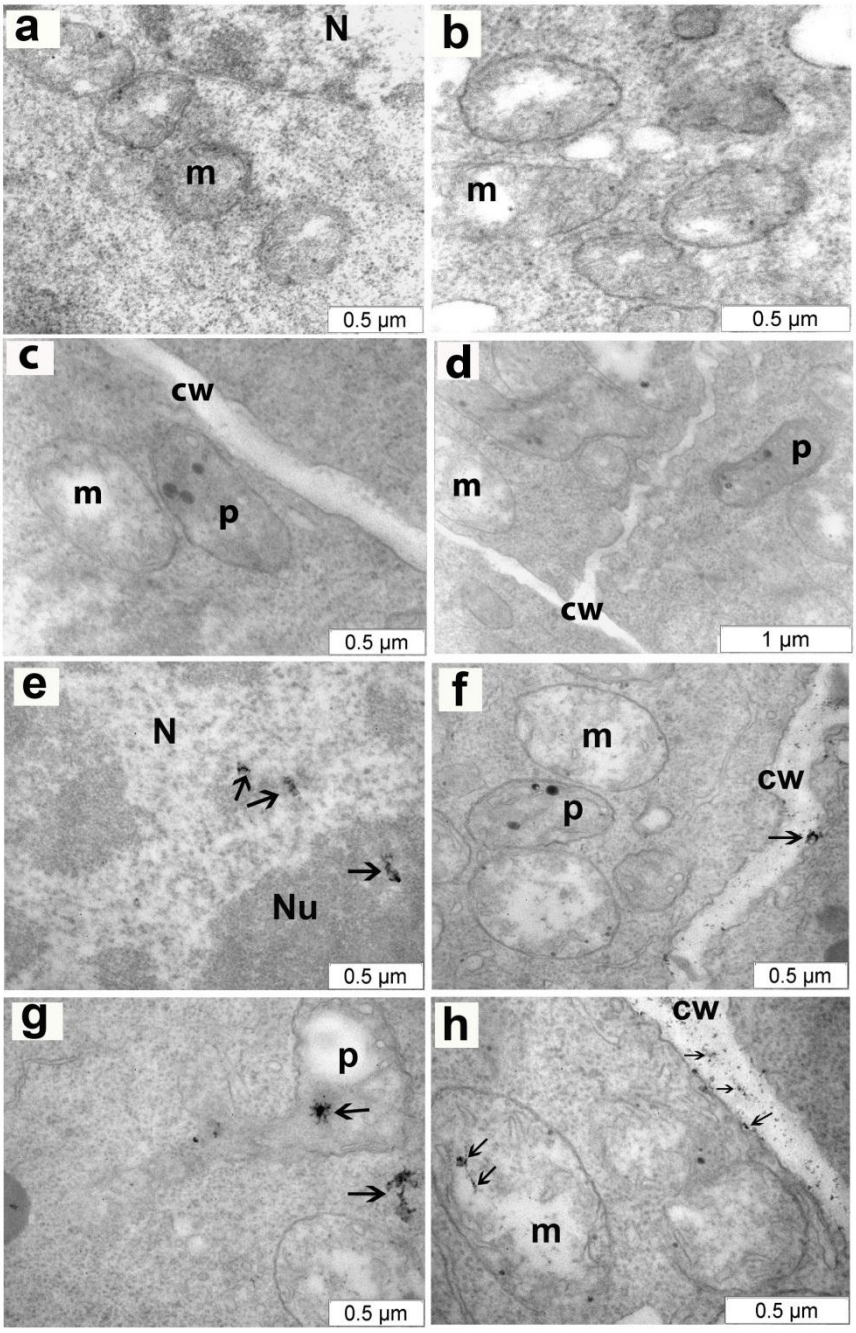
3.5. Intracellular localisation of hydrogen peroxide (H_2O_2) in *A. coranica* embryos exposed to the various procedural steps involved in cryopreservation

In the context of the viability data discussed above (Tables 3.2-3.4) it was evident that the recalcitrant zygotic germplasm of all three of the species presently investigated was extremely sensitive to the preconditioning (cryoprotection, partial dehydration, and their combination) and cooling steps involved in their cryopreservation. Recent studies (Varghese *et al*., 2011; Sershen *et al*., 2012c) have shown that oxidative stress is associated with the loss of viability frequently accompanying the preconditioning and subsequent cooling of recalcitrant zygotic germplasm. Based on these suggestions, and the poor maintenance of post-cryo viability in the present study, an aspect of the possible effect of oxidative stress during the procedural steps involved in the cryopreservation of *A. coranica* was investigated. These studies involved exposing *A. coranica* axes to the various procedural steps and thereafter assessing the intracellular production of H_2O_2, via ultrastructural localisation of perhydroxide precipitates produced as a consequence of the oxidation of cerium chloride ($CeCl_3$; Bestwick *et al*., 1997) applied exogenously to the explant, after each treatment.

Whole embryos were exposed to the treatment but the ultrastructural studies were confined to the root meristematic cells.

Hydrogen peroxide can be used as a marker of changes in the oxidative status of plant tissues (Oracz *et al.*, 2007), as higher concentrations of non-dismutated H_2O_2 are particularly detrimental intracellularly (Riley, 1994) and can provoke the onset of cell death (Gechev and Hille, 2005). This molecule has a relatively long life in comparison with other ROS (Bienert *et al.*, 2006). In most cases, H_2O_2 production is the result of a two-step reduction of molecular oxygen (the first step leading to the superoxide radical), with this ROS being formed after the reduction of superoxide radicals catalysed by superoxide dismutase. Simultaneous with its production, a network of antioxidants is constantly 'on the alert' for rising H_2O_2 concentrations and provides effective scavenging (Apel and Hirt, 2004; Gechev *et al.*, 2006; Miller *et al.*, 2010). The biological effect of H_2O_2 is mostly dependent on its concentration, but also on the site of production, the developmental stage of the plant and previous exposures to different kinds of stress (Gechev and Hille, 2005). Additionally, its small size allows it to traverse cellular membranes and migrate to different compartments, which facilitates its signalling functions (Bienert *et al.*, 2006). Generally, at low concentrations it is known to act as a signalling molecule, while at higher concentrations it provokes the onset of cell death (Gechev and Hille, 2005).

Figure 3.11: Subcellular aspects of the root meristem of freshly excised zygotic embryos of *A. coranica* (water content 3.12±0.51 g g^{-1}). Images **a**–**d**, show material not incubated in $CeCl_3$ (control material), while images **e**–**h**, show the situation after incubation in $CeCl_3$ for localisation of hydrogen peroxide (H_2O_2). The cerium perhydroxide precipitates (electron-opaque [dark] deposits) indicating H_2O_2 localisation are indicated by arrows. *Abbreviations*: n, nucleus; Nu, nucleolus; cw, cell wall; m, mitochondrion; p, plastid.


a
N
m
0.5 µm
b
m
0.5 µm
c
cw
m
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0.5 µm
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m
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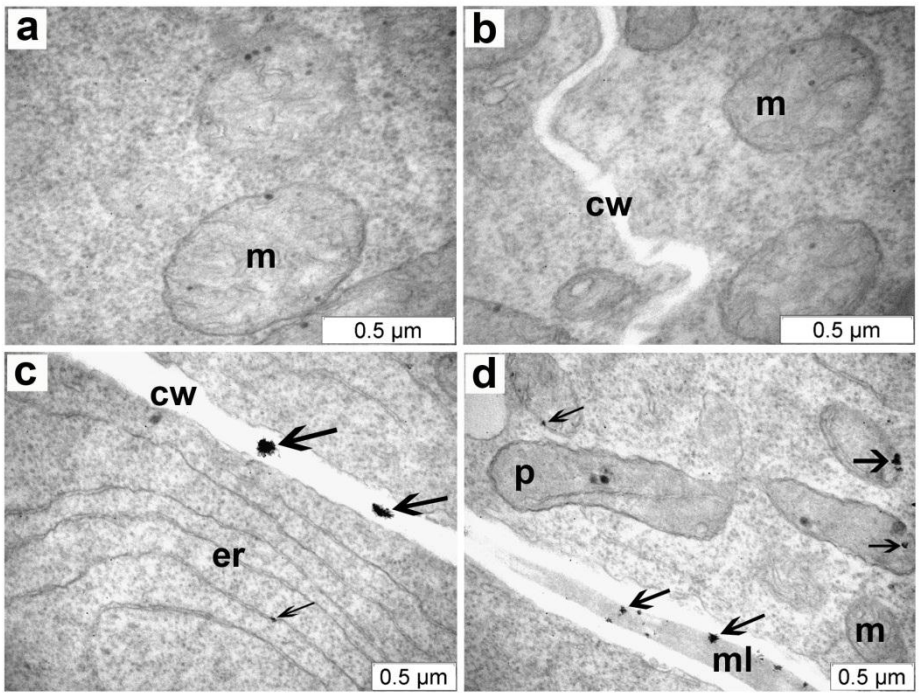
e
N
Nu
0.5 µm
f
m
p
cw
0.5 µm
g
p
0.5 µm
h
cw
m
0.5 µm

The results showed cerium perhydroxide precipitates to be located extracellularly associated with the cell walls, and intracellularly, in root meristematic cells of freshly excised (undried and uncooled) *A.coranica* embryos (Fig. 3.11e-h). Extracellular deposition was seen as dark deposits scattered along the apoplast and occasionally aggregating near the plasma membrane (indicated by arrows in Figs 3.11f and h). Cerium perhydroxide precipitates were also evident in some mitochondria as aggregates in the matrix, and in the nuclei (Figs 3.11 h and e, respectively). Furthermore, aggregates of the precipitates were also seen in the cytomatrix (Fig. 3.11g). The production of ROS in newly-excised embryos from the hydrated stored recalcitrant *A. coranica* seeds was not surprising as other authors have observed their production in embryos excised from newly-harvested recalcitrant seeds (Varghese *et al*., 2011) or those stored hydrated (Sershen *et al*., 2012c). The occurrence of ROS is a characteristic feature of fresh immature and mature non-orthodox seeds (Chaitanya and Naithani, 1994) and appears to be a natural consequence of respiration (Francini *et al*., 2006). The production of ROS in hydrated stored recalcitrant seeds, as shown for *A. coranica* in this study, is suggested to have been the consequence of a mild water stress that develops in recalcitrant seeds as a consequence of their on-going metabolism and germinative development (Pammenter *et al*., 1994; Sershen *et al*., 2012c). However, as in *A. coranica* (Fig. 3.11), presence of this ROS appears harmless in amaryllid embryos (e.g. those of *Amaryllis belladonna* and *Haemanthus montanus*) for short periods of time, as antioxidant enzymes are active (Pukacka and Ratajczak, 2006; Sershen et al., 2012c). Additionally, H_2O_2 in mitochondria (Petrov and Van Breusegem, 2002; Turrens, 2003) and along the cell walls can be expected (Bolwell *et al.*, 2002; Agrawal *et al.*, 2003). The consistent localisation of H_2O_2 in the apoplast of all cells observed could be explained by the suggestion that the main source of this ROS is a class of cell membrane NADPH-dependent oxidases (Agrawal *et al*., 2003) and cell wall-associated peroxidases (Bolwell *et al.*, 2002). Additionally, its presence within the mitochondria and cytomatrix was consistent with other studies that have indicated that in plants, and particularly in seeds, ROS may originate from the mitochondrial respiratory chain or be produced through the action of enzymes such as NADPH oxidase (Bailly, 2004) and H_2O_2 can easily migrate to different cell compartments (Bienert *et al*., 2006).

Figure 3.12: Subcellular inspection for hydrogen peroxide (H_2O_2) in the root meristem of glycerol-cryoprotected (images **a**, **b**) and DMSO-cryoprotected (images **c**, **d**) zygotic embryos of *A. coranica*, at 2.43±0.73 and 4.74±0.56 g g^{-1}, respectively, visualised after staining with $CeCl_3$. The cerium perhydroxide aggregated precipitates (electron-opaque [dark] deposits) indicating H_2O_2 localisation, observed in DMSO-cryoprotected embryos, are indicated by arrows. *Abbreviations*: cw, cell wall; er, endoplasmic reticulum; m, mitochondrion; p, plastid; ml, middle lamella.


a
m
0.5 µm
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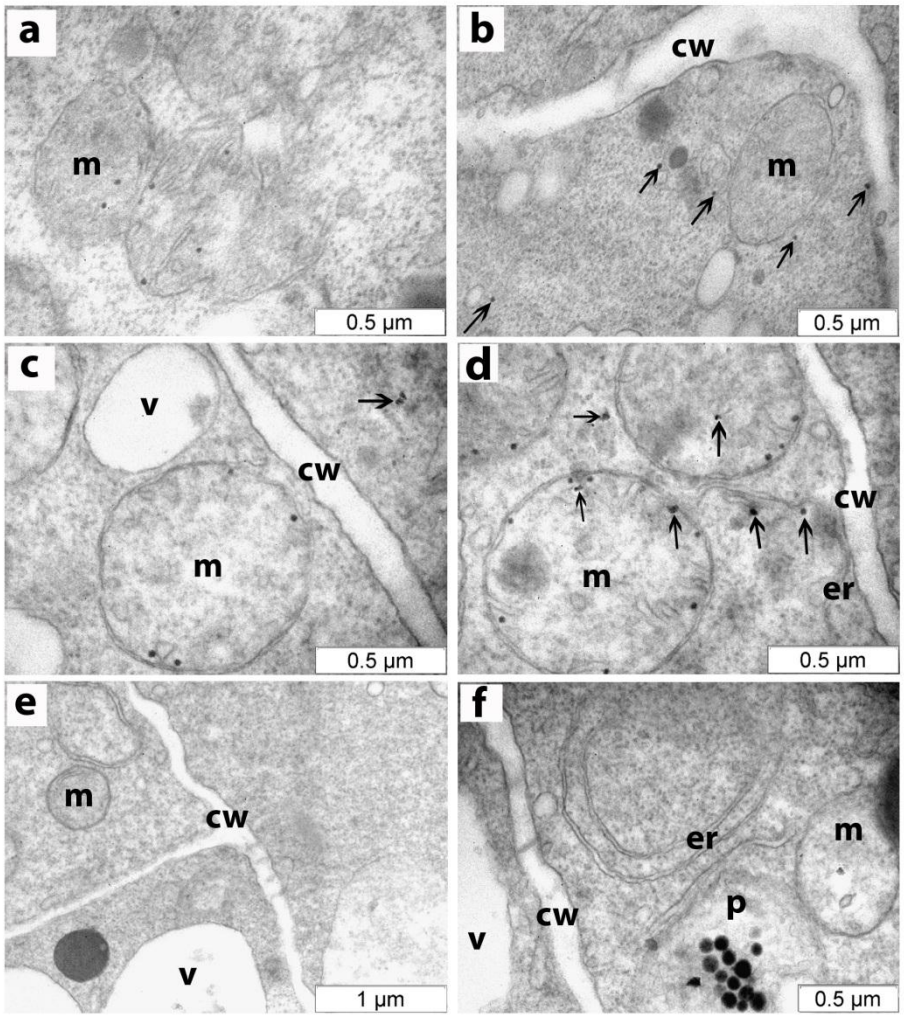
cw
er
0.5 µm
d
p
m
ml
0.5 µm

Interestingly, whilst no cerium perhydroxide precipitates were detected in the root meristematic cells of embryos cryoprotected with glycerol (Fig.3.12a and b), precipitate aggregates were detected both intra- and extracellularly in those cryoprotected with DMSO (Fig. 3.12c and d). The extracellular aggregation of the precipitates detected was in the apoplast (arrows, Fig. 3.12c) while the intracellular aggregation was mainly associated with organelles, with aggregates found in some mitochondria and plastids (arrowed, Fig. 12d). This evidence of H_2O_2 accumulation supports earlier suggestions that DMSO was toxic to the embryos investigated in this study and may explain why more embryos treated with DMSO lost viability than those cryoprotected with glycerol when flash-dried to comparable WCs (Fig. 3.5). If H_2O_2 dismutation does not occur rapidly it can be involved in the formation of hydroxyl radicals via the Fenton reaction, $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + HO^{-}$ (Benson and Bremner, 2004), which are the most damaging ROS in biological tissues (Halliwell, 2006). The absence of precipitates in the glycerol-cryoprotected material may possibly be explained by enhanced activity of some antioxidant defence mechanisms, as shown by Sershen *et al*. (2012c) for glycerol-cryoprotected, dried embryos of *A. belladonna* and *H. montanus*. Even though, a decline in the activity of some antioxidant enzymes was observed in that study (i.e. CAT and GR activity), the presence of ascorbate peroxidase (AsPX) in all cell compartments was believed to have had this impact because of its known high affinity for H_2O_2 (Pukacka and Ratajczak, 2006) which might have led to the enzymatic detoxification of H_2O_2.

Figure 3.13: Subcellular inspection for hydrogen peroxide (H_2O_2) in the root meristem of flash-dried (images **a**, **b**), flash-dried and cooled (images **c**, **d**), and cathodically flash-dried and cooled (images **e, f**) zygotic embryos of *A. coranica*; at 0.43±0.13, 0.43±0.13 and 0.35±0.05 g g^{-1}, respectively, visualised after staining with $CeCl_3$. The cerium perhydroxide precipitates (electron-opaque [dark] deposits) indicating H_2O_2 localisation, observed in flash-dried and cooled embryos, are indicated by arrows. *Abbreviations*: cw, cell wall; er, endoplasmic reticulum; m, mitochondrion; p, plastid; v, vacuole.


a
m
0.5 µm
b
cw
m
0.5 µm
c
v
cw
m
0.5 µm
d
cw
m
er
0.5 µm
e
m
cw
v
1 µm
f
er
m
cw
p
v
0.5 µm

No evidence of hydrogen peroxide accumulation was detected in the apoplasts of root meristem cells of embryos that were flash-dried and cooled (Fig. 3.13). However, one cannot rule out extracellular free radical production, and possibly oxidative stress, during these procedural steps, as ROS other than H_2O_2 (e.g. the superoxide anion/radical, hydroxyl radical, or the non-radical species, singlet oxygen) could have been produced. Sershen *et al*. (2012c), for example, showed both partial dehydration and cooling to result in extracellular superoxide-radical-based oxidative stress. The absence of H_2O_2 in the apoplast of the root meristematic cells of embryos, assessed in this study, after the drying (Fig. 3.13a and b) and cooling (Fig. 3.13c and d) treatments may also be partially attributed to the reduction in the water content of the tissues, limiting the activity of enzymes (Bailly, 2004), such as the NADPH-dependent oxidases (Kranner *et al*., 2010) and extracellular peroxidises (Minibayeva *et al*., 2009) that could have contributed to the initial extracellular deposition in hydrated specimens (Fig. 3.11e-h). It must therefore be considered that assessment of such embryos in a re-hydrated state after flash drying could yield a considerably different result. Alternatively, the absence of apoplastic cerium perhydroxide precipitates could indicate either the dismutation of H_2O_2 or – what might be more likely – its involvement in Fenton chemistry thus producing highly damaging hydroxyl radicals. The latter suggestion is in line with the loss of viability of *A. coranica* embryos after flash drying for longer than 120 min without cathodic protection (Fig. 3.3) and of cooling after such flash drying (Table 3.2). An absence of H_2O_2 was also observed for embryos that were exposed to glycerol as a cryoprotectant (Fig. 3.12a and b). In this case, however, it may be pertinent that glycerol cryoprotection resulted in a 32% reduction in embryo water content (Fig. 3.1), which could have limited activity of enzymes associated with the plasma membrane and cell walls. Some cerium perhydroxide deposition indicating the presence and localisation of H_2O_2 was detected in the cytomatrix and in mitochondrial matrices of embryos that were flash-dried and those that were cooled after drying (arrows, Fig. 3.13b, c and d), but the embryos that had been cathodically protected during flash drying showed no deposition after cooling (Fig. 3.13e and f). The lack of residual H_2O_2 in the cells of these embryos is suggested to have resulted from the reduction of ROS by means of the negative electric charge applied during cathodic flash drying (refer to Fig. 2.5). Consequently, incorporation of cathodic protection (by provision of cathodic water as a reducing solvent [Berjak *et al.*, 2011b]) in the other procedures of cryopreservation which are known to contribute towards ROS accumulation,

should also be explored in an attempt to ameliorate oxidative stress. Such measures could be applied during embryo excision, and exposure to cryoprotectants: Naidoo (2012) showed that the use of cathodic water as a solvent to make up the cryoprotectants, DMSO and glycerol, facilitated a 40% survival of *T. dregeana* excised axes, when none retained viability when the solvent used was distilled water. This was suggested to have resulted from the ameliorative effects of the highly reducing cathodic water, as ROS reduction, particularly a significantly reduced H_2O_2 accumulation, was noted (Naidoo, 2012).

The present results contribute to the growing body of knowledge suggesting that oxidative stress during preconditioning steps (including surprisingly, exposure to some conventionally constituted cryoprotectant solutions) resulting in ROS production could predispose axes/embryos to even greater damage during cooling. These observations need to be supplemented by further work, to compare the amount of H_2O_2 accumulated in the embryos during recovery after each step by biochemical quantification of this ROS (Oracz *et al.*, 2007), in order to determine the oxidative status of the cells. Furthermore, quantitative assays indicating whether (or not) hydroxyl radical generation occurs, will be invaluable in positioning the present observations on the occurrence of H_2O_2 in the context of the damaging effects of $^{\bullet}OH$ and viability loss. Oxidative stress is suggested to have been one of the (probably major) contributory factors to the poor post-cryo survival obtained for the species investigated in this study, especially embryos that were exposed to DMSO before further treatment, and provides even more motivation for studies on the amelioration (particularly by cathodic protection) of oxidative stress during the cryopreservation of recalcitrant zygotic germplasm (Berjak *et al*., 2011b).

Chapter 4

4. Overview and recommendations

The results of this study suggest that each procedural step applied in the cryopreservation protocol for recalcitrant zygotic embryos of *Ammocharis coranica*, *Brunsvigia grandiflora* and *Haemanthus albiflos* induced one or more stresses, so compromising viability. Embryos of all three species proved to be tolerant to initially moderately rapid water loss in the short term, with >60% retaining germinability at water contents (WCs) ≥0.5 g g^{-1}. However, further dehydration (particularly to WCs below 0.2-0.3 g g^{-1}), which required necessarily to be prolonged, proved to be highly detrimental. These results confirmed the desiccation sensitivity (seed recalcitrance) of the three species investigated here (as originally suggested by von Fintel, 2006) which ultimately contributed to their cryo-recalcitrance. Seedling regeneration after cryopreservation was achieved for only 30% of glycerol cryoprotected, rapidly cooled *A. coranica* embryos flash-dried to 0.36±0.10 g g^{-1}; but similar recovery was not achieved for cryopreserved embryos of the other two species investigated. Survival (even though scored as abnormal development) was achieved of *H. albiflos* embryos, but none of the *B. grandiflora* embryos survived cooling. Although partially successful, the limitation in the numbers of embryos surviving for at least two of the species (*viz. A. coranica* and *H. albiflos* [callus production by 10% of embryos]) is thought to be mainly due to the stresses imposed during the prolonged periods of physical dehydration (i.e. flash drying) needed to achieve water contents considered amenable for cooling (0.2-0.4 g g^{-1} [Wesley-Smith *et al*., 2001a; Normah and Makeen, 2008; Sershen *et al*., 2012b]). The detrimental effects of partial drying also appeared to be exacerbated by cooling and re-warming, which is in line with suggestions made in other studies on recalcitrant zygotic germplasm (Wesley-Smith *et al*., 2001a; 2004a).

With reference to the results obtained in this study, the present chapter highlights some of the factors that may have contributed to the viability loss induced during procedures required for cryopreservation of *A. coranica*, *B. grandiflora* and *H. albiflos* zygotic embryos. An appraisal of approaches (e.g. cathodic protection) that could potentially improve post-cryo survival in these species is also made. Recommendations for future studies are provided: these are motivated primarily by the findings of this and the other studies discussed in previous chapters.

4.1. The influence of drying rates and explant size

The post-harvest WCs of the zygotic embryos of *A. coranica*, *B. grandiflora* and *H. albiflos* were 3.28±0.52, 2.55±0.22, 4.48±0.92 g g^{-1}, respectively, which are typical of recalcitrant seeds that are shed highly hydrated at a range of WCs (Pammenter and Berjak, 1999b; Berjak and Pammenter, 2004b; 2008; Sershen *et al*., 2007). Slow drying, in extending the time that recalcitrant seed tissues spend at intermediate WCs, is believed to lead to an accumulation of metabolism-linked damage (Pammenter and Berjak, 1999b; Walters *et al*., 2002a). The embryos of all the species investigated here were dried using a method that is presently regarded as the most rapid way to dehydrate recalcitrant zygotic germplasm partially, *viz*. flash drying (Berjak *et al.*, 1990). However, embryos of *A. coranica* and *H. albiflos* (in particular) dried to WCs suitably low for cryopreservation relatively more slowly than other amaryllid species that have been cryopreserved successfully (Sershen *et al*., 2007; 2008; 2011). For instance, *Amaryllis belladonna* was found to require only 30 min of flash drying to reach water contents <0.4 (from 4.67) g g^{-1} (Sershen *et al.*, 2011; 2012a). However, *A. coranica* and *H. albiflos* embryos reached WCs in this range only after at least 180 min, whereas those of *B. grandiflora* required 30 min. The drying rate for *B. grandiflora* embryos is suggested to have been primarily a consequence of their being relatively smaller compared with those of the other two species. However, at this stage other factors (e.g. characteristics of the cuticle) limiting the drying rate of *A. coranica* and *H. albiflos* embryos cannot be discounted. Differences in the rate at which zygotic embryos lose water during flash drying has been shown to influence desiccation- and cryo-sensitivity in a number of amaryllid species (Sershen *et al.*, 2008, 2012b). An increase in the rate at which water is lost has been reported to permit viability retention to lower WCs in several recalcitrant-seeded species (Farrant *et al*., 1993; Berjak and Pammenter, 1997; Pritchard and Manger, 1998; Liang and Sun, 2002). Since the idea that desiccation rates influence the degree of water loss tolerated in recalcitrant germplasm is well accepted (Pammenter and Berjak, 1999b; Wesley-Smith *et al*., 2001a; Liang and Sun, 2002; Berjak and Pammenter, 2004b; 2008), there is now a need to increase the rate at which inherently 'slow-drying' recalcitrant embryos (e.g. those of *A. coranica* and *H. albiflos*) are dehydrated, in an effort to improve post-cryo survival. This prompted modification of the flash-drying apparatus by Sisunandar *et al*. (2010) to improve the dehydration rate of coconut embryos which enhanced their cryo-survival, but such positive effects need to be confirmed, especially since the response to dehydration-induced stress appears to be primarily species-specific. Thus in future work on *A. coranica* and *H.*

albiflos attempts should be made towards increasing the rapidity of dehydration during flash drying. Flash drying under vacuum is one approach that demands further investigation, as was briefly introduced in this study. The food industry has developed innovative applications of vacuum to aid the dehydration of fruits (Sagar and Suresh, 2010), which might potentially be adapted in order to optimise the conditions: these include according particular attention to the temperature, and to the pressure) in order to shorten the flash drying time required to reach WCs amenable for cryostorage.

Reduction of the size of the explants also deserves further investigation as smaller explants would allow for more rapid, and possibly more homogenous, drying (Muldew *et al.*, 2004; Hor *et al.*, 2005). The explant size and WC contribute to the thermal mass of a specimen (Wesley-Smith *et al*., 2004b), such that an increase in such factors lowers the probability of achieving a uniform rate of cooling, a decrease favours non-injurious cooling in liquid nitrogen (Pammenter *et al*., 1998). Uneven water distribution has been recorded for recalcitrant seed tissues (e.g. Pammenter *et al.*, 1998; Tompsett and Pritchard, 1998; Wesley-Smith *et al.*, 2001b), with meristems likely to be at higher WCs than other parts of the axes. Attaining even drying of explant tissues which are initially at different WCs is an important consideration when attempting to improve existing flash drying techniques. The phenomenon of species-specific drying rates is probably due to differing tissue characteristics (Ferrando and Spiess, 2001), particularly the nature of the cuticle and its thickness. A good starting point is suggested to be characterising the embryo tissue architecture and composition in relation to the rate at which water is lost under the same flash drying conditions, of a number of recalcitrant-seeded species as part of the effort towards improving drying rates; and, where possible, opting to use tissue of uniform (i.e. meistematic regions) rather than using those of varying cell architecture (i.e. whole embryos). In the case of the embryos of *B. grandiflora*, the results appear anomalous in terms of the benefits of rapid dehydration. All embryos of *B. grandiflora* retained viability after dehydration to WCs <1.0 >0.5 g g^{-1} achieved in the first 15 min, but 30-40% had lost viability when WC was reduced to <0.5 g g^{-1} after further flash drying for 15 min, suggesting their extreme desiccation sensitivity. Unfortunately, limitation of *B. grandiflora* seed numbers precluded refinement of the experiments, although the results of cathodic flash drying indicate that, with modification of other parameters (e.g. reducing explant size), successful germplasm cryopreservation of this species may be achievable.

4.2. The use of cryoprotectants

von Fintel (2006) initially reported the use of glycerol to be associated with subsequent stunted growth and abnormal development in the species presently re-investigated. Sershen *et al*. (2007) also pointed out similar effects on embryos of other amaryllids. Whilst exposure to the cryoprotectant (glycerol or DMSO) without subsequent drying and cooling did not severely compromise viability or induce abnormal growth in this study, cryoprotection may have been one of factors underlying the loss of viability after cryopreservation as it did increase desiccation sensitivity. Glycerol induced a substantial amount of water loss (osmotic dehydration) in the embryos presently investigated, prior to the evaporative dehydration induced by flash drying. This initial dehydration was suspected to have maintained the embryos at potentially damaging intermediate WCs for an extended duration (2 h) before the actual flash drying. The degree of metabolic damage accrued by recalcitrant embryos at intermediate WCs appears to be increasingly proportional to the time spent at these water contents (Walters *et al*., 2002a). The potentially beneficial effects of cryoprotection cannot be discounted though, and it is imperative that we investigate ways of avoiding cytoxicity and minimising the effects of the osmotic dehydration they bring about. This is often a challenge in vitrification-based cryopreservation protocols for recalcitrant seed germplasm as cryoprotectants, as applied to vegetative explants, have to be used in high concentrations (Fuller, 2004; Volk and Walters, 2006). The efficacy of cryoprotectants depends on various factors, among which the concentration and temperature at which they are applied, are key factors. Recent concepts of aqueous glasses have described the complexity of temperature responses in supercooled, super-viscous solutions (Angell, 2002; Walters, 2004; Capaccioli and Ngai, 2011) associated with the use of CPAs in cryopreservation and how these affect the propensities of glass formation. However, these findings cannot be generalised across species because of various other factors which would need to be ascertained for cryoprotectants and individual species, such as those used in this study.

Future studies should focus on realising the benefits of glycerol cryoprotection whilst reducing the time spent at intermediate WCs. The application of cryoprotectants such as glycerol under vacuum or at higher concentrations for shorter exposure times are approaches worthy of investigation. The results of the present study do, however, indicate that DMSO was cytotoxic. Other studies on tropical recalcitrant-seeded species also suggest that the embryos of such species may be more sensitive than temperate representatives (Kistnasamy

et al., 2011; Naidoo, 2012). Cytotoxicity of CPAs has been shown to increase with time, temperature and concentration (Finkle *et al*., 1985; Fuller, 2004; Berejnov *et al*., 2006; Wang *et al.*, 2007). Lawson *et al*. (2011) suggest that CPA cytotoxicity may not be additive and that combining CPAs may increase cytotoxicity synergistically. The effect of each cryoprotectant in a mixture needs to be determined as it may differ with species, cell type, temperature, and other solution components (Fahy *et al.*, 1984; Finkle *et al.*, 1985). The effects of glycerol and DMSO, for example, are known to differ (Bhandal *et al.*, 1985) and their interactive effects are not fully understood, arguing for future studies on both the individual and interactive effects of cryoprotectants on various explant types. This could possibly be achieved for the embryos presently investigated by the use of radio-labelled CPA components (as suggested by Sershen, 2010) to reveal relative uptake, and interaction with intracellular components.

4.3. Cooling rates

Since it has been shown that damage incurred during drying can influence post-cryo survival (Walters *et al.,* 2008), it was therefore conjectured that this may have been the case in this study. However, it must also be said that embryos in this study were almost always exposed to cryogenic temperature at WCs at which intracellular ice formation was possible (Wesley-Smith *et al*., 1992; 1999; 2004a). This has been shown to be the predominant cause of intracellular damage in cryopreserved plant tissues (Mycock *et al.*, 1995; Pearce, 2004; Sakai, 2000; 2004). Most cryopreservation protocols that achieve any success focus on facilitating vitrification in order to protect against such damage. Slow cooling rates were particularly detrimental to the embryos of *A. coranica*, *B. grandiflora* and *H. albiflos*. This is possibly because slow cooling allows ample time for extracellular ice nucleation and growth (Mazur, 1990; 2004), this would be accompanied by further dehydration (Karow, 1969; Engelmann, 2004). Although slow cooling rates have been successfully applied to shoot apices (Brison *et al*., 1995) and some embryonic tissues (Ford *et al.*, 2000a; b; Kistasamy *et al.*, 2011), Sershen *et al*. (2007) showed that slow cooling rates are inappropriate for the zygotic embryos of various recalcitrant-seeded amaryllid species. As in this study, those authors showed rapid non-equilibrium cooling rates to be far more suitable. Faster cooling rates limit extracellular ice nucleation and attendant dehydration and promote supercooling of the cell interior with production of small, uniformly distributed ice crystals (Franks, 1985; Carrington *et al.*, 1996). Future studies that aim to develop cryopreservation protocols for the recalcitrant embryos investigated here could focus on optimising the rapid cooling method presently used. If

embryos can be successfully cooled at somewhat higher WCs than presently used, this may also limit the extent of any freeze-induced dehydration occurring (Engelmann, 2004). However, it is known that embryos/axes at higher WCs require more rapid rates of cooling to restrict ice crystallisation and associated freezing damage (Walters *et al*., 2008), so it is important that the smallest explants possible are used to promote rapid cooling (Muldew *et al*., 2004). As the specimens of *A. coranica*, *B. grandiflora* and *H. albiflos* were cooled at hundreds of °C s^{-1} in nitrogen slush, the effects of paring down the explant to the minimum size and ascertaining the least amount of drying that would facilitate post-cryo seedling establishment, should be ascertained.

4.4. Cathodic protection

Recent studies (Varghese *et al*., 2011; Sershen *et al*., 2012c) have shown that oxidative stress accompanying the procedural steps involved in cryopreservation may contribute significantly to the loss of viability usually associated with the preconditioning and subsequent cooling of recalcitrant zygotic embryos/axes. Oxidative stress from unregulated free-radical-mediated processes, predominantly those resulting from unbalanced metabolism, have been implicated as among the most detrimental stresses associated with dehydration (Franca *et al*., 2007) and cryopreservation of recalcitrant seed-derived germplam (Roach *et al.*, 2008; Varghese and Naithani, 2009; Whitaker *et al.*, 2010; Pammenter *et al.*, 2011). In the present study, cathodic protection was applied to the explants during flash drying via the metal mesh on which the embryos were placed (adapted from Pammenter *et al.*, 1974), in attempt to ameliorate the oxidative stress usually accompanying the dehydration of recalcitrant embryos. Additionally, some of the explants were re-hydrated in cathodic water (Berjak *et al*., 2011b). Cathodic protection during flash drying seemed to be beneficial, for *A. coranica* and *H. albiflos* embryos, facilitating survival to significantly lower WCs than those tolerated by embryos flash-dried in the absence of cathodic protection. The use of cathodic water, on the other hand, did not seem to be beneficial which may have been due to the fact that it was not applied during other procedural steps in this study that have been shown to induce ROS production: e.g. explant excision (Berjak *et al*., 2011b) and cryoprotection (particularly with DMSO [Naidoo, 2012]). Interestingly, intracellular H_2O_2 was not detected in glycerol-cryoprotected material which, based on findings by Sershen *et al*. (2012c), could suggest that glycerol either quenched the ROS produced or enhanced the activity of endogenous antioxidants in the embryos investigated here. These conjectures demand further

investigation. Possible avenues include scavenging of ROS (Shirahata *et al.*, 1997), including selectively quenching H_2O_2 (Lee *et al.* 2006), and/or ascertaining whether endogenous antioxidant activity is enhanced (Hanaoka, 2001). Furthermore, the production of other potent ROS species (such as the hydroxyl radical and singlet oxygen [Sharma *et al*., 2012), in conjunction with their possible quenching, should also be investigated.

4.5. Conclusions

The zygotic embryos and axes of the amaryllid species investigated (*Ammocharis coranica*, *Brunsvigia grandiflora* and *Haemanthus albiflos*) showed a high degree of cryo-recalcitrance, but the results obtained have helped to identify a number of interventions that could be used to minimise the damage incurred during the various procedural steps involved in cryopreservation. Efforts to ameliorate the effects of oxidative stress, improve cryoprotection and reduce the damage incurred during flash drying appear to be the major priorities as many cryopreservation studies on embryos/axes of recalcitrant-seeded species report poor survival (Krishnapillay, 2000; Hajari *et al*., 2011; Naidoo *et al*., 2011; Essack, 2012; Walters *et al*., 2013). Also, for species in which zygotic embryos and axes appear to be cryo-intolerant, the use of alternative explants such as meristems (Varghese *et al*., 2009; Normah *et al*., 2011), or even as a last resort, somatic embryos [Gonzalez-Arnao *et al*., 2008]), should be explored. Meristems do, after all, have the ideal characteristics (compact cells with a large nuclear:cytoplasmic ratio) for cryopreservation (Engelmann, 2011). Furthermore, meristems excised from bulked-up material derived from seedlings offer the same genetic diversity as do the seeds from which they develop. Finally, while the quest to optimise and establish successful cryopreservation protocols for recalcitrant-seeded species continues, complementary means of *ex situ* conservation to secure the germplasm should be practiced, including *in vitro* slow growth and establishment of field genebanks

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Appendix

Table A1: Average monthly temperatures and total monthly rainfall during the seed development period of *A. coranica* over two fruiting seasons in Queenstown (Rietvlei, Cathcart), South Africa.

	2010			2011		
Month	**Rainfall (mm)**	**Min Temp (°C)**	**Max Temp (°C)**	**Rainfall (mm)**	**Min Temp (°C)**	**Max Temp (°C)**
Nov	5.2	11.9	26.3	60.6	12.0	26.6
Dec	34.6	13.0	28.6	54.6	13.4	26.2
Jan	98.6	15.4	27.5	109.2	15.8	27.9
Feb	1.0	15.9	29.9	102.2	16.3	28.1
Average		14.1±1.9	28.1±1.5		14.4±2.0	27.2±0.9
Total	139.4			326.6		

Table A2: Average monthly temperatures and total monthly rainfall during the seed development period of *B. grandiflora* over two fruiting seasons in Queenstown (Rockford Bridge, Cathcart), South Africa.

	2010			2011		
Month	**Rainfall (mm)**	**Min Temp (°C)**	**Max Temp (°C)**	**Rainfall (mm)**	**Min Temp (°C)**	**Max Temp (°C)**
Jan	38.8	15.4	27.5	109.2	15.8	27.9
Feb	91.8	15.9	29.9	102.2	16.3	28.1
Mar	25.6	14.0	28.7	79.8	15.1	27.4
Apr	26.4	9.6	23.9	6.4	9.5	21.1
Average		13.7±2.9	27.5±2.6		14.2±3.2	26.1±3.4
Total	182.6			297.6		

Table A3: Average monthly temperatures and total monthly rainfall during the seed development period of *H. albiflos* over one fruiting season in East London (Dune Forest, Kei Mouth), South Africa.

	2011		
Month	**Rainfall (mm)**	**Min Temp (°C)**	**Max Temp (°C)**
Apr	78.4	14.8	23.7
May	82.2	12.7	21.5
Jun	196.0	10.4	19.7
Jul	220.8	9.2	18.6
Average		11.8±2.5	20.9±2.2
Total	577.4		