Differing Responses of Embryonic Axes of Four Recalcitrant-Seeded Species from Temperate and Tropical Provenances to the Procedures Involved in Cryopreservation

By

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Abstract

Cryopreservation is the most convenient and cost-effective method of conserving recalcitrant-seeded species in the long term. There are a series of steps required before explants can be exposed to cryogenic temperatures, these include: excision itself; exposure to cryoprotectants; flash- (very rapid) drying; cooling in liquid nitrogen, thawing after cryostorage and regeneration. All these steps are potentially injurious, yet recalcitrant embryonic axes from seeds of temperate origin withstand the stresses more successfully than do those from seeds of tropical origin. It was therefore the aim of this study to determine differing responses of seeds from different provenances to cryopreservation and all steps involved therein. To achieve this, two recalcitrant-seeded species of tropical origin (*Castanospermum australe* and *Trichilia emetica*) and two recalcitrant-seeded species of temperate origin (*Acer pseudoplatanus* and *Quercus robur*) were used and their survival after each pre-treatment was compared.

Excision did not affect the viability of the embryonic axes of all four recalcitrant-seeded species prior to any other manipulations, regardless of provenance. Though the recalcitrant-seeded species from tropical provenance had higher initial water contents than those from temperate provenances, the time taken to reach the intended water contents amenable for cryopreservation upon dehydration as well as the drying rates varied greatly across the species. However, viability after dehydration to lower water contents (*c* 0.3 gg^{-1}) was higher for the temperate species than for the tropical species. Cryoprotection with glycerol (Gly) prior to dehydration decreased the water contents of the embryonic axes except for *T. emetica* which exhibited an increase in water content. The time taken to reach 0.3 gg^{-1} was greatly reduced (halved) upon dehydration post cryoprotection with Gly for embryonic axes of the temperate species whilst that of the tropical species was unaffected. This proved to be favourable during cryopreservation as those embryonic axes of the recalcitrant-seeded species from temperate provenance had survival cryopreservation.

Cryoprotection was significant as survival post cryopreservation was greatly enhanced when embryonic axes were cryoprotected with Gly after excision. Higher cooling rates coupled with lower water contents yielded higher survival after cryopreservation for temperate recalcitrant-seeded species. Water content was especially significance for cryopreservation of embryonic

axes of *A. pseudoplatanus* as shoot production was observed when the water content was 0.3 gg^{-1}. However, survival, where obtained, was always limited to root production, radicle elongation or callus formation for embryonic axes of *Q. robur, C. australe,* and *T. emetica*, respectively.

Thermal analysis of intracellular water further emphasized the effect of variation displayed by recalcitrant-seeded species. It also showed that cryoprotection with Gly increases the amount of non-freezable water for temperate species. The difference in genotypes between species may potentially be the major underlying factor for the varying responses to cryopreservation displayed by recalcitrant-seeded species. Future recommendations include using biomarkers such as electrolyte leakage and ethane production after each pre-treatment step to determine the extent to which each treatment stresses the embryonic axes. This would help design cryopreservation protocols for each individual species as it would identify where each species is less stressed and more amenable to cryopreservation.

Preface

The experimental work described in this dissertation was carried out in the School of Life Sciences, University of KwaZulu-Natal, Durban, under the supervision of Professors Patricia Berjak and Norman Pammenter.

This study represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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Thembela Mathews Mshengu Prof N. W. Pammenter (Supervisor)

February 2018

Declaration 1 – Plagiarism

I, Thembela Mathews Mshengu, 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 person' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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Signed:

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I would also like to thank all the members of the Plant Germplasm Conservation Research Group, most of whom have become life time friends over the years shared together.

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I am eternally grateful to my family, who even when everything wasn't going according to plan, never gave up on me. Your unconditional love and support propelled me forward and thus I never gave up. To my daughter Weziwe, one day all this will make sense to you, I love you.

I am also sincerely grateful to all my friends, especially the CoAF. You guys continually push one another to be the best they can be. You made sure I never lost sight of the ultimate goals, really honoured to call you my brothers.

I am most grateful to God for the people mentioned above, for the opportunity and ability granted to me to pursue this degree.

Dedication

I would like to dedicate this dissertation to the late Prof Pat Berjak whom without I would've never pursued this MSc degree. She groomed me from a very young age as an undergrad with no ambitions beyond completing his junior degree. She installed the love and respect for science that I now hold.

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Abbreviations and Symbols

ANOVA	Analysis of variance
$CaCl_2$	Calcium chloride
$CaCl_2.2H_2O$	Calcium chloride dihydrate
CaMg	Calcium magnesium
CP	Cryoprotection
dmb	Dry mass basis
DM	Dry mass
DMSO	Dimethyl sulphoxide (Me_2SO)
DR	Drying rate
DSC	Differential scanning calorimetry
E	East
Gly	Glycerol
H_2O	Water
IWC	Initial water content
LEA	Late embryogenesis abundant protein
LN	Liquid nitrogen
LSD	Lowest significant mean difference
$MgCl_2$	Magnesium chloride
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
MS	Murashige and Skoog
NaOCl	Sodium hypochlorite
PVS2	Plant vitrification solution 2
RFO	Raffinose family oligosaccharides
ROS	Reactive oxygen species
S	south
WC	Water content
w/v	Weight per volume

Units of Measurements

%	Percent
°C	Degree Celsius
°C min^{-1}	Degree Celsius per minute
G	Gram(s)
gg^{-1} dmb	g H_2O per g of dry matter, dry mass basis
gg^{-1} min^{-1}	g H_2O per g of dry matter per minute
hr	Hour(s)
J	Joule(s)
J/g	Joules per gram
L	Litre(s)
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
ml L^{-1}	Millilitres per litre
mm	Millimetre(s)
mM	Millimolar
PPM	Parts per million
µM	Micromolar

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Declining Plant Biodiversity

With global climate change prevailing, especially in Africa, it is imperative that much of the genetic variability of plants is conserved for future purposes (Berjak *et al.,* 2010). The number of critically endangered plant species has increased by 60% (Engelmann, 2012). Apart from global warming, various other factors contribute to the loss of plant biodiversity most of which are of anthropogenic origin. These include deforestation for urbanization, and land alteration to plant medicinal crops and other agricultural purposes (Berjak and Pammenter, 2001; Berjak *et al.,* 2010; Engelmann, 2012). Another natural factor which affects plant biodiversity is the invasion of alien species on indigenous lands (Berjak and Pammenter, 2001). According to Thuiller *et al.* (2005), man-induced climate changes are mostly responsible for the change in distribution, physiology and phenology of plant species. In their model study, Thuiller *et al.* (2005), assuming that there will be no migration of any plant species, postulated that over half of the existing species now might be extinct by the year 2080 in Europe alone.

1.2 Plant Germplasm Conservation Strategies

Generally, conservation of plant germplasm can be categorized into two strategies, *ex situ* and *in situ* conservation. *Ex situ* conservation generally entails conservation/preservation of biological material outside its natural ecosystem whilst *in situ* conservation generally means preservation of biological material within its natural habitat, effectively requiring habitat conservation (Engelmann, 2011; 2012). *In situ* conservation generally conserves a whole plant as it is and usually protects it from outside disturbances whilst keeping it in its natural ecosystem such as in forest reserves and parks (Engelmann, 2012). *Ex situ* conservation can be a whole plant as in botanic gardens, a part of the germplasm such as seeds in genebanks, or even just a piece of tissue of a plant as in *in vitro* conservation (Engelmann, 2011; Engelmann, 2012). Even though *in situ* conservation might seem ideal as it also allows natural gene diversity of species, it still leaves the species vulnerable to natural disasters and might sometimes be limited by geographic boundaries (Engelmann, 2012). *Ex situ* on the other hand offers a more pathogen-safe environment and does not use larger amount of land which would generally require extensive funding (Engelmann, 2012).

1.3 Seed Storage and Conservation

Higher plants produce seeds which contain all the genetic information required for the succeeding generation, thus these seeds are the simplest form in which the genetic resources of plants can be conserved. Seeds even offer the most convenient way in which plant species can be transported and distributed as they require less space and effort whilst the plants' genetic material is kept intact. Even crop plant species are easier delivered to farmers as seeds, and research scientists can obtain seeds from longer distances over longer periods of time.

1.3.1 Seed Types and How They Differ

Seeds are characterized as recalcitrant or orthodox (Roberts, 1973). Orthodox seeds are desiccation tolerant, meaning that they can lose ≥ 80% of their water content but still retain viability (Pammenter and Berjak, 1999; Roberts and King, 1980). This desiccation tolerance facilitates the storage of orthodox seeds in the dry state, which is best achieved under conditions of low temperature and low relative humidity (Pammenter and Berjak, 1999; Roberts and King, 1980). Recalcitrant seeds on the other hand are desiccation sensitive, i.e. they lose viability when subjected to very little water loss, thus rendering them unstorable under conditions used for orthodox types (Pammenter and Berjak, 1999; Roberts and King, 1980). The extent to which seeds are desiccation tolerant or sensitive differs from one species to another (Berjak and Pammenter, 2008). There is a third class of seeds called intermediate seeds and these are less sensitive to dehydration and chilling than recalcitrant seeds but do not undergo the same developmental stages as orthodox seeds, thus cannot dry very low (Hong and Ellis, 1996).). Unlike orthodox seeds, intermediate seeds are shed at high water contents from the parent plant because they are not subject to natural dehydration (Berjak and Pammenter, 2008). Seed desiccation tolerance is more of a continuum than a discrete entity as recalcitrant seeds are reported to be able to withstand dehydration to about 0.2 gg^{-1} water content (under special conditions) whilst orthodox seeds can go below 0.07 gg^{-1} and intermediate seeds would fall in between (Walters, 2015The seeds themselves contain a zygotic embryo, viz. an embryonic axis and cotyledon(s), and endosperm may or may not be present at maturity. The genetic information for the new plant is actually inherent in the embryonic axes, whilst the cotyledons and/or endosperm serve as storage reserve vital for germination of the seed and seedling establishment.

1.3.2 Development and Desiccation Tolerance in seeds

Unlike recalcitrant seeds, after maturation orthodox seeds undergo a further developmental stage termed maturation drying during which they acquire desiccation tolerance (Berjak, 2006; Pammenter and Berjak, 1999). The majority of the plant's cell space is occupied by a fluid-filled vacuole which is susceptible to shrinking upon desiccation (Pammenter and Berjak, 1999). During maturation drying this fluid is replaced via the accumulation of insoluble reserve material (Pammenter and Berjak, 1999). As described by Farrant *et al.* (1997), the more the desiccation tolerant the seeds are, the greater will be the accumulation of insoluble material whilst the vacuole size decreases. According to Farrant *et al.* (1997) and, Berjak and Pammenter (2008), during later stages of development, some recalcitrant seeds also accumulate dry mass. However, this dry matter is soluble and is not accompanied by water reduction, thus it has no implication on desiccation tolerance (Berjak and Pammenter, 2008; Farrant *et al.,* 1997).

One of the most deleterious effects of dehydration is unregulated metabolism (see section 1.6.3) which recalcitrant seeds undergo once shed from the mother plant(Pammenter and Berjak, 1999). Orthodox seeds, on the other hand, have the ability to lower, even shut-off, their metabolism upon desiccation (Pammenter and Berjak, 1999). Orthodox seeds undergo metabolic shut-off during maturation drying (desiccation) thus they are able to be stored at conditions of low temperature and low relative humidity for long periods (Pammenter and Berjak, 1999; Roberts and King, 1980). Recalcitrant seeds remain metabolically active even upon shedding, at no point during their development do recalcitrant seeds halt their metabolism thus they are always susceptible to metabolism-induced damage during desiccation (Berjak and Pammenter, 2008; Pammenter and Berjak, 1999).

Other structural changes implicated in desiccation tolerance that orthodox seeds acquire during maturation drying include DNA conformational change and intracellular organelle de-differentiation. In their study, Farrant *et al.* (1997) found that the size of the mitochondria negatively correlates to the degree of desiccation tolerance with desiccation sensitive seeds having larger mitochondria occupying the cell. Various endogenous amphipathic molecules have

been observed to migrate into the membranes of orthodox seeds upon dehydration and this is suggested to maintain the integrity and stability of the membranes during desiccation stress (Pammenter and Berjak, 1999).

Additionally, some of the compounds that are present in orthodox seeds and are said to be involved in the induction of desiccation tolerance are also present in recalcitrant seeds but these seeds die long before they can reach the water content at which all these compounds become effective (Berjak and Pammenter, 2002). This may be because of structural changes that orthodox seeds undergo during maturation drying and recalcitrant seeds do not.

1.4 Cryopreservation

Various methods are employed in the conservation of plant genetic resources of recalcitrant-seeded species: these include *in vitro* conservation and cryopreservation (cryostorage) (Engelmann, 2011). However, according to that author, *in vitro* storage including slow growth is costly, requires highly trained staff and also offers storage for a limited time span. Cryopreservation, on the other hand, is the most convenient and cost-effective method of storing biological material especially in the long term (Engelmann, 2011). Theoretically, cryopreservation offers a time-limitless storage of germplasm in a contamination free state and consequently does not affect/alter the germplasm (Engelmann, 2011). This involves storing material at cryogenic temperatures (defined as ≤ -80°C) with the use of liquid nitrogen (LN) or its vapour phase (-196 and -160°C, respectively). Theoretically, during cryopreservation all cellular divisions and metabolic activity are halted therefore ensuring the recovery of the plant tissue at the state it was stored in (Engelmann, 1991). Cryostorage over long period of time would generally require constant filling up with liquid nitrogen so as to maintain the storage conditions. Most recalcitrant seeds are relatively large in size thus embryonic axes rather than whole seeds are utilized on their behalf (see section 1.6.1).

Classical cryopreservation techniques dehydrate the freezable intracellular water prior to exposure to liquid nitrogen (Engelmann, 2004). This is done via various methods, one of which

involves formation of extracellular ice whilst the cellular components are supercooled (Engelmann, 2004). This creates a water potential gradient across the cell membrane as the solute concentration increases extracellularly and as a result water moves from the cells to the extracellular milieu. According to Engelmann (2004), though the amount of water lost cannot be quantified, almost all the freezable water can be lost this way thus precluding water crystallization. According to Engelmann (2004), modern cryopreservation techniques involve dehydration of the specimen prior to exposure to liquid nitrogen. Dehydration may be carried out either using cryoprotective compounds (e.g. dimethyl sulphoxide, glycerol, and sucrose) or exposure to dry air, rapidly (flash drying) or relatively slow via alginate beads (Engelmann, 2004). With modern cryopreservation techniques the most critical point is the survival of the specimen post-dehydration, whilst with the classical techniques the critical point is the survival post-cooling (Engelmann, 2004). Thus now the dehydration rate also plays a significant role in cryopreservation of biological tissues especially recalcitrant seeds. There are seven different modern cryopreservation procedures that can be employed to induce vitrification (meaning explained below) in the samples, namely: (i) encapsulation-dehydration; (ii) vitrification; (iii) encapsulation-vitrification; (iv)dehydration; (v) pregrowth; (vi) pregrowth-dehydration; and (vii) droplet freezing. More insight on these procedures is provided by Engelmann, 2004.

1.5 The Glassy State

The thermal properties of water change with a change in the water content of the axes as a function of the viscosity and density of the intracellular solution (Engelmann, 2011). Solutes such as the late embryogenesis abundant (LEAs) proteins and sugars, specifically sucrose and raffinose family oligosaccharides (RFO), if present in a favourable ratio, inhibit ice crystal formation whilst promoting vitrification (Koster and Leopold, 1988, Kuo *et al.,* 1988). Vitrification is a process in which water changes into an amorphous, highly viscous, glassy state (Berjak, 2006). It is the establishment of the glassy state that is thought to be a key factor in the survival of biological material upon cryopreservation as it inhibits physical damage of tissue (Berjak, 2006; Engelmann, 2011). Chemically, vitrification is the establishment of an amorphous (glassy) material that lacks structural definition observed in a solid material and also the alignment of molecules is more random compared to that of liquid molecules (Buitink and Leprince, 2004). Thus the glassy state is more like an interphase between the solid and fluid states.

The concept of possible glass formation was first proposed by Burke (1986) when he assessed the ability of cytoplasmic solutes such as sugars to deter denaturation of molecules with survival of seeds in the dry state. Williams and Leopold (1989) were the first authors to report evidence of glass transitions in maize embryonic axes using the differential scanning calorimeter (DSC). They went on to postulate that the establishment of the glassy state, among others, offered advantages such as preventing the crystallisation of cell constituents, and consequently mobilisation and reactions. Vitrification also helps prevent cell collapse upon dehydration as the glass fills the cellular space (Koster, 1991). Removal of water from a solution results in an increase in the solute concentration and concomitantly in the viscosity of the solution (Buitink and Leprince, 2008). This therefore forms the basis for the establishment of the glassy state in the cytoplasm upon dehydration. According to those authors, as water is removed, cohesive forces between the cytoplasmic molecules increase thereby limiting molecular mobility within the cytoplasm.

Buitink *et al.* (2000) used impatiens and bell pepper seeds to show that the viscosity, therefore molecular mobility, of the cytoplasm increases with an increase in temperature and/or relative humidity. This was later on re-emphasized by Buitink and Leprince (2008) when they stated that the water content at which the glassy state can be obtained, upon drying, strongly relies on temperature such that drying at lower temperatures increases the water content at which vitrification can occur. In a model study using DSC, Koster (1991) had showed that the glass transition temperature varies depending on the amount of water in the tissue. However, as Buitink and Leprince (2008) stated, model studies of sugars alone cannot be reliable in depicting the formation of the glassy state but model studies of sugars in association with LEA proteins produce situations which better mimic the glassy state in desiccation tolerant biological tissues which might include interactions with amino acids, salts and organic acids.

1.6 Factors Influencing Cryopreservation Success of Recalcitrant-Seeded Plant Species

Various factors are believed to affect the success of cryopreservation of recalcitrant seeds. These range from physical to biological all the way to chemical and it can be either just one or a combination of these factors that alters post cryogen exposure success of the recalcitrant seeds.

1.6.1 Explant Type and Size

Seeds are often seen as the most obvious forms in which plants can be conserved. Recalcitrant seeds are usually large and this would result in different thermal properties in tissues of the same seed upon exposure to cryogenic temperatures (Noor *et al.,* 2011). According to Whitaker *et al.* (2010) a whole recalcitrant seed dries up at a much slower rate which promotes cellular deterioration and spread of pathogen before desired water contents for cryopreservation can be reached. However, embryonic axes offer more compound structures which differ in the sensitivity to dehydration and freezing (Engelmann, 2011; Noor *et al.,* 2011). Embryonic axes of some species display an increased degree of sensitivity to dehydration than the whole seed (Engelmann, 2004). Success or failure of cryopreservation of these axes would not necessary reflect properties of the whole seeds.

Embryonic axes of some species show better survival post cryopreservation when they are attached to a piece of a cotyledon (Naidoo *et al.,* 2011). Embryonic axes of some species are themselves too large to be used as cryopreservation explants whilst some species do not have well defined embryonic axes (Engelmann, 2004). Walters *et al.* (2008) and Berjak *et al.* (2011) stated that for successful cryostorage, it is imperative that the explants be small so as to prevent various imbalanced processes such as different drying and cooling rates. The size of an explant is vital as the smaller the explant, the faster the dehydration and cooling rates can be (Walters *et al.*, 2008). Working on mint and garlic shoot tips, Volk and Walters (2006) observed that it took six times longer for PSV2 to have the same desired effect on garlic as it did on mint shoot tips and this was attributed to the astonishing size difference between the two species (1.5 and 0.1 mg, respectively). According to Noor and colleagues (2011), other explants for cryopreservation

include meristematic tissues such as shoot tips, and pollen. Choosing an explant is thus important and in some instances may underlie the success of the study.

1.6.2 Explant Development Status

It is also vital that the seeds (therefore embryonic axes) be sampled at their optimum developmental stage as this crucially affects the success of cryopreservation (Engelmann, 2004;; Goveia *et al.,* 2004). Working on *Landolphia kirkii* seeds*,* Berjak *et al*. (1992), found that the level of desiccation sensitivity increased with maturity. These authors also found that the enthalpy of melt for immature seeds were higher than for other developmental stages and so was the water contents at which freezing and melting transitions were no longer detectable. It is therefore vital that seeds are harvested at the correct stage of maturity.

1.6.3 Water Content and Drying Rate

There has been a limited success in the cryostorage of axes/embryos from recalcitrant seeds as there is a significant variation in their water content between provenances and also between harvest seasons (Berjak *et al.,* 2010; Engelmann, 2011). For successful cryostorage of recalcitrant seeds, dehydration and cooling rates must be higher than the resulting rate of damage (Walters *et al.,* 2008). There are two major types of damages that embryonic axes are susceptible to upon exposure to drying i.e. desiccation damage and metabolism-linked damage (Berjak and Pammenter, 2002).

Desiccation damage in the strict sense occurs when axes are dehydrated to such an extent that non-freezable water (structure-associated), which stabilizes intercellular structures, is lost. Dehydration beyond the structure-associated water results in structural collapse (Berjak and Pammenter, 2002). According to those authors, the cellular structure of the embryonic axes is complex such that whole axes does not lose water at the same rate. This may result in metabolic imbalances which may result in production of free radicals and the resulting damage is termed metabolism-linked damage. In a single embryonic axes, the shoot apex might exhibit a different

reaction to dehydration than the root apex region and this might explain the variable post cryo-exposure response (Engelmann, 2012). A window of opportunity at which its water content is reduced enough to avoid freezing injury whilst also avoiding desiccation damage, is necessary for successful cryopreservation of such embryonic axes (Volk and Walters, 2006; Ballesteros *et al.,* 2014).

1.6.4 Use of Cryoprotectants

Cryoprotection involves using solutions to establish the glassy state thereby inhibiting ice crystallisation (Benson, 2008). The mode of action of cryoprotection depends of the size of the cryoprotectants such that small enough cryoprotectants will enter the cells and those are said to be penetrating or permeating whilst relatively larger solutes are termed non-penetrating or non-permeating cryoprotectants (Benson, 2008; Berjak, 2006). Cryoprotection is applied prior to introduction of explants into liquid nitrogen and successful cryopreservation involves inhibition of ice crystallization during both cooling and warming (Benson, 2008).

Glycerol and dimethyl sulfoxide (DMSO) are classical examples of penetrating cryoprotectants. When explants are imbibed in these cryoprotectant solutions, they readily take them up (Benson, 2008). The mode of action of penetrating cryoprotectants is not fully understood. Glycerol, for example, is said to exhibit the same solvent properties as water and thus postulated that seeds will lose water more readily in the presence of glycerol (Benson, 2008). Penetrating cryoprotectants are advantageous as they counter any volume changes that may be associated with water loss (Benson, 2008). According to that author, penetrating cryoprotectants also restrict ice crystal growth whilst increasing viscosity. However, the restriction of ice formation and growth may also be due to the destabilisation of the crystalline structure and not the limited mobility/interaction of the water molecules. Special attention must be paid to the concentration of the cryoprotectant as when present at high concentrations, the cryoprotectant may be toxic (Benson, 2008). Benson (2008) also speaks of colligative damage which may result after dehydration, leaving a high concentration of various solutes which alter cell pH.

Non-penetrating/non-permeating cryoprotectants include solutes relatively large in size, such as sucrose, which are therefore unable to enter into the cells (Benson, 2008). According to that author, non-penetrating cryoprotectants are particularly useful for cells with large vacuoles. When explants are imbibed in a non-penetrating cryoprotectant solution an osmotic gradient is created with the inside of the explant having less solutes and the outside having more solutes (Benson, 2008). Consequently, water then diffuses from the region of high concentration (inside) to a region of low concentration (outside). The mode of operation of the non-penetrating cryoprotectants is therefore dehydration which results in an increase in viscosity of the cytoplasm (Benson, 2008). As more water diffuses to the extracellular milieu, extracellular ice crystallization may occur which also facilitates further dehydration (Benson, 2008). Non-penetrating cryoprotectants may also result in cellular volume changes which are undesirable for the survival of biological material. Working on two amarylid recalcitrant-seeded species, Sershen *et al.* (2012) found that cryoprotection of zygotic embryos with glycerol followed by partial dehydration prior to cryopreservation increased post-cryostorage viability. This corresponded with a decrease in lipid peroxidation upon treatment compared with untreated embryos. Lipid peroxidation upon dehydration of embryonic axes results from a lowered antioxidant/enzyme activity but can be limited through cryoprotecting with glycerol (Sershen *et al.,* 2012). Cell type, temperature and species may alter the mode in which the cryoprotectant penetrates and also functions, whilst using a cryoprotecting solution with various components (for example PSV2) can enhance the mode of operation of a cryoprotectant (Volk *et al.*, 2006).

Evidence that some cold-resistant plants produce some of the cryoprotectant compounds upon temperature drop suggests that the role of cryoprotectants is more than just the manipulation of water content (Volk and Walters, 2006). Working on garlic shoot tips, Volk and Walters (2006), found that exposure to PVS2 solution prior to exposure to LN caused a decline in water content but there was no change in the fresh mass of the shoot tips, re-emphasizing the role of the cryoprotectant solution in mimicking the role of the water in biological tissue.

1.6.5 ROS Production

In their chapter on acquisition and loss of desiccation tolerance, Vertucci and Farrant (1995) produced a hydration level diagram in which they give systematic chemical and mechanical

events which occur in seeds at each hydration level. Those authors suggested that, at hydration level III (corresponding to the water content of 0.25-0.45 gg^{-1} on a dry mass basis) unregulated metabolism prevails. This entails an increasing free radicals, particularly reactive oxygen species (ROS), production. Free radicals are chemical species (atoms/molecules) with one or more unpaired electron. These molecules are highly reactive because of their potential to readily donate the unpaired electron (Kranner and Birtić, 2005). Reactive oxygen species is a term that is used to describe nitric oxide and hydrogen peroxide as well as oxygen free radicals which include superoxide, hydroxyl radical and singlet oxygen etc (Berjak, 2006; Kranner and Birtić, 2005). Reactive oxygen species are considered lethal as they cause oxidative damage to protein, lipids and nucleic acids (Berjak *et al.,* 2007).

Reactive oxygen species are produced in metabolic pathways in the mitochondria and chloroplast under strict control by the antioxidant system (Kranner and Birtić, 2005). However, any form of stress, including desiccation, disrupts the action of the antioxidant and results in an upregulated ROS production (Kranner and Birtić, 2005). Although ROS have their beneficial roles in the normal functioning of cells (most prominent being cell signalling and protection against pathogens), they are most known for their destructive work inside cell (Berjak *et al.,* 2007; Kranner and Birtić, 2005). These include lipid peroxidation, which results in membrane appression which in turn leads to death of recalcitrant seeds (Kranner and Birtić, 2005).

Orthodox seeds are able to survive desiccation because, according to Kranner and Birtić (2005), upon dehydration (and maturation drying) they convert glutathione, an antioxidant, to glutathione disulphide. Glutathione disulphide binds and protects SH-groups against oxidation and denaturation. Upon rehydration (germination) glutathione disulphide is converted back to glutathione (Kranner and Birtić, 2005). Recalcitrant seeds also possess antioxidants, however, upon desiccation these become damaged and lose their ability to scavenge free radicals (Berjak, 2006; Kranner and Birtić, 2005; Varghese et al 2011). *Acer saccharinum* seeds pre-imbibed in sodium selenite showed more viability after dehydration compared with untreated seeds as selenium has been shown to improve activity of glutathione peroxidase in treated embryonic axes (Pukacka *et al.,* 2011). According to those authors, glutathione peroxidase prevents lipid peroxidation by promoting the reduction of organic peroxidases and hydrogen peroxide.

1.6.6 Cooling

Noor *et al.* (2011) attributes the limited success of cryopreservation of recalcitrant material to the susceptibility of the explants to cooling and dehydration injury. Successful cryostorage strongly relies on the ability of the axes to survive desiccation to a point at which the remaining water cannot form ice crystals. As much as ice crystal formation underlies cryopreservation failure, it is the localization of the ice crystal within the cell compartments that is more important (Wesley-Smith *et al.*, 1992, 2015). Increasing the cooling rate by hundreds of degrees Celsius might result in supercooling of the intracellular water thereby increasing the non-freezable water content, and further cooling of this water would result in vitrification and no ice crystallization (Volk and Walters, 2006). A study on *T. emetica* (Kioko *et al.,* 2006) revealed that just after 10 days of storage at 6°C, seeds started to show ultrastructural damage which can be attributed to chilling sensitivity, typical of recalcitrant seeds from tropical provenances.

1.6.7 Sugars

In their paper, Bryant *et al.* (2001) stated that cellular membrane integrity is affected by its composition, temperature, water content as well as the complexity of the surrounding solution, and that upon dehydration, membrane-bound proteins are lost and the membrane phospholipids undergo a phase transition from fluid phase to the gel phase. The presence of simple sugars such as sucrose, glucose, sorbitol and trehalose around the membrane phospholipids inhibits the phase transition thereby maintaining the membrane integrity (Bryant *et al.,* 2001; Pammenter and Berjak, 1999). This may happen in one of two ways, the first is by increasing the osmotic pressure with increasing solute concentration as water is being lost and this increase in osmotic pressure around the membrane prevent any further loss of water (Bryant *et al.,* 2001). Or this is explained via the water replacement hypothesis which states that specific sugars maintain the correct lipid head-group spacing by binding to polar ends of macromolecules via hydrogen bonds thereby preventing phospholipid phase transition (Crowe *et al*, 1992).

Koster and Leopold (1988) explained the water replacement hypothesis as that hydroxyl groups can bind to polar head-groups, providing the hydrophilic interactions necessary for membrane structure and stability. They also stated that sucrose is vital for desiccation tolerance whilst its own crystallization is prevented by larger oligosaccharides, such as raffinose and stachyose, by promoting the establishment of the glassy state. This argument was supported by the loss of desiccation tolerance in orthodox seeds following the disappearance of the larger oligosaccharides. In their study on seeds of ten coffee species, Chabrillange *et al.* (2000) found that the ratio of oligosaccharides to sucrose was higher in orthodox seeds compared to recalcitrant ones. The water replacement hypothesis was further supported by the findings of Volk and Walters (2006), where these authors obtained evidence that the mass of cryoprotectants taken in by plant material is equivalent to that of the water lost by the same material.

Trehalose has been previously shown to offer protection to yeast cytoplasm against dehydration *in vivo* whilst sucrose has been shown to protect enzymes against salt-induced damage *in vitro* (Blackman *et al.*, 1992). In their study on soybean embryonic axes, those authors found that drying induced an increase in raffinose family oligosaccharides (RFO) production. Although they could not specify, Blackman *et al.* (1992) found that stachyose had a role in desiccation tolerance as it increased drastically with development of soybean seeds and concomitantly acquisition of desiccation tolerance. Koster (1991) also showed, using a differential scanning calorimeter, that sucrose alone (in the absence of oligosaccharides) did not promote vitrification, therefore re-iterating the importance of raffinose and stachyose in preventing sucrose crystallization.

Buitink *et al.* (2000) questions the role of oligosaccharides as in their study these authors observed that an increase in sucrose concentration but decrease in oligosaccharide levels upon priming (induced aging) had no effect on the establishment of the glassy state in bell pepper and impatiens seeds. Subsequently, in a study using desiccation tolerant rotifers, Lapinski and Tunnacliffe (2003) deduced that there are probably several ways of achieving vitrification as those organisms did not show any sugar accumulation upon dehydration but still provided evidence for the establishment of the glassy state.

1.6.8 Lipids

In their study on tropical species producing recalcitrant seeds, Touchell and Dixon (1994) found that seed survival after exposure to liquid nitrogen correlated with the lipid content of the seeds such that seeds with greater lipid content had better survival post cryopreservation. Those authors also stated that seeds with high lipid content turn out to be less susceptible to injury upon rapid freezing rates. However, the actual role of lipids during cryopreservation is not yet fully understood (Buitink *et al.,* 2000; Touchell and Dixon, 1994). It is postulated that a specific ratio of saturated to unsaturated fatty acids is required to promote post cryopreservation survival of seeds, however, this ratio is species-specific (Buitink *et al.,* 2000; Touchell and Dixon, 1994). The lipid composition in cell membrane plays a critical role in the rate of temperature dispersal thereby controlling intracellular ice formation (Buitink *et al.,* 2000; Touchell and Dixon, 1994).

In a study comparing species from the same genus (*Acer)* but with differing seed desiccation responses, Pukacka and Czubak (1998) found a decrease in content of all phospholipids in the recalcitrant seeds upon dehydration whilst the orthodox counterparts showed an increase in main phospholipids and the others remained constant. Also in the same study, the recalcitrant seeded *A. pseudoplatanus* showed a decline in the unsaturated fatty acids and also the ratio of unsaturated/saturated fatty acids but the orthodox *A. platanoides* increased unsaturated fatty acids whilst the ratio was unchanged.

1.7 Provenance as a Limiting Factor to Cryopreservation Success of Recalcitrant-Seeded Species

More than 8% of higher plants produce either recalcitrant or intermediate seeds and these account for more than 45% of tropical rainforest plant species (Joet *et al.,* 2016). Tropical rainforest habitats are mega-diverse and contain many recalcitrant-seeded species (Ashmore *et al.*, 2011). According to those authors these habitats are under threat of extinction mainly due to their susceptibility to climate change. Tropical plant species produce a higher proportion of recalcitrant seeds than do temperate species (Berjak and Pammenter, 2002; Engelmann, 1991;

Noor *et al.,* 2011; Touchell and Dixon, 1994). Berjak *et al.* (2011) as well as Engelmann (2011) stated that it is known, but not understood, that recalcitrant seeds of species from temperate regions tend to react better to manipulations which offer success post-cryostorage when compared with those from tropical provenances. This led to the conclusion that cryopreservation protocols used for temperate species cannot be readily applied for tropical species (Noor *et al.,* 2011). According to Walters *et al.* (2008), recalcitrant seeds from tropical provenance generally lack dormancy and should therefore be cryostored immediately after harvest unlike those of temperate origin which retain viability for months after harvest. According to those authors, recalcitrant seeds from temperate provenances hold an advantage over those from tropical provenance as they are generally exposed to overwintering weather conditions and thus rendering them less sensitive to chilling stress. Farrant and Moore (2011) also stated that a possible account for the lack of desiccation tolerance in tropical recalcitrant seeds might be the fact that these species occur in environments with conditions conducive for germination upon shedding.

Berjak and Pammenter (2002) suggested that the degree of dehydration that recalcitrant seeds/axes can tolerate is strongly dependent on the water content at which the seed is shed which is in turn dependent on the amount of water that is lost or accumulated during late seed development. Concomitantly, the amount of water lost/accumulated during late seed development should be, amongst other factors, dependent on the water available in the environment and this might be the underlying difference in cryopreservation success between the seeds /axes from temperate and tropical provenances. Various interconnected processes are said to confer desiccation tolerance and lack of one or more of these processes may result in the varied degree of sensitivity displayed by the recalcitrant seeds/axes (Berjak and Pammenter, 2002). According to those authors, late embryonic abundant (LEA) proteins are suggested to be involved in dehydration tolerance and these proteins have been detected in some recalcitrant seeds from temperate regions but not in those from tropical provenances.

1.8 The Present Study

There are a series of steps required before explants can be exposed to cryogenic temperatures and then successfully recovered. These steps include: excision; exposure to cryoprotectants;

flash- (very rapid) drying; cooling in LN, warming after cryostorage and regeneration. All these steps are potentially injurious, yet recalcitrant axes from seeds of temperate origin withstand the stresses more successfully than do those from seeds of tropical origin (Berjak, 2006; Engelmann, 2011; Walters *et al.,* 2008). Recalcitrant seeds are highly variable and lack the ability to be stored, and there is inadequate biochemical and physiological information to explain the wide variation in the behaviour of these seeds (Engelmann, 2012). It is the combination of these factors, and others, that contribute to the limited success when it comes to cryopreservation of these species. It was therefore the aim of this study to determine differing responses to cryopreservation and all steps involved therein. To achieve this, two recalcitrant seeded species of tropical origin (*Castanospermum australe* and *Trichilia emetica*) and two recalcitrant seeded species of temperate origin (*Acer pseudoplatanus* and *Quercus robur*) were compared. These four recalcitrant seeded species underwent the same treatments and conditions and their responses to each treatment were recorded and compared.

Some of these species are of economic and ecological importance. For instance, the bark of *Trichilia emetica* tree has medicinal properties, whilst the seeds can be used for cosmetic purposes as well as food (Kioko *et al.,* 2006). Birds and monkeys feed on the flowers of *T. emetica* whilst monkeys and baboons feed on the fruit (Neuwinger, 2000). The bark, roots and fruits of *T. emetica* have medicinal properties and can be used against stomach ailments, fevers and skin problems and the bark is also used for dye manufacture (Neuwinger, 2000). The wood is used as timber for various tradition carvings in Southern Africa (Neuwinger, 2000).

The *genus Acer* is very complex as it includes both orthodox and recalcitrant-seeded species, maybe working on species from the same genus but different water properties provided better insight on the matter. *Acer pseudoplatanus* flowers are visited by bees in abundance as they produce nectar in copious amount (Rushforth, 1999). The wood is also used as timber for making furniture, flooring, parquetry and also in the manufacturing of musical instruments (Rushforth, 1999). *Quercus robur* is not in danger of extinction but is of great ecological importance as it harbours over 400 insects and the acorns are fed upon by sunbirds (White, 1995). *Quercus robur* is cultivated because its wood is used for furniture making (White, 1995). However, a worldwide decline of *Quercus sp.* has been observed over decades due to environmental stresses such as drought and pollution as well as infection by the fungus *Phytophtora ramoram* which results in

rapid death (Plitta *et al.,* 2014). *Castanospermum australe* seeds are poisonous to humans when ingested raw but can be processed into flour for baking (Elliot and Jones, 1982).

With respect to cryopreservation of recalcitrant seeds or their embryonic axes, cryoprotection of explants is expected to increase the temperature at which vitrification occurs, with the first order transitions becoming reduced whilst the second order transitions are more apparent (Volk and Walters, 2006). Use of one of the common cryoprotectants (e.g. glycerol) should establish whether there are differences in response to cryoprotection of recalcitrant seeds from different provenances. Using differential scanning calorimetry (DSC), it should be possible to determine the water content and temperature at which the intracellular water freezes and whether vitrification occurs. Using the data obtained using the DSC it should be possible to develop a species-specific cryopreservation protocol. Water content has a major influence on the success or failure of cryopreservation and thus proper assessment of the behaviour of water upon drying and/or cryoprotection is important. Differing responses to the cryopreservation protocol steps (such as flash drying) by seeds of different provenance might help better understand results previously obtained. On the other hand, minor differences might at least help point to one of the other steps in the cryopreservation protocol. Seeds of one of the tropical species were collected from plants occurring in the natural habitat, seeds of the other species being collected from plants growing outside their native region (had been planted). The situation was the same for *A. pseudoplatanus* and *Q. robur*. This could give pointers as to how much of an effect, if any, a region of origin and of growth have on the storage behaviour of recalcitrant seeds.

CHAPTER 2: MATERIALS AND METHODS

2.1 Seed Collection

2.1.1 Tropical Species

Mature fruits of *Trichilia emetica* were collected at Mtunzini (28.9502° S, 31.7577° E), North of Durban, KwaZulu-Natal, South Africa. The fruits were collected straight from the trees and none were collected from the ground. The fruits were transported to Durban the same day. Upon arrival the seeds were removed from the fruits and decoated. They were then surface sterilized

first for 20 minutes in 1% sodium hypochlorite (3:1 dilution of domestic bleach). The seeds were then rinsed three times with deionised water before being soaked for one hour in a 1L fungicide cocktail containing 2.5 ml l^{-1} Previcur N (active ingredient, propamocarb-HC; AgrEvo, South Africa) and 0.5 ml l^{-1} early Impact (active ingredients, triazole and benzimidizole; Zeneca Argrochemicals, South Africa). The seeds were again rinsed three times before being left to air dry down to the original weight on a laboratory benchtop. The seeds were dusted with a benomly-based fungicide, Benlate (active ingredient: benomyl/benzimidazole; Dupont, USA) prior to being placed on a plastic grid suspended over a moistened paper towel and placed in plastic storage buckets for hydrated storage. Both the buckets and the grid were pre-sterilized by soaking in 1% NaOCl for at least 30 minutes and then allowed to air dry overnight. The seeds were stored in hydrated storage at 16 °C with the lids of the buckets lined with a layer of paper towel so as to prevent any condensate dripping down on to the seeds.

Castanospermum australe A. Cunn. and *C. Fraser (Fabaceae)* is an Australian legume that grows in coastal rainforests and beaches, and can grow as tall as 40 m and have a trunk diameter of 1.2 m (Myers *et al.,* 1987). Mature (brown) pods of *Castanospermum australe* were collected rom trees on the road side in Pietermaritzburg (29.6159° S, 30.3945° E), Midlands of KwaZulu-Natal, South Africa. All the fruits were collected from the trees and none from the ground. The pods were transported, via road, same day to Durban. Upon arrival the pods were stored in hydrated at 16 °C as they were and seeds were taken out of the pods only when needed.

2.1.2 Temperate Species

Mature seeds of *Quercus robur* were collected from the ground in Himmeville, midlands of KwaZulu-Natal, South Africa. The seeds were transported, via road, within a day of collection. Upon arrival in Durban, the seeds were sorted and those deemed 'healthy' were then surface sterilized by soaking for one hour in 1% NaOCl. The seeds were then rinsed three times with deionized water and left to return to original water content (pre-determined through weighing of seeds as they dried down) through air drying on the lab desktop overnight. The following day the seeds were dusted with a benomly-based fungicide and then placed in a pre-sterilized container on a paper towel and closed with a lid lined with paper towel so as prevent any water dripping on to the seeds and then stored at 3°C room.

Mature seeds of *Acer pseudoplatanus* L. were collected form the city of Kornik in Poland. The seeds were transported via aeroplane to South Africa, Durban within 5 days after collection in a neutral coloured zip-lock plastic bag and put in a box. Prior to transportation of these seeds from Poland, their water content was reduced to about 60% of their initial water content so as to minimise chances of fungal contamination. Upon arrival the seeds were removed from their pods but the seed coat left intact. The seeds were surface sterilized first for 20 minutes in 1% sodium hypochlorite (NaOCl). The seeds were then rinsed three times with deionised water before being left to air dry down to original water content (pre-determined through weighing of seeds as they dried down) on a laboratory benchtop. The seeds were dusted with a benomly-based fungicide, Benlate (Active ingredient: benomyl/benzimidazole; Dupont, USA) prior to being suspended in a plastic grid suspended over a moistened paper towel and placed in plastic storage buckets. Both the buckets and the grid were pre-sterilized by soaking in 1% NaOCl for at least 30 minutes and then allowed to air dry overnight. The seeds were then stored in hydrated storage at 6°C with the lids of the buckets lined with a layer of paper towel so as to prevent any water dripping down on to the seeds.

2.2 Drying and Germination Curves

2.2.1 Partial Dehydration

Because of size, none of the tests was performed on whole seeds; embryonic axes instead were used. The embryonic axes were excised b removing the pulp rom the seeds using a blunt scalpel. This also enabled the analysis of the effect of excision on the vigour and viability of embryonic axes. The embryonic axes of these four species varied greatly in size with the smallest being those of *T. emetica* (*c*. 50 mm) and the largest being *C. australe* (*c.* 3 cm). To test the effect of dehydration on the viability and vigour of the embryonic axes, the excised embryonic axes were subjected to dehydration using a flash drier (Berjak *et al.,* 1989) to various water contents. The embryonic axes were evenly spaced on a mesh that was suspended over activated silica gel and was subjected to the dry air generated by a computer fan. Subjecting the embryo to various times in the flash drier gave information of the drying rate of the embryos, whilst culturing embryos after each interval gave an indication of the viability of the embryos. After each drying

interval ten embryos were used to test for the water content and 15 were cultured to determine viability. Water content was determined by weighing individual embryos before and after oven-drying over silica gel for 48 hours at 80°C, and quantitatively expressed on a dry mass basis (dmb) as g H_2O per g dry matter (g g^{-1}).

2.2.2 Rehydration and Recovery

In an attempt to employ cathodic protection so as to limit aggressive action of ROS whilst improving viability retention of the embryonic axes after flash drying, rehydration of the embryos was done in cathodic water rather than the usual calcium magnesium (CaMg) solution (Berjak *et al.*, 2011). Cathodic water was produced by electrolysing a dilute solution of $CaCl_2$ and $MgCl_2$ (1:1 aqueous solution of 0.5 µM $CaCl_2.2H_2O$ and 0.5mM $MgCl_2.6H_2O$ [Mycock, 1999]) with the anode and cathode in separate chambers. Electrolysis was at a potential of 60V for 60 minutes. Immediately after drying the embryos were soaked in cathodic water in the dark for 30 minutes prior to culturing under aseptic conditions. The embryonic axes were surface decontaminated with 5000 ppm calcium hypochlorite (Mediclor, Merck, Germany) then rinsed three times with sterile deionized water. Prior to culturing on 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 embryos were blot-dried on sterile filter paper. Five embryonic axes were plated per 90 mm diameter Petri dish. To minimize the risk of photo-oxidative-induced free radical damage, the Petri dishes were placed in a dark cupboard for at least 72 hours and then removed to a growth room with 16h light/ 8h dark photoperiod and a temperature of 28°C for further growth. The most obvious point of growth was recorded as the extension of the radicle.

2.3 Thermal Properties of Tissue Water

All Differential Scanning Calorimeter (DSC) tests were done on embryonic axes for each respective species except for those of *C. australe* which are too big to fit in a cryo-pan and therefore embryonic shoot apices were used. The water thermal properties during drying and different steps of cryopreservation were assessed using a sub-ambient DSC (TA Q2000) to ascertain the propensity for vitrification on cooling (Pammenter *et al*., 1991). The DSC does not

only allow for the study of water properties alone, but also water properties and its interaction with soluble molecules such as sugars (Vertucci, 1990). The DSC measures the energy associated with phase transitions. Two DSC pans were placed in the DSC instrument, the one were empty (reference pan) and the other contained the sample (embryonic axis). Embryonic axes were subjected to flash drying to various water contents prior to DSC measurements. Upon running the DSC scan, temperature was first equilibrated at 25°C then reduced at 10°C per minute to -150°C. It was maintained at this temperature for two minutes before ramping up at 10°C per minute to 40°C. The energy released or absorbed by the sample is compared to that of the reference pan. After getting the dry weight, consequently the water content, of the sample the enthalpy was calculated and expressed as J g^{-1}.

2.4 Cryopreservation

2.4.1 Dehydration

Prior to cryopreservation, embryonic axes were dehydrated to two specific water contents using the flash drier, namely ≈1 gg^{-1} and ≈0.3 gg^{-1} (n=15 per treatment). The water content ≈1 gg^{-1} represents the lowest water content before significant loss of viability is observed in the embryonic axes of the four species upon dehydration. The water content ≈0.3 gg^{-1} was chosen because it is the water content just before unregulated metabolism prevails and the cellular processes are still intact, even if temporally (Vertucci and Farrant, 1995). The approximate time needed to dehydrate the respective embryos was worked out using the information obtained in section 2.2.1. As another treatment, embryonic axes were cryopreserved at their initial water content this was used as a control treatment.

2.4.2 Cooling

Immediately after dehydration, the embryos were subjected to liquid nitrogen exposure, but there were three different cooling rates used.

2.4.2.1 Slow Cooling

Slow cooling was achieved by cooling individual embryonic axes using the DSC and cooling at 10°C/min to -150°C. The embryos were enclosed in cryovials and immediately transferred to liquid nitrogen with cryocanes in Dewars to be thawed at a later stage.

2.4.2.2 Rapid Cooling

Rapid cooling was achieved by putting 15 embryos in three cryovials (per water content treatment) after dehydration in 2 ml polypropylene cryovials (Greiner^{TM}) which were subsequently submerged straight into liquid nitrogen. This approximates to a cooling rate of up to -200°C per minute (Vertucci, 1989; Varghese et al., 2009). These were then stored in the liquid nitrogen freezer (in liquid phase) till thawing.

2.4.2.3 Ultra-Rapid Cooling

Ultra-rapid cooling was achieved by exposing embryonic axes to nitrogen slush (liquid nitrogen sub-cooled to -210°C under vacuum) immediately after dehydration. Nitrogen slush was prepared in a 100 mm x 50 mm diameter polystyrene container and this achieved a cooling rate approximating hundreds of °C per second. After exposure to slush, the embryos were transferred to a liquid nitrogen freezer in cryovials for storage until thawing.

2.4.3 Thawing

Storage of embryonic axes at cryogenic temperatures lasted a minimum of 24 hr, after which they were retrieved from the cryogen and individual embryos were soaked in cathodic water at 40 °C for 30 minutes in the dark. These were then cultured for survival assessment as described above (Section 2.2.2).

2.5 Cryoprotection

To ascertain if cryoprotection had any effect on vigour and viability of embryonic axes, embryonic axes were subjected to soaking in 5% glycerol (w/v) for 30 minutes and then in 10% glycerol (w/v) for 30 minutes. This on its own had dehydration properties prior the embryos being subjected to steps mentioned above. This was done for embryonic axes of all four recalcitrant seeded species.

2.6 Data Analysis

Statistical analyses were performed using SPSS 23.0 (version 23.0, SPSS Inc., Chicago, Illinois, USA) and GenStat 12.0, using the critical p = 0.05. All the germination data is categorical therefore it was arcsine transformed prior to any analysis. The two-way analysis of variance (ANOVA) was used to determine the effect of partial dehydration, cryoprotection and cooling rate on the success rate of embryonic axes of recalcitrant-seeded species from different provenances. The assumption of normality of the distribution of the residuals was tested for using 1-sample Kolmogorov Smirnov test, and the residuals were normally distributed. The assumption of equality of variances of the residuals was tested for using the Levene's test and the assumption was satisfied. The lowest significant mean difference (L.S.D.) was also determined so as to create subsets. For graphs depicting the effect of partial dehydration on germination (n=15), standard deviations are represented by error bars. A regression equation was calculated for each of the four recalcitrant seeded species with enthalpy (Jg^{-1}) as function of water content (gg^{-1}). The slope of the regression signified the enthalpy of the cellular water in the embryonic axes whilst the x intercept signified the amount of non-freezable water in the embryonic axes.

CHAPTER 3: RESULTS

The present study investigated the responses of embryonic axes of four recalcitrant-seeded species two each from tropical and temperate provenances to various steps involved in

cryopreservation of biological material. This chapter describes the viability responses of the embryonic axes of the recalcitrant-seeded species to: (1) excirsion, (2) partial dehydration, (3) partial dehydration and subsequent cryopreservation, and (4) cryoprotection followed by partial dehydration and subsequent cryopreservation. Results are generally presented for all four species (*Acer pseudoplatanus, Castanospermum australe, Trichilia emetica* and *Quercus robur*) in order to draw comparisons across species. However, due to variation in seasonal seed availability, some experiments/treatments such as cryoprotection and cryopreservation were performed on embryonic axes collected only in the second season (the experiment was repeated twice). Although there are a number of different cryoprotectants that previously have been used for the cryopreservation of embryonic axes (Benson, 2008; Plitta *et al.,* 2014; Teixeira, *et al.,* 2014), the choice of Gly was based on the beneficial effects in recalcitrant seed material which include enhanced antioxidant enzyme activity, reduced lipid peroxidation and thereby improving post cryopreservation survival (Sershen *et al.,* 2012).

3.1 Partial Dehydration

Castanospermum australe embryonic axes had significantly higher initial water content (2.58±0.43 gg^{-1}) than all the other species with *A. pseudoplatanus* having the lowest (1.3 gg^{-1}). It took 15-30 minutes for the water content of embryonic axes of all four species to dry down to around 1.0 gg^{-1} from their respective initial water contents (Figure 1). However, time taken to reach the water content around 0.3 gg^{-1} ranged from 90 minutes for *A. pseudoplatanus* to 240 minutes for *Q. robur* (Figure 1).

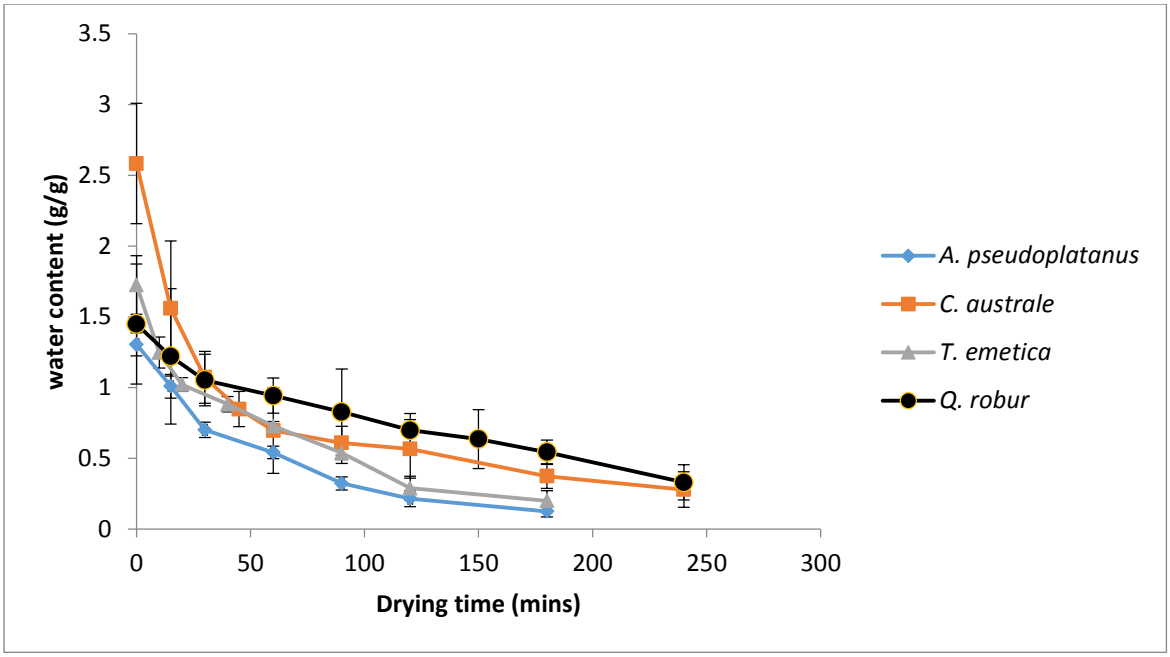

Figure 1: Effects of flash drying on water content of embryonic axes (n=10) of the four recalcitrant-seeded species (replication =2). Error bars represent the standard deviation from the mean.

All four species exhibited 100% germination at the initial water content and there was a significant drop when axes were rapidly dried to a water content of 1.0 gg^{-1} except for *A. pseudoplatanus* which started to decline in germination only at water contents below 0.54 gg^{-1} (Figure 2). Around water contents of 0.3 gg^{-1} *A. pseudoplatanus* exhibited 67% germination, followed by *Q. robur* with 40% germination and *C. australe* and *T. emetica* at 27% and 30% viability, respectively (Figure 2).

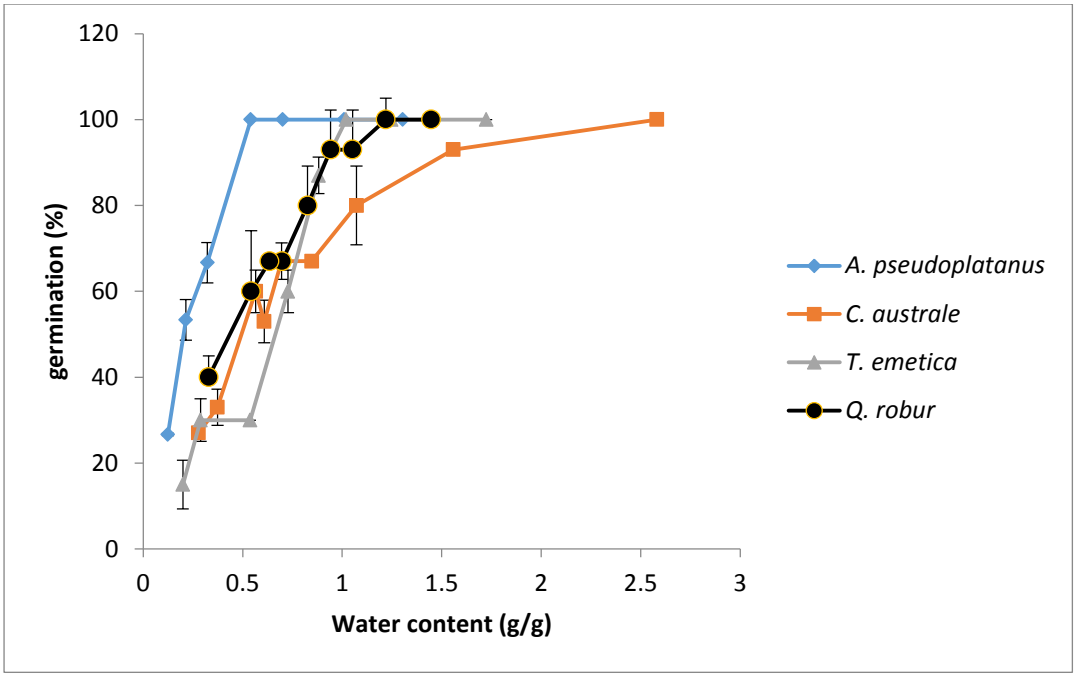

Figure 2: Effect of flash drying on germination as a function of water content of embryonic axes (n=15) of four recalcitrant-seeded species (replication=2). Error bars represent the standard deviation from the mean.

3.2 Cryoprotection

Upon cryoprotection with Gly *Q. robur, C. australe* and *A. pseudoplatanus* lost water and had initial water content of 1.09, 2.03 and 1.18 gg^{-1}, respectively whilst *T. emetica* exhibited an increase in water content after cryoprotection with Gly, 1.94 gg^{-1} (figure 3). After cryoprotection, it took *T. emetica* and *C. australe* 40 and 60 minutes, respectively, to reach water content of 1 gg^{-1} when subjected to flash drying, whilst *A. pseudoplatanus* and *Q. robur* took 5 and 15 minutes, respectively, after cryoprotection with glycerol (Figure 3). To reach water contents around 0.3 gg^{-1} took 120 minutes for both *C. australe* and *Q. robur* whilst *A. pseudoplatanus* was shorter with 30 minutes and *T. emetica* longer with 240 minutes after cryoprotection with glycerol (Figure 3).

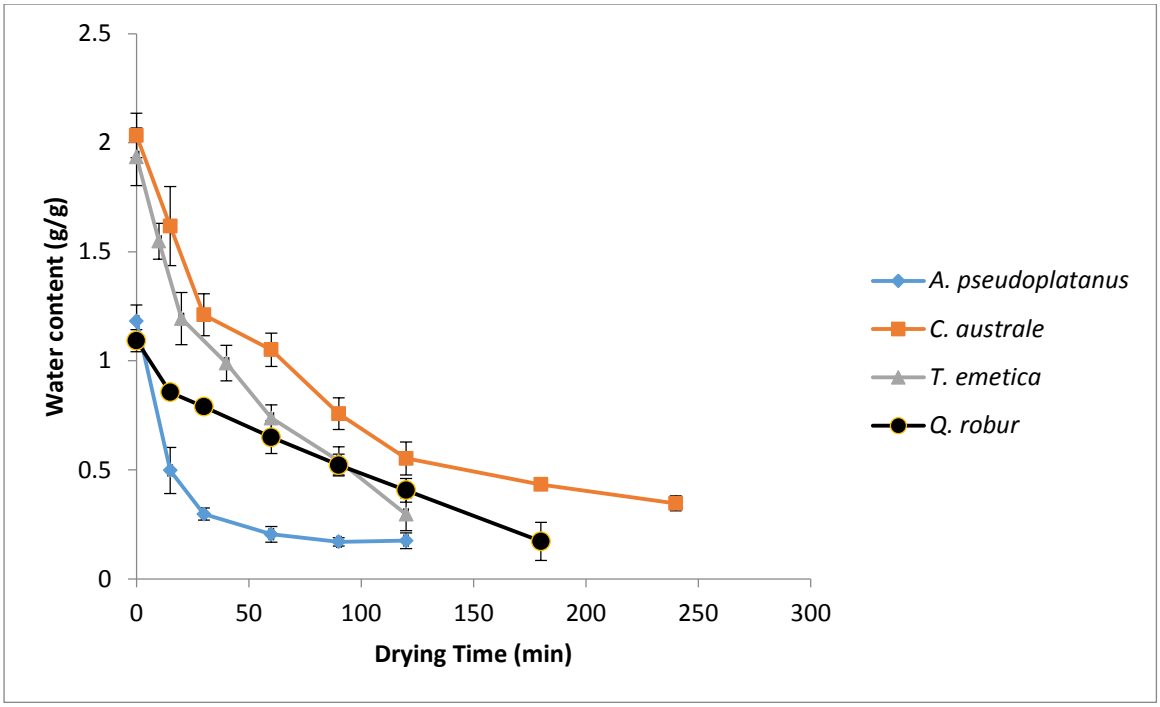

Figure 3: Effects of cryoprotection with Gly and subsequent flash drying on water content of embryonic axes (n=10) of the four recalcitrant-seeded species (replication=2). Error bars represent the standard deviation from the mean.

Germination percentage was 100 for embryonic axes of all four species post cryoprotection but prior to dehydration (figure 4). This declined to 73% and 67% for *T. emetica* and *C. australe*, respectively after drying embryos to around 1.0 gg^{-1}, post cryoprotection with glycerol, whilst germination of both *A. pseudoplatanus* and *Q. robur* were still 100%. After flash drying to around 0.3 gg^{-1}, *A. pseudoplatanus* and *Q. robur* exhibited viability of 93% and 60% respectively whilst *C. australe* and *T. emetica* had 30% and 20% viability, respectively, post cryoprotection with glycerol (Figure 4).

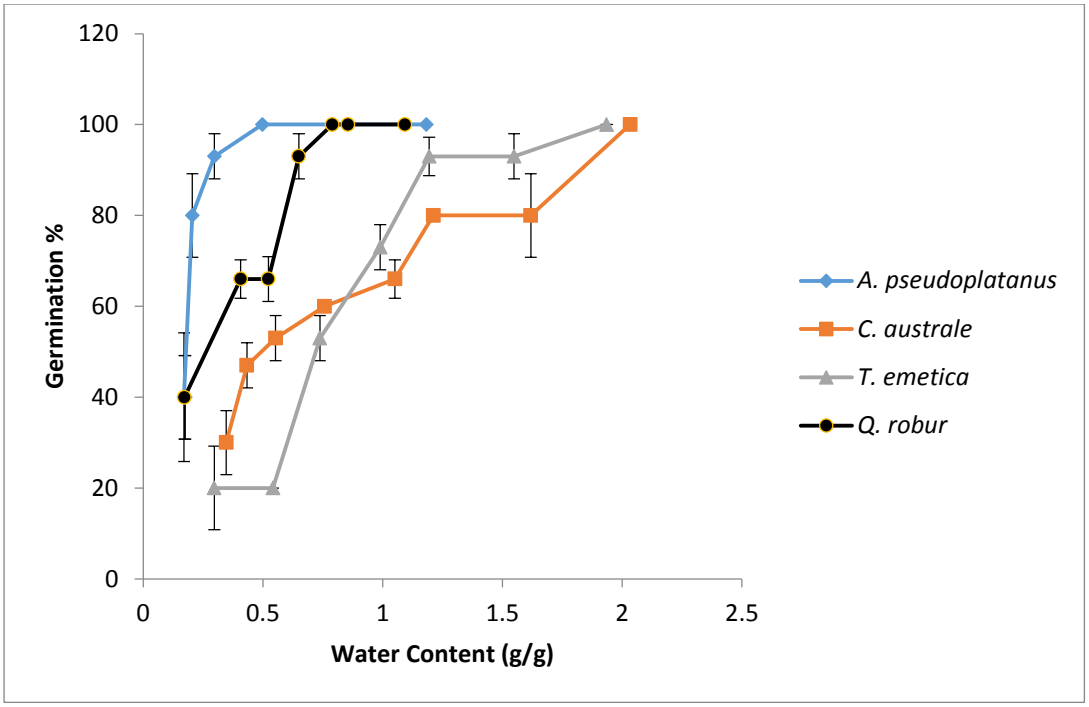

Figure 4: Effects of cryoprotection with Gly and subsequent flash drying on germination (n=15) of embryonic axes of the four recalcitrant-seeded species (replication=2).

Table 1 shows that cryoprotection with glycerol resulted in a decline of water contents of all embryonic axes except for *T. emetica* where water content increased after cryoprotection but prior to flash drying. Concomitantly, this resulted in an increase in time taken for the embryonic axes of *T. emetica* to be flashed dried to ≈1.0 gg^{-1}. The time it took for embryonic axes of *C. australe* to be flash dried to ≈1.0 gg^{-1} also increased, though there was a decline in water content of axes post cryoprotection with glycerol. This increase in time may have resulted in a decline in germination for both these species post cryoprotection with glycerol and flash drying to ≈1gg^{-1} (Table 1).

Furthermore, for all four species, the percentage of water lost by the embryonic axes (wth respect to time) from the initial water content to ≈1.0 gg^{-1} was greater when the axes were not cryoprotected compared to when they were cryoprotected with glycerol regardless of the time taken to dry down to that particular water content. The time taken for embryonic axes of the two species from tropical provenance to dry down to ≈0.3 gg^{-1} was the same regardless of whether they were cryoprotected or not prior to flash drying, whilst the two species from temperate provenances took a much shorter time when cryoprotected with glycerol compared

to when not cryoprotected. However, *T. emetica,* when cryoprotected with Gly, had the highest drying rate (0.05 gg^{-1} min^{-1}) to reach ≈1.0 gg^{-1} whilst the drying rate of *Q. robur* was unchanged (0.001 gg^{-1} min^{-1}) regardless of cryoprotection with Gly. At 0.028 $gg^{-1}min^{-1}$ cryoprotected *A. pseudoplatanus* embryonic axes had the highest drying rate to reach ≈0.3 gg^{-1} (Table 1).

Table 1: Summarised results from cryoprotecting with Gly and partial dehydration to specific water contents on the germination of four recalcitrant-seeded species as well as their drying characteristics (CP=Cryoprotection [No Gly=no cryoprotection; Gly=cryoprotected with glycerol]; WC= water content [g H_2O g^{-1} dry mass]; DR=drying rate at a specific range [gg^{-1} per minute]).

Species	CP	Initial water content			≈ 1.0 gg^{-1}				≈ 0.3 gg^{-1}			
		WC	% germination	DR	% WC lost	% germination	DT (min)	DR	% WC lost	% germination	DT (min)	
A. pseudoplatanus	No Gly	1.3	100	0.02	22.31	100	15	0.009	75.38	67	90	
A. pseudoplatanus	Gly	1.18	100	0.03	14.41	100	15	0.028	74.58	93	30	
C. australe	No Gly	2.58	100	0.05	58.53	80	30	0.004	89.15	27	240	
C. australe	Gly	2.03	100	0.02	48.28	67	60	0.004	85.22	30	240	
T. emetica	No Gly	1.72	100	0.04	70.70	100	20	0.007	83.14	30	120	
T. emetica	Gly	1.94	100	0.02	48.9	73	40	0.009	84.54	20	120	
Q. robur	No Gly	1.45	100	0.01	27.59	93	30	0.004	79.31	40	240	
Q. robur	Gly	1.09	100	0.01	5.94	100	5	0.005	59.41	67	120	

3.3 Cryopreservation

To determine the responses of axis to partial dehydration only, and cryoprotection with Gly and subsequent partial hydration on the viability post cryopreservation, embryonic axes of the four recalcitrant-seeded species were exposed to LN using three different cooling rates at three different water contents. The three different water contents were initial water content (undried [control], written as IWC), those rapidly dried to ≈1.0 gg^{-1} and ≈0.3 gg^{-1} dmb. The three cooling rates were achieved via direct exposure to LN slush (written as slush), putting in cryovials then expose to LN (written as vial) and by cooling using DSC, written as DSC (Table 2).

Cryoprotecting with Gly then drying down to ≈0.3gg^{-1} water content prior to subsequent rapid cooling via LN slush yielded the highest germination upon retrieval for both *A. pseudoplatanus* (66.5%) and *Q. robur* (60%) (Table 2). Embryonic axes of these species were able to germinate and produce roots and shoots upon retrieval from LN (Figure 6). Embryonic axes of both species from tropical provenance (*C. australe* and *T. emetica*) did not respond well to any of the treatments prior to and including cryopreservation (Table 2). The second highest germination for *Q. robur* was 36.25% when the embryonic axes were not cryoprotected but dried down to ≈0.3 gg^{-1} water content then rapidly cooled using slush (Figure 6). The second highest germination of *A. pseudoplatanus* (33.5%) was when the embryonic axes were cryoprotected with glycerol, flash dried to ≈0.3gg^{-1} prior to slow cooling via the DSC (Table 2). The only time when there was callus formation on one embryonic axes of *T. emetica* when cryoprotected with glycerol, flash dried to ≈0.3gg^{-1} prior slow cooling via the DSC whilst for *C. australe (*6.5%*)* there was an extension of the radicle when cryoprotected with glycerol, flash dried to ≈0.3gg^{-1} prior rapid cooling (Figure 7).

Table 2: Mean germination (%) for all species under different treatments after retrieval from LN with each superscript denoted to a subset group (compared means across the four species within a particular treatment) should the means differ by more than the calculated LSD value (n=15; replication=2).

Cryoprotection	Cooling rate	Water content	Species			
			A. pseudoplatanus	*C. australe*	*T. emetica*	*Q. robur*
None	DSC	IWC	0^{a}	0^{a}	0^{a}	0^{a}
None	DSC	≈1.0 gg^{-1}	0^{a}	0^{a}	0^{a}	0^{a}
None	DSC	≈0.3 gg^{-1}	13.5^{b} roots	0^{a}	0^{a}	0^{a}
None	Vial	IWC	0^{a}	0^{a}	0^{a}	0^{a}
None	Vial	≈1.0 gg^{-1}	0^{a}	0^{a}	0^{a}	0^{a}
None	Vial	≈0.3 gg^{-1}	0^{a}	0^{a}	0^{a}	0^{a}
None	Slush	IWC	0^{a}	0^{a}	0^{a}	0^{a}
None	Slush	≈1.0 gg^{-1}	0^{a}	0^{a}	0^{a}	0^{a}
None	Slush	≈0.3 gg^{-1}	16.5^{b} roots	0^{a}	0^{a}	36.25^{c} roots
Gly	DSC	IWC	0^{b}	0^{a}	0^{a}	0^{a}
Gly	DSC	≈1.0 gg^{-1}	0^{a}	0^{a}	0^{a}	0^{a}
Gly	DSC	≈0.3 gg^{-1}	33.5^{c} shoots	0^{a}	3.5^{a} callus	10^{b}
Gly	Vial	IWC	0^{a}	0^{a}	0^{a}	0^{a}
Gly	Vial	≈1.0 gg^{-1}	0^{a}	0^{a}	0^{a}	0^{a}
Gly	Vial	≈0.3 gg^{-1}	16.5^{b} shoots	0^{a}	0^{a}	0^{a}
Gly	Slush	IWC	16.5^{b} roots	0^{a}	0^{a}	3.5^{a} roots
Gly	Slush	≈1.0 gg^{-1}	13.5^{b} roots	0^{a}	0^{a}	3.5^{a} roots
Gly	Slush	≈0.3 gg^{-1}	66.5^{d} shoots	6.5^{b}roots	0^{a}	60^{c} roots

LSD at 5% significance level = 5.9

All treatments significant with $p<0.0005$

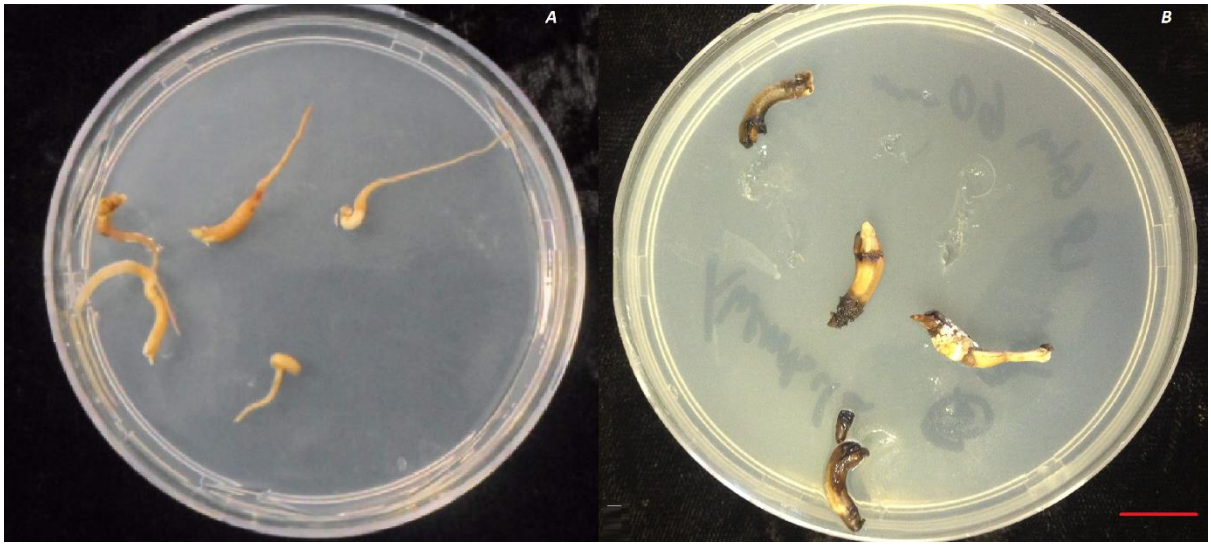

Figure 5: Embryonic axes of (a) *A. pseudoplatanus*; and (b) *Q. robur* showing radicle elongation (rooting) after six and four weeks, respectively, in culture following flash drying to ≈0.3 gg^{-1} and subsequent ultra-rapid cooling using LN slush, without cryoprotection with Gly. Scale bar = 10 mm.

Figure 6: Embryonic axes of (a) *A. pseudoplatanus* showing rooting and shooting; and (b) *Q. robur* showing rooting after eight and seven weeks, respectively, in culture following cryoprotection with Gly prior to flash drying to ≈0.3 gg^{-1} and subsequent ultra-rapid cooling using LN slush. Scale bar = 10 mm.

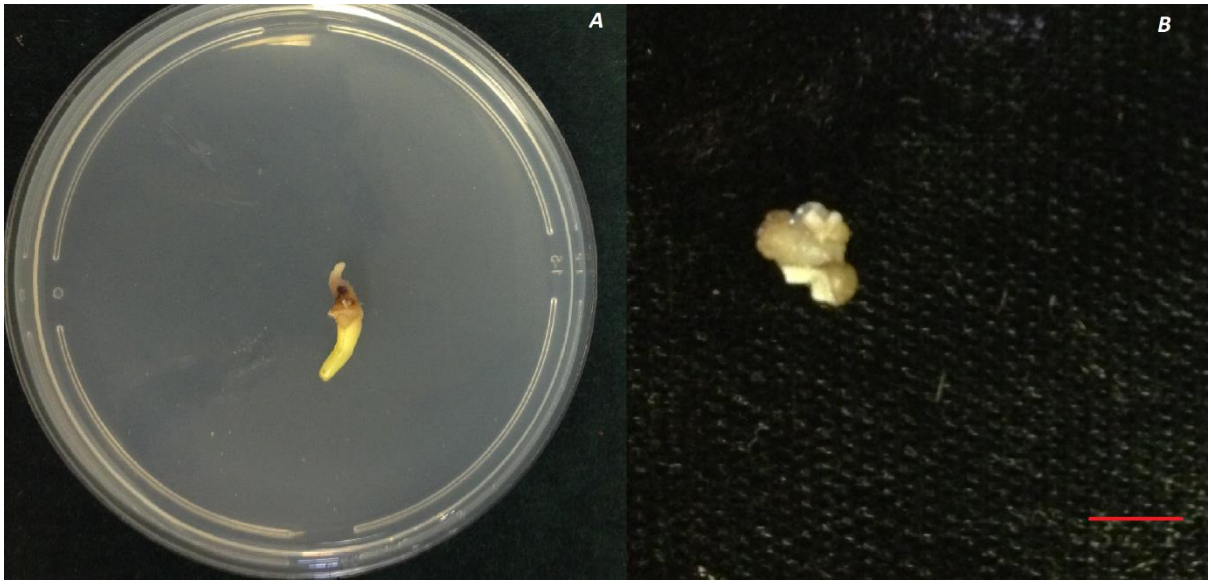

Figure 7: Embryonic axes of (a) *C. australe* showing radicle elongation after six weeks in culture following cryoprotection with Gly prior to flash drying to ≈0.3 gg^{-1} and subsequent ultra-rapid cooling using LN slush and (b) *T. emetica* showing callus development after four weeks in culture following cryoprotection with Gly prior to flash drying to ≈0.3 gg^{-1} and subsequent slow cooling using the DSC. Scale bar = 10 mm.

3.4 Thermal Properties of Water

With water content, cryoprotection and the individual species all significant contributors to the observed germination after cryostorage (Table 2), it then became a logical next move to investigate water behaviour upon dehydration, with or without cryoprotection for each individual species. This was done by subjecting embryonic axes of the four species to Differential Scanning Calorimetry (DSC). Firstly, embryonic axes were flash dried then subjected to DSC for thermal analysis. Another set of axes were cryoprotected with Gly and then flash dried prior to DSC measurements. First order (melting) transitions were detected for all four species regardless of cryoprotection and dehydration. Second order (vitrification) transitions were detected only for embryonic axes of *A. pseudoplatanus* that had not been cryoprotected with Gly but had been flash dried to water contents of 0.38-0.27 gg^{-1}. This transition occurred at a temperature ≈-70°C (Figure 8). All first order transitions were detected at -10 to -15°C for *A. pseudoplatanus* embryonic axes which were flash dried but not cryoprotected. For the axes that had been cryoprotected then subsequently flash dried first order transitions were detected around -20°C but kept on declining with decreasing water content until -40°C for the embryonic axes flashed dried to ≈0.3 gg^{-1} (Table 3). Table 3 also shows that all first order transitions for embryonic axes of *Q. robur* and *T. emetica* were detected to have peaked at 0°C

regardless of water content and cryoprotection with glycerol. This was of course with the exception of uncryoprotected but flash dried to ≈0.3 gg^{-1} embryonic axes of *T. emetica* (-10°C) and cryoprotected then subsequently dried to ≈0.3 gg^{-1} embryonic axes of *Q. robur* (-30°C). First order transitions of embryonic axes of *C. australe* were detected to have peaked around -5°C at initial water content but further decreased to around -10°C when dried down to around ≈0.3 gg^{-1} uncryoprotected (Table 3). When cryoprotected with Gly the first order transitions of *C. australe* peaked around -10°C regardless of water content (Table 3). According to Table 3, for all species, with the exception of *T. emetica,* the enthalpy was relatively higher for the uncryoprotected embryonic axes across the respective water contents and kept decreasing with a decline in water content. The DSC results also show that the enthalpy for *T. emetica* was higher when cryoprotected than when not cryoprotected.

Table 3: Specific enthalpy of the four recalcitrant-seeded species at the three water contents of interest, with and without cryoprotection with glycerol, as well as the temperature at which the transitions were detected (CP=Cryoprotection [No Gly=no cryoprotection; Gly=cryoprotected with glycerol]).

Species	CP	Initial Water Content		≈1.0 gg^{-1}		≈0.3 gg^{-1}	
		Temperature (°C)	Enthalpy (Jg^{-1})	Temperature (°C)	Enthalpy (Jg^{-1})	Temperature (°C)	Enthalpy (Jg^{-1})
A. Pseudoplatanus	No Gly	-10	388	-10	189	-15	25
A. Pseudoplatanus	Gly	-20	259	-30	201	-40	11
C. Australe	No Gly	-5	740	-5	324	-10	99
C. Australe	Gly	-10	620	-10	306	-10	114
T. emetica	No Gly	0	450	0	240	-10	80
T. emetica	Gly	0	576	0	278	0	162
Q. robur	No Gly	0	463	0	321	0	118
Q. robur	Gly	0	277	0	237	-30	63

Figure 8 shows that the embryonic axes of *A. pseudoplatanus* have the ability to vitrify when dehydrated to water contents below 0.4 gg^{-1} without cryoprotection with glycerol. All the second order transitions were detected to have peaked around -60°C. This nevertheless was not enough to

enhance survival of those embryonic axes when uncryoprotected but flash dried to ≈0.3 gg^{-1} as survival was below 20% regardless of the cooling rate used (Table 2). However, cryoprotection with Gly precluded any glass transition as none were detected when the embryonic axes of *A. pseudoplatanus* were cryoprotected with Gly (Figure 9). Conversely, it was when the embryonic axes of *A. pseudoplatanus* were cryoprotected with Gly then subsequently flash dried to ≈0.3 gg^{-1} that their survival post cryopreservation was enhanced (Table 2). Lipid content was undetectable using the DSC measurements as when the embryonic axes where dehydrated down to unfreezable water contents (<0.15 gg^{-1}) no transition peaks were detected (Figure 9).

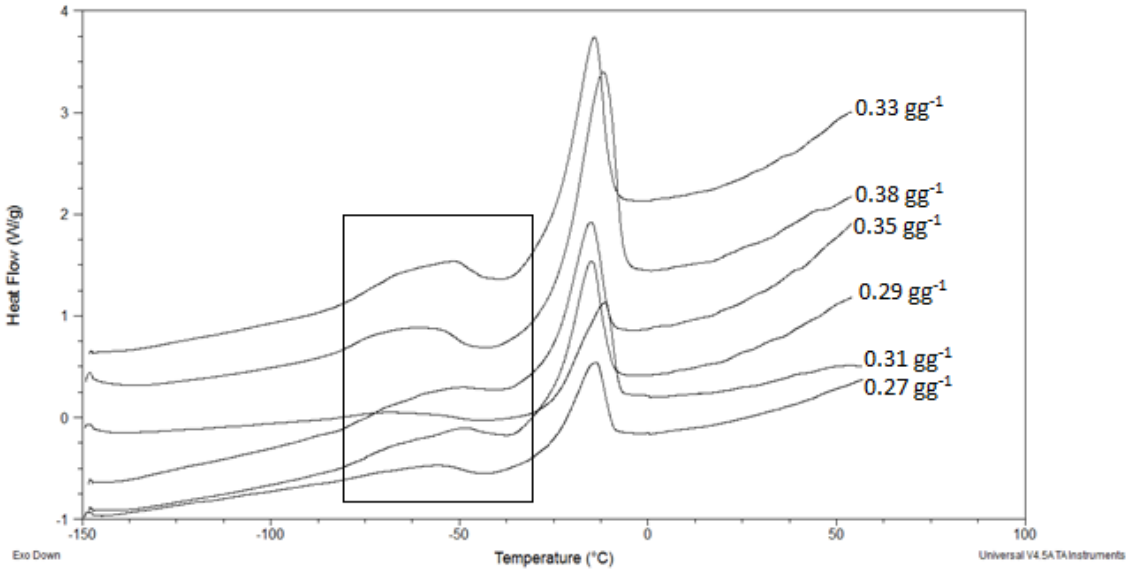

Figure 8: DSC heating thermograms showing glass transition (in the rectangle) in the embryonic axes of *A. pseudoplatanus* when flash dried to water contents of 0.38-0.27 gg^{-1} (shown on the right next to each line)without any cryoprotection with glycerol, scanned at 10°C min^{-1}.

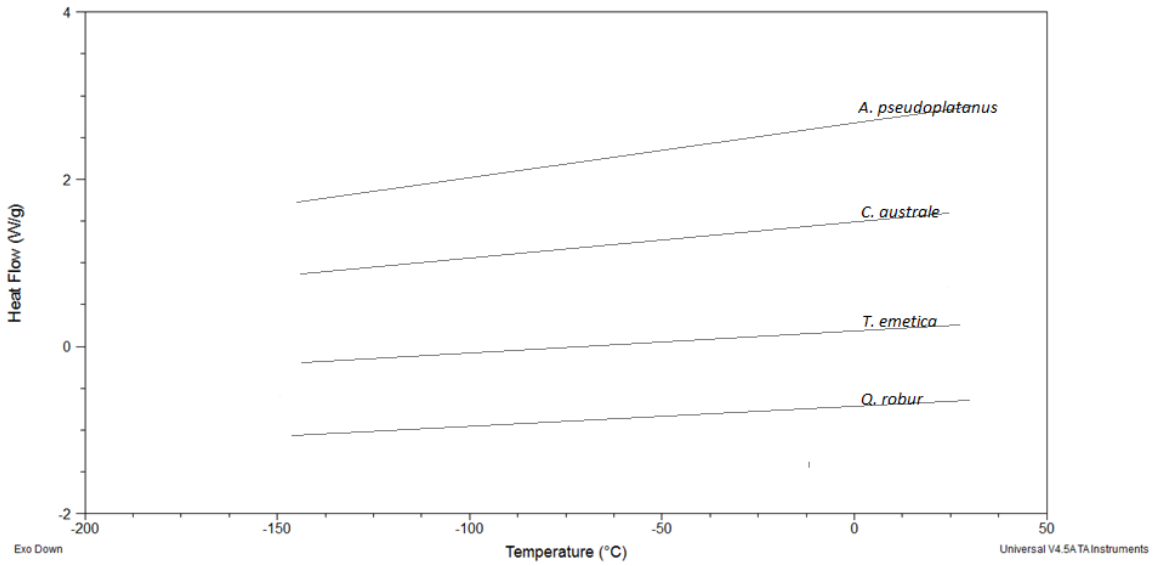

Figure 9: DSC heating thermograms of embryonic tissues of all four recalcitrant-seeded species flash dried down to < 0.15 gg^{-1} scanned at 10°C min^{-1} showing no sign of lipid transitions.

The enthalpy of melt for *A. pseudoplatanus* and *T. emetica* increased from 243.9 J/g (R^2=0.994) and 238.5 J/g (R^2=0.999), respectively when flash dried only, to 272.3 J/g (R^2=0.988) and 273.9 J/g (R^2=0.997), respectively, when cryoprotected with Gly then subsequently flash dried (Figures 10 and 11). The enthalpy of melt of *Q. robur* and *C. australe* decreased from 314.9 J/g (R^2=0.999) and 301.9 (R^2=0.999), respectively when flash dried only, to 288.3 J/g (R^2=0.989) and 282.5 J/g (R^2=0.999) respectively, when cryoprotected with Gly then subsequently flash dried (Figures 10 and 11). The amount on non-freezable water for *T. emetica* remained constant (0.1 gg^{-1} dmb) regardless of cryoprotection with Gly whilst that of *C. australe* decreased from 0.1 gg^{-1} to 0.05 gg^{-1} dmb after cryoprotection with Gly (Figures 10 and 11). The amount of non-freezable water of *A. pseudoplatanus* and *Q. robur* increase from 0.25 gg^{-1} and 0.1 gg^{-1} dmb respectively without cryoprotection with Gly to 0.4 gg^{-1} and 0.2 gg^{-1} dmb respectively, when cryoprotected with Gly (Figures 10 and 11). Also figures 10 and 11 show that upon cryoprotection with Gly, the amount of non-freezable water in the embryonic axes of *A. pseudoplatanus* and *Q. robur* increases, compared to the uncryoprotected axes, which in turn increase survival chances upon cryostorage. Also regardless of whether the enthalpy of melt for each species increased or decreased upon cryoprotection with Gly, all went to the same range of 272-288 J/g. This means that Gly potentially has the same effect on thermal properties of water regardless of the species but some other confounding factors act differently on each species.

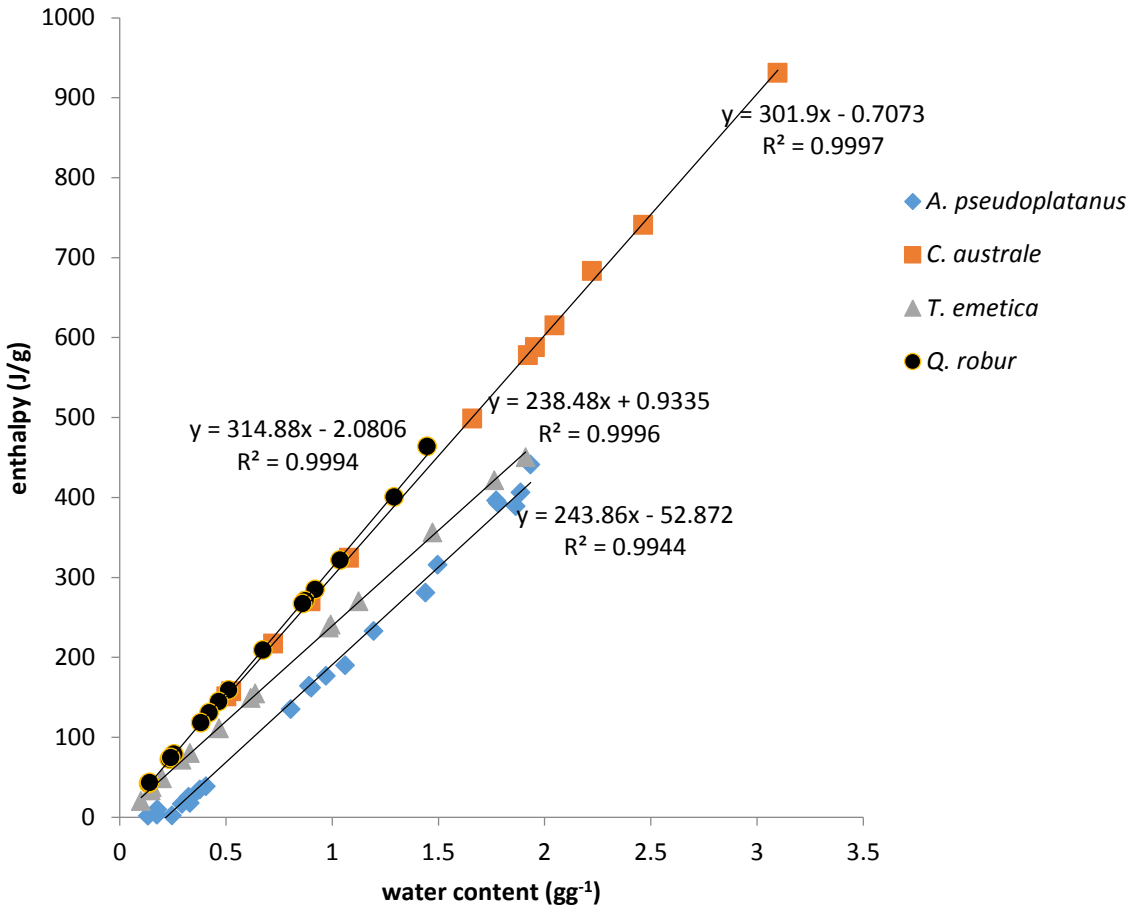

Figure 10: Enthalpy of melt for embryonic axes of four recalcitrant-seeded species after flash drying to various water contents cooled and warmed at 10 °C min^{-1} without cryoprotection with Gly.

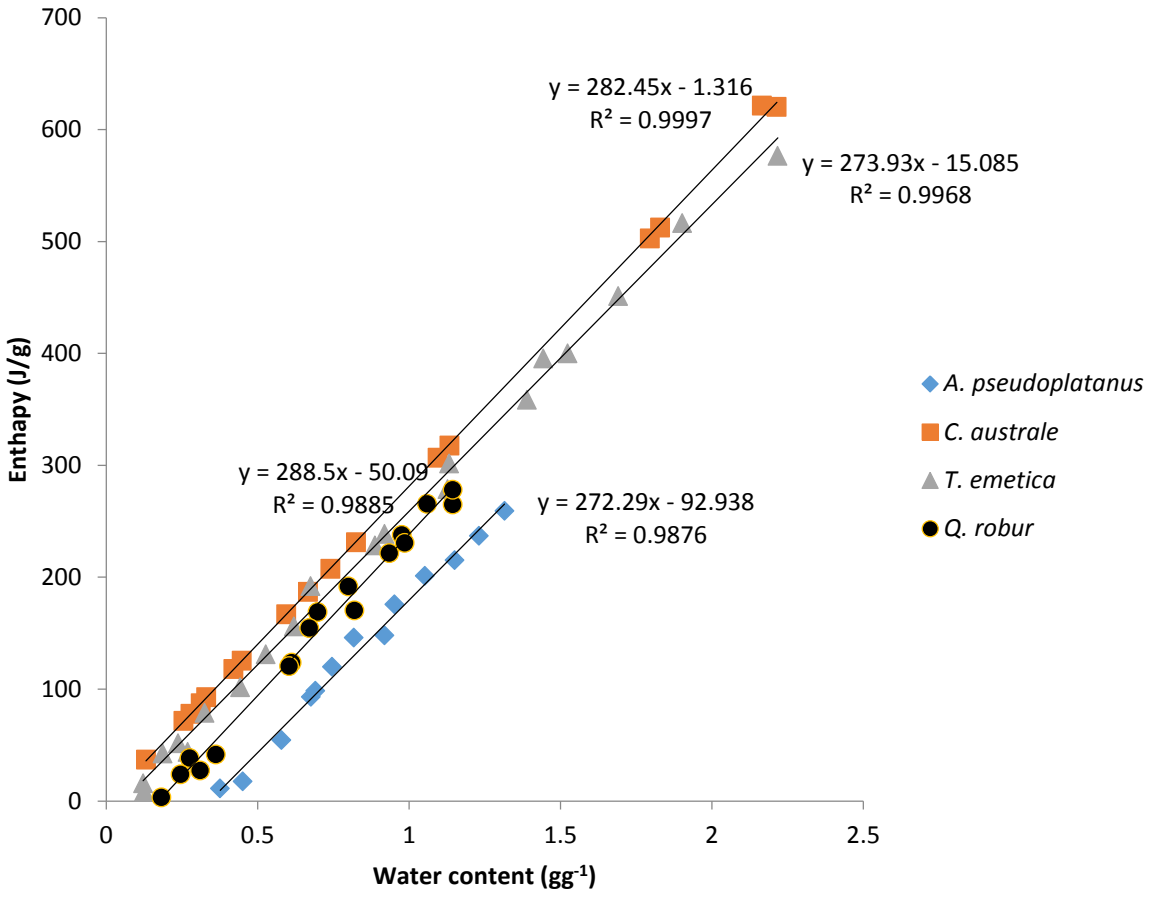

Figure 11: Enthalpy of melt for embryonic axes of four recalcitrant-seeded species after cryoprotection with Gly, flash dried to various water contents, cooled and warmed at 10 °C min^{-1}.

CHAPTER 4: DISCUSSION

Seed variability is one of the most limiting factors when it comes to conservation of recalcitrant-seeded material. However, recalcitrant-seeded material from temperate provenances tend to respond better to cryopreservation and the steps involved therein compared with those from tropical provenances. It was then the purpose of the present study to investigate where and how these differ using two recalcitrant-seeded species from temperate provenances (*Acer pseudoplatanus* and *Quercus robur*) and two from tropical provenances (*Castanospermum australe* and *Trichilia emetica*). The four species were subjected to various steps involved in cryopreservation including excision, cryoprotection, partial dehydration and exposure to LN retrieval and regeneration.

4.1 Excision

Size of an explant is of paramount importance when it comes to exposure to LN as the smaller the size of the explant the higher the cooling rate (Ballesteros *et al.,* 2014; Finch-Savage, 1992; Pukacki and Juszczyk, 2014). This also shortens the time during which some parts of the explants are cooled thus halting metabolism whilst other parts would still be undergoing (Ballesteros *et al.,* 2014; Finch-Savage, 1992; Pukacki and Juszczyk, 2014). Smaller sized explants also allow better and/or easier manipulations (Ballesteros *et al.,* 2014; Benson, 2008). . Excision of recalcitrant-seeded species is vital as larger dry mass reduces the cooling rate whilst rapid cooling rates are necessary for successful cryopreservation (Ballesteros *et al.*, 2014). A whole recalcitrant seed dries at a much slower rates which promotes cellular deterioration and spread of pathogen before desired water contents are reached (Whitaker *et al.,* 2010). Excision to embryonic axes is therefore inevitable for most recalcitrant-seeded species as the seeds of these species are usually fleshy and big whilst, for instance in *Q. robur,* 98% of their seed weight is made up by cotyledons (Finch-Savage *et al.,* 1996). However, excision itself may result in ROS burst thus immediately after excision prior any other manipulations, embryonic axes of *A. pseudoplatanus, C. australe, T. emetica* and *Q.* robur were set for germination in media. Since undehydrated embryonic axes of all four species produced 100% percent germination it can be deduced that excision alone, though leading to ROS production, does not have detrimental effects on those recalcitrant seeds regardless of provenance

4.2 Dehydration

Water content is of paramount importance during cryopreservation as it must not be too high as it would promote ice crystallisation but also not too low as that would result in cellular damage (Wesley-Smith *et al.*, 1992). Embryonic axes of recalcitrant-seeded species can have vacuoles occupying as much as 60% of their cellular space and this results in an increase in dehydration sensitivity (Umarani *et al.,* 2015). Those embryonic axes are therefore susceptible to mechanical damage upon dehydration as the vacuoles collapse resulting in volume reduction (Umarani *et al.,* 2015). The cytoskeletal elements undergo conformational changes upon dehydration and the failure of the cytoskeletal elements to reassemble upon rehydration contributes towards mechanical damage (Umarani *et al.,* 2015). According to those authors, the inability of cellular membranes to recover upon rehydration also contributes towards mechanical damage via cellular leakage. Another damage that embryonic axes of recalcitrant-seeded species are susceptible to is metabolism-linked

damage which is a consequence of ROS burst coupled with ineffective antioxidant mechanisms (Walters *et al.*, 2001).

To test if dehydration resulted in altered responses in species from different provenances, embryonic axes of *A. pseudoplatanus, C. australe, T. emetica* and *Q.* robur were flash dried to various water contents and set for germination in media. When checking water content immediately after excision prior to any manipulation, the two recalcitrant-seeded species from tropical provenances exhibited higher initial water contents compared with those from temperate provenances. Generally, tropical provenances have higher relative humidity than temperate ones and this might be the influence behind such a difference in initial water contents (Sun, 1999). Time taken for loss of viability upon flash drying to become apparent varied across species, ranging from 15 minutes for *T. emetica* to 90 minutes for *A. pseudoplatanus*. Sershen *et al.* (2016) suggested that respiration activity declines in embryonic axes of recalcitrant-seeded species upon dehydration, but that is associated with metabolic impairment rather than controlled metabolic down-regulation.

There was no immediate obvious trend associated with provenances observed as for all four species, the initial drying rate to get to around 1 gg^{-1} was relatively higher than that required to reach around 0.3 gg^{-1} water contents. However, to reach water contents around 0.3 gg^{-1}, embryonic axes of the two species from tropical provenance lost a higher percentage of water than those from temperate provenances and concomitantly, the percentage survival was higher for the temperate species than for tropical recalcitrant-seeded species. This was irrespective of that both *C. australe* and *Q. robur* had the lowest drying rates whilst *A. pseudoplatanus* had the highest drying rate. This means that drying rate did not necessarily have an influence on the percentage water lost and concomitantly, viability after dehydration. Furthermore, the results were also consistent with findings of Sershen *et al.*, (2016) that shedding water content does not have an effect of the drying rate. Finch-Savage *et al.* (1996) and, Pukacki and Juszczyk (2014) reported evidence of lipid peroxidation upon dehydration of embryonic axes of *Q. robur* and *A. pseudoplatanus*. However, those two species were able to display better survival upon dehydration than their tropical counterparts. Finch-Savage *et al.* (1994) reported on the synthesis of late embryogenesis accumulating proteins (LEAs) which offer protection against desiccation damage in embryonic axes of *A. pseudoplatanus* and *Q. robur* upon dehydration. Dehydrin proteins are understood to bind to micromolecular structures thereby preventing damage during dehydration (Finch-Savage *et al.*, 1994). The presence of the dehydrin

proteins in the embryonic axes of *A. pseudoplatanus* and *Q. robur* is only enough to confer limited protection upon dehydration but not tolerance against desiccation (Finch-Savage *et al.*, 1994).

4.3 Cryoprotection

One of the major effects of cryoprotection is partial dehydration (Berjak *et al.*, 2010; Engelmann, 2012) However, it is not the water content *per se* that declines, but a decline in the water to solutes ratio as there is an increase in solute concentration and this results in a decline in the calculated water content of the embryonic axes (Berjak *et al.*, 2010; Engelmann, 2012). However, for embryonic axes of *T. emetica*, cryoprotection with Gly was accompanied by an increase in water content. All the embryonic axes of the other three recalcitrant-seeded species had a decline in water content upon exposure to Gly. One possible explanation for the increase in water content of the embryonic axes of *T. emetica* is that some of the solutes in these axes are soluble in Gly thus decreasing the solutes concentration which in turn increases the water content. Glycerol is a non-toxic polar organic solvent capable of dissolving inorganic salts, enzymes, acids, bases as well as other organic compounds which are poor miscible in water (Wolfson *et al.,* 2007). However, identification of intracellular solutes was beyond the scope of the present study.

Imbibition in Gly did not affect the viability of the embryonic axes for all the four recalcitrant-seeded species after excision but prior to flash drying. Cryoprotection with GLY did not change the time it took for partial dehydration of embryonic axes of *C. australe* to start losing viability, whilst that of *T. emetica* and *A. pseudoplatanus* were decreased by 10 and 30 minutes, respectively. However, cryoprotection with Gly increased the time it took for embryonic axes of *Q. robur* to start losing viability by 60 minutes thereby displaying the protective qualities of Gly. Concomitantly, the rate of water loss declined post cryoprotection with Gly for the two recalcitrant-seeded species from tropical provenances whilst those from temperate provenance were increased. This is in agreement with Benson (2008) finding that seeds readily lose water upon dehydration when prior imbibed in Gly. Dehydration to water contents ≈1 gg^{-1} post imbibition in Gly resulted in relatively lower viability values for the embryonic axes from tropical provenances whilst the time taken to reach this water content doubled for both *C. australe* and *T. emetica*. However, further dehydration to water contents ≈0.3 gg^{-1} did not have any significant effect on the viability of tropical embryonic axes whilst those from temperate provenances increased in viability. This might be explained by the fact the time taken by the two tropical species to reach ≈0.3 gg^{-1} was not affected by Gly whilst that of

the temperate species was greatly reduced. As previously explained, reducing the time taken by embryonic axes to reach a particular water content reduces the time the axes experiences dehydration damage thereby enhancing their chances of survival (Ballesteros *et al.,* 2014; Engelmann, 2012). However, Berjak and Pammenter (2013) stated that the use of cryoprotectants can result in ROS burst rather than combating ROS and this has been shown for embryonic axes of certain recalcitrant-seeded species from tropical provenances.

4.4 Cryopreservation

With all the different steps that embryonic axes are subjected to prior to cryopreservation having run their course, it was time to see the effect of the actual exposure to LN of the surviving embryonic axes of the four different recalcitrant-seeded species. There was no survival recorded for embryonic axes of the tropical species post cryopreservation when they were not cryoprotected with Gly prior to cryopreservation. Even after cryoprotection with Gly, survival of those species was limited to callus formation and radicle elongation for *T. emetica* and *C. australe*, respectively. For both species the survival percentage was lower than 10%. On their study on embryonic axes of *T. dregeana*, a tropical recalcitrant-seeded species in the same genus as *T. emetica*, Whitaker *et al.* (2010) attributed low survival post cryopreservation to a burst of ROS which were bound to the cell wall. This ROS burst was recorded throughout all steps up to and including cryopreservation (Whitaker *et al.*, 2010). Kioko *et al.* (2006) reported ultrastructural damage during storage of *T. emetica* seeds and associated this damage with chilling sensitivity, typical of recalcitrant-seeded species from tropical provenances. Cell membranes are most susceptible to cryo-injury mostly through the disruption of the lipid-protein structure which can occur in several ways during exposure to LN (Pukacki and Juszczyk, 2014).

There was survival observed for axes of temperate species when they were exposed to LN without prior cryoprotection with Gly though that survival was limited to shoot formation only. Cryoprotection with Gly prior to dehydration and cooling enhanced survival rates post cryopreservation of the embryonic axes from the temperate species. Berjak and Pammenter (2014) stated that rapid cooling is effective only when the viscosity of the cytoplasm is increased i.e. when axes are cryoprotected prior to exposure to LN. Ballesteros *et al.* (2014) also supported this by stating that slower cooling rates promote ice crystallization.

Wesley-Smith *et al.* (2015) reported that rapidly cooled embryonic axes are more susceptible to damage during thawing as they would have relatively more ice crystals than the slow cooled axes. However, there still was no shoot formation observed for embryonic axes of *Q. robur* whilst shoots formed on embryonic axes of *A. pseudoplatanus* that were dehydrated to ≈0.3gg^{-1} post cryoprotection but prior to cryopreservation. Though Gly might confer only limited physical and biochemical protective mechanisms during cooling in *C. australe* and *T. emetica*, it also altered the drying characteristics for *A. pseudoplatanus* and *Q. robur*. At higher water contents, survival of *A. pseudoplatanus* and *Q. robur* embryonic axes were limited to only root formation even when they were cryoprotected with Gly. Failure to produce shoots may be a result of excision-related injury which results in ROS burst and consequently the necrosis of the apical meristem (Berjak and Pammenter, 2014). Those authors suggested that an extra step in which an antioxidant is added on the embryonic axes after excision was required so as to quench the reactive oxygen species. However, this was thought not to be necessary for the recalcitrant-species used in the present study as excision alone did not result in any loss of viability.

The production of reactive oxygen species is not always deteriorative but their concentrations have to be tightly controlled. Beneficial effects of extracellularly produced ROS include combats against pathogens, assistance in wound healing as well as intracellular cell signalling (Whitaker *et al.,* 2010). Working on embryonic axes of *A. saccharinum,* Wesley-Smith *et al.* (2015) reported subcellular damage and autophagic activity around the shoot apices and as a result the was no shoot production post cryopreservation of those embryonic axes. Those authors further suggested that formation of ice crystals during cooling acted as a signal for programmed cell death and might not be as responsible for the perceived organelle physical damage.

On their study on two *Acer* species displaying different dehydration sensitivity, Pukacki and Juszczyk (2014) showed an increase in malondialdehyde, a by-product of lipid peroxidation, in embryonic axes of *A. pseudoplatanus* upon dehydration and concomitantly cryopreservation. The increase in malondialdehyde concentration coincided with loss of viability. However, like in the present study, those authors reported better survival results for *A. pseudoplatanus* embryonic axes when they were dehydrated to about 35% of their initial water content (Pukacki and Juszczyk, 2014). This also coincided with the DSC results that dehydrating these axes to 35% of their initial water content hindered any ice nucleation events but glass formation was observed (Pukacki and Juszczyk, 2014). Working on one recalcitrant-seeded and one orthodox-seeded species of *Spartina*, Chappell *et al.*

(2015) reported no significant difference in electrolyte leakage in the two species post dehydration, however, viability after dehydration was <20% and >95% for the recalcitrant and orthodox-seeded species, respectively. These further illustrate the lack of desiccation tolerance mechanisms afforded by orthodox-seeded species but not recalcitrant-seeded species even when in the same genus.

Unlike their tropical counterparts, recalcitrant seeds from temperate provenances can display dormancy as they can persist in the soil for long periods after shedding before germination (Joet *et al.*, 2016). This dormancy displayed by the recalcitrant-seeded species from temperate provenances is suggested to be influenced by unfavourable weather conditions rather than the seeds defence mechanisms themselves (Joet *et al.*, 2016). Pawloski and Staszak (2016) reported on dormancy displayed by *A. pseudoplatanus* seeds whilst Nuc *et al.* (2016) reported on dormancy of *Q. robur* seeds post shedding prior to germination. In their study on the influence of environmental conditions on the recalcitrant seed behaviour, Joet *et al.* (2016) reported that seed water content was directly proportional to cumulative rainfall and drought duration whilst high water content was negatively correlated with high temperatures. This would partially account for the higher water contents for recalcitrant-seeded species from tropical provenances reported in the present study. Finch-Savage *et al.* (1996) and, Pukacki and Juszczyk (2014) reported evidence of lipid peroxidation which decreased viability upon dehydration of embryonic axes of *Q. robur* and *A. pseudoplatanus*. However, those two species were able to display better survival upon dehydration than their tropical counterparts and survival was enhanced upon cryopreservation with Gly further indicating antioxidant abilities of glycerol.

4.5 Thermal Properties of Water

There was a steady decline in the height and the breadth of the ice nucleation peaks associated with a decline in water content due to flash drying of embryonic axes of all four recalcitrant-seeded species from two different provenances. The same trend was also observed when the embryonic axes of all the four species were cryoprotected with Gly prior to dehydration and subsequent DSC thermal measurements. Concomitantly, the temperature at which the ice nucleation occurred subsequently declined with the decline in water content except when the two tropical recalcitrant-seeded species were cryoprotected with Gly. Working with the embryonic axes of the temperate recalcitrant-seeded *A. pseudoplatanus*, Pukacki and Juszczyk (2014) also noted that decreasing the water content decreased the ice nucleation temperature until the water content was below the

freezable water content after which no ice nucleation was observed. This was the same for Nuc *et al.* (2016) when they were working on *Q. robur* embryonic axes. The decrease in water content results in an increase in the viscosity of the cells' milieu and consequently, the amount of energy needed to freeze the system increases thus a decline in the temperature needed for ice nucleation (Benson, 2008; Buitink and Leprince, 2004). By this concept, cryoprotection with glycerol should lower the ice nucleation temperature even further. However, in the present study, the ice nucleation temperature did not change for the embryonic axes of *C. australe* and *T. emetica* post cryopreservation after cryoprotection followed by with Gly and flash drying. In fact, embryonic axes of *T. emetica* increased in water content after imbibition in Gly and consequently, enthalpy of the ice nucleation peaks was higher for when cryoprotected with Gly then when not at their respective water contents.

This observed difference is not seemingly understood but it is not the consequence of the cooling rate as Teixeira *et al.* (2014) demonstrated using five different vitrification solutions that their respective vitrification temperatures were not a consequence of the cooling rate but their thermal characteristics differed significantly upon introduction to biological explants. Cryoprotection with Gly resulted in the decline in the height of the ice nucleation peaks for all four species, even though the water properties were not the same in each species after cryoprotection with Gly. Al Zoubi and Normah (2015) reported a decrease in mean onset temperature and enthalpy peak with an increase in drying rate which also increased the amount of non-freezable water and concomitantly survival post cryopreservation.

Vitrification was observed only when *A. pseudoplatanus* embryonic axes were not cryoprotected with Gly but flash dried to water contents from 0.38-027 gg^{-1}. However, these vitrification peaks were also accompanied by ice nucleation peak. It is possible that the glass formation was a consequence of the ice nucleation as Engelmann (2010) stated that ice formation results in the removal of water from the surroundings which in turn increases the viscosity therein making conditions conducive for glass formation. Cryoprotection with Gly did not result in glass formation for the embryonic axes of any of the four recalcitrant-seeded species. Figures 10 and 11 show the amount of non-freezable water in each species without and with Gly cryoprotection, respectively. There was no benefit in this respect for the recalcitrant species from tropical provenance, whilst those from temperate provenances increased their non-freezable water upon cryoprotection with Gly. The increase in the amount of non-freezable water in *A. pseudoplatanus* and *Q. robur* upon cryoprotection with Gly increased the chances of survival of the embryonic axes of these species

upon cryopreservation. This should decrease the amount of time the embryonic axes are subjected to flash drying thereby limiting the potential ROS burst and other dehydration induced injuries.

However, the same does not apply for the recalcitrant-seeded species from tropical provenances and the difference in response to cryoprotection is not fully understood but might be one of the underlying factors for the observed difference in survival after cryopreservation in LN for recalcitrant-seeded species from different provenances. When looking at the enthalpy values of the *A. pseudoplatanus* and *T. emetic*a embryonic axes with and without Gly cryoprotection, the water thermal properties were similar. Thermal properties of water in embryonic axes of *C. australe* and *Q. robur* were also similar with and without cryoprotection with Gly. However, the effect of glycerol on the water content of these species greatly differs. Such differences can be partially attributed to the difference in provenances of these species.

When completely dehydrated embryonic axes were examined under the DSC no transitions were detected for all four recalcitrant-seeded species and this indicate low oil contents in embryonic axes. Seed oil content, and so that of the embryonic axes, is important for survival under stressful conditions as high oil content exacerbates ROS deteriorative actions (Kumar *et al.*, 2016). Soluble oil and sugar content, as well as cuticle thickness are some of the factors influencing the drying rate of embryonic axes (Ballesteros *et al.,* 2014). Oil content can be counted out as one of the possible explanations for the observed difference between the drying rate of the embryonic axes of the recalcitrant-seeded species from tropical and those from temperate provenances.

CHAPTER 5: Conclusions and Recommendations for Future Studies

Survival of embryonic axes was limited, in both percentage and quality, when they were not cryoprotected with Gly, signifying the importance of cryoprotection prior to cryopreservation. Survival was also limited when slower cooling rates were used which further emphasize the importance of rapid cooling rate upon cryopreservation so as to enhance post cryopreservation survival. Water content is also vital during cryopreservation of recalcitrant-seeded species as survival post cryopreservation was enhanced when embryonic axes were flash dried to lower water content prior exposure to LN. The findings of the present study clearly indicate that embryonic axes of

recalcitrant seeds from temperate provenances respond better to steps involved in cryopreservation than those from tropical provenances. The reasons are not fully understood but might be associated with harsh weather conditions that the temperate species experience and therefore survive as this makes them more amenable to cryopreservation conditions. The difference in genotypes between species may potentially be the major underlying factor for the varying responses to cryopreservation displayed by recalcitrant-seeded species (Pukacki and Juszczyk, 2014).

The effect of variation in recalcitrant seeds cannot be stressed enough. Recalcitrant seeds are not shed with the same water contents even when they are from the same parent tree and the standard deviation from when water contents are measured testify for such (Ballesteros *et al.,* 2014). Although sometimes negligible, the variation in water content might have consequences when the embryonic axes are dried to certain specified average water contents as some axes will have more and others less than the specified content. Those that have less than the specified water content are likely to retain viability post cryopreservation provided they were not killed by removal of structure-associated water. The embryonic axes which had more than the intended water content are likely to be killed through ice crystallisation. Both these instances can confound results of the study.

Various compounds have been reported as reliable biomarkers for desiccation stress in recalcitrant-seeded plants. These include ethane and malondialdehyde both of which are by-products of lipid peroxidation (Finch-Savage *et al.,* 1996; Pukacki and Juszczyk, 2014); reactive oxygen species; electrolyte leakage as well as cellular vacuolation (Sershen *et al.,* 2016); and heat shock proteins as well as raffinose family oligosaccharides, and conformational changes (Kumar *et al.*, 2016). Future recommendations include using at least two of these biomarkers and testing for their presence after all of the steps involved in cryopreservation. This would give insight as to whether recalcitrant-seeded species from temperate and tropical provenances experience and deal with desiccation the same and if not, where or how do they differ. This would also help determine appropriate manipulations that each individual species can tolerate and the point at which each species is more amenable to cryopreservation thereby ensuring better post cryopreservation success. Enzymatic antioxidants can also be added to thawing solution as these act by detoxification mechanisms which include dismutation of ROS molecules to water and oxygen (Kumar *et al.,* 2016)

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