

UNIVERSITY OF KWAZULU-NATAL

**Studies on the behaviour of intracellular water during
dehydration and cryopreservation of embryos
and shoot tips of *Trichilia emetica***

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**Studies on the behaviour of intracellular water during
dehydration and cryopreservation of embryos and shoot tips
of *Trichilia emetica***

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As the candidate's supervisors, we have approved this dissertation for submission.

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Preface

This dissertation is being submitted as a fulfilment of the requirements for the degree of Master of Science at the University of KwaZulu-Natal. The work described herein was conducted at the Plant Germplasm Conservation Research laboratory, School of Life Sciences, University of KwaZulu-Natal (UKZN), on the Westville Campus. This research was supervised by Prof. N.W Pammenter, Prof. P Berjak (deceased), and Dr. B Varghese, all from the University of KwaZulu-Natal, South Africa.

This dissertation is to the best of my knowledge original, except where acknowledgements are made to previous studies. Neither this dissertation, nor any other substantially similar dissertation has been or is being submitted to any other university for a similar purpose.

Part of this research was orally presented at the College Research Day, College of Agriculture, Engineering and Sciences, University of KwaZulu-Natal, Pietermaritzburg Campus in September 2015.

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Thabiso Peacemaker Thabethe

January 2017

Declaration

I, Thabiso Peacemaker Thabethe declare that:

1. I am fully aware that plagiarism is wrong. Plagiarism is considered a contravention of Rule 9(e) (i) (ff) of the UKZN Student Disciplinary Rules Handbook (2014).
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I am extremely grateful to Late Prof. P Berjak for the opportunity she gave to me, believing in me, as she first took me as a research assistant and later as her student, which opened a new door in my life to be able to pursue my interest in science of recalcitrant seeds.

Thanks are due to the University of KwaZulu-Natal for providing the resources (School of Life Sciences, and Microscopy and Microanalysis Unit [MMU]) in conducting this research project.

Lastly, my sincere gratitude to my family (Mom, “Smangele” and Ntokozo) for their love, patience and the support they have shown to me.

Dedication

To God: Colossians 1:17 “And He Himself existed and is before all things, and in Him all things hold together. (His is the controlling, cohesive force of the universe).”

(AMPLIFIED BIBLE)

Abstract

Cryopreservation of plant germplasm at ultra-low temperatures usually in liquid nitrogen (LN) (at -196 °C) or in the vapour phase of LN (-130 °C to -160 °C) is the most promising method for the long-term conservation of germplasm of recalcitrant-seeded species. Developing successful cryopreservation protocols for such germplasm, which is usually challenging due to their sensitivity to desiccation and low temperatures, depend on optimising of several procedural steps. The content and status of the water in plant tissues prior to and during exposure to ultra low temperatures is a critical factor that determines the ability of plant germplasm to survive after cryopreservation. Studies have shown that many of the lethal events occur during cooling and warming steps of cryopreservation. Hence, cryoprotective agents and cooling rates, which avoid lethal ice crystallisation and dehydration damage are applied for successful cryopreservation of plant germplasm. Over the past years, considerable attention has been given in understanding and optimising partial dehydration, freezing and thawing protocols for germplasm from various plant species producing recalcitrant seeds. However, whole embryonic axes of *Trichilia emetica* (a recalcitrant-seeded species) were reported previously to not survive cryopreservation, whilst, shoot tips have proved to be suitable explants as they survive cryopreservation.

It is for this reason that the aim of the current investigation was to gain fundamental understanding on why shoot tips excised from *in vitro* cultured seedlings survive cryopreservation whilst embryonic axes of *T. emetica* with or without cotyledonary segments attached do not survive exposure to LN. In this study, physical properties of water in cryoprotected and physically dried embryonic axis shoot segments and shoot tips (without physical drying) of *T. emetica* were assessed using differential scanning calorimetry (DSC). Embryonic axis shoot segments and shoot tips from *in vitro* cultured seedlings were pre-cultured for 3 d, cryoprotected with either 5% followed by 10% glycerol or plant vitrification solution (PVS 2) and then subjected to either slow cooling or directly plunged in LN for relatively rapid cooling. The behaviour of water in these cryopreserved explants was assessed using DSC. For viability assessments, embryonic axes with cotyledonary segments attached were used to determine retention of viability following retrieval from cryogenic conditions.

Findings of this study showed that prior to cryopreservation, viability of embryonic axes, with cotyledonary segments attached, was relatively higher in the absence of pre-culture (100%) than in the presence of pre-culture ($\geq 65\%$), across fresh (non-cryoprotected control) and two cryoprotection treatments. Retention of viability declined in these axes with a decrease in water content resulting from physical dehydration; in the absence of pre-culture, $> 55\%$ viability was retained when axes were rapidly dried to WCs $\geq 0.45 \text{ g g}^{-1}$ across control and treatments. When axes were pre-cultured, $> 55\%$ viability was retained after dehydration to WCs $\geq 1.0 \text{ g g}^{-1}$ (and *c.* 0.9 g g^{-1} in PVS2), across all cryoprotection treatments. Below these WCs, retention of absolute viability was lost across all axes. Moreover, embryonic axes with cotyledonary segments attached, dried to *c.* 0.45 g g^{-1} did not survive exposure to cryogenic conditions, regardless of pre-culture (0.3 M sucrose + 0.5 M glycerol), cryoprotectant solutions (glycerol and PVS2), and the cooling rates (slow and direct plunge in liquid nitrogen [LN]). On the other hand, viability in freshly excised shoot tips of *T. emetica* before cooling was $> 75\%$ across all treatments, and only under slow cooling procedure shoot tips excised from seedlings established in culture survived cryopreservation (68% survival; WC = 1.4 g g^{-1}), when pre-cultured for 3 d and pre-treated with PVS2.

Differential scanning calorimetry assessments revealed that cryoprotectants had differing effects on the properties of water in embryonic axis shoot segments and shoot tips; the enthalpies of melt in both explants were different from that of pure water ($333 \text{ J g}^{-1} \text{ H}_2\text{O}$). Results show that pre-culture for 3 d followed by glycerol cryoprotection increased the WC of both embryonic axis shoot segments and shoot tips, thus, promoting ice crystallisation and higher melt enthalpies $>150 \text{ J g}^{-1} \text{ H}_2\text{O}$, a point considered to signify naturally acquired protection in plant tissues. In contrast, PVS2 treated explants exhibited melt enthalpies $<150 \text{ J g}^{-1} \text{ H}_2\text{O}$, irrespective of the cooling rates, suggesting that these values might fall in the ranges of limited intermediate endogenous protection. In both embryonic axis shoot segments and shoot tips, slow cooling increased the point where unfreezable water was observed, which might be suitable for cryo survival, whilst the opposite was true for rapidly cooled explants. PVS2 cryoprotection promoted formation of amorphous glass in pre-cultured embryonic axis shoot segments and shoot tips, which potentially limit ice

crystallisation. The formation of glass especially in shoot tips treated with PVS2 and subjected to slow cooling may be responsible for better survival (68%) in cryogenic conditions; rapid cooling of these shoot tips exhibited no survival. Similarly, embryonic axes, with cotyledonary segments attached, treated with PVS2 and dried to *c.* 0.45 g g⁻¹ DW also showed no survival after rapid cooling. This is probably a result of extreme desiccation and/or dehydration associated toxicity by PVS2, which was aggravated possibly by cooling. Many other studies also have shown that the mode of function for cryoprotective agents can differ with the type of germplasm.

In conclusion, shoot tips of *T. emetica* were more amenable to cryopreservation which could be explained by their higher potency compared with embryonic axes, glass formation during cooling and higher unfreezable water when cryoprotected with PVS2. Although the advantages of embryonic axes cryoprotection prior to rapid dehydration and cooling is species-specific, shoot tips generated from seeds of recalcitrant-seeded species may not survive cryopreservation unless treated with cryoprotective solutions (e.g. PVS2); physical drying of shoot tips may incur metabolism-linked or mechanical damage.

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Abbreviations and symbols

BAP: Benzylaminopurine

CaMg: Calcium magnesium (1:1 solution of 0.5 μ M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)

CPA: Cryoprotective agent

DNA: Deoxyribonucleic acid

DW: Dry weight

EPR: Electron Paramagnetic Resonance

GA₃: Gibberellic acid

H₂O: Water

H₂O₂: hydrogen peroxide

H₀: Null hypothesis

IUCN: International Union for Conservation of Nature

KCl: Potassium chloride

LEA: Late embryogenesis abundant

MS: Murashige & Skoog, 1962

MgCl₂: Magnesium chloride

RPM: Revolutions per minute

SA: South Africa

SANBI: South African National Biodiversity Institute

SPSS: Statistical Package for the Social science

TA: Thermal Analysis

T_g: Glass Transition Temperature

TSDC: Transition Thermally Stimulated Depolarization Currents

V: Version

WC: Water content

WPM: woody plant medium

Units of measurement

μ	: micro
μM	: micro molar
$\mu\text{mol m}^{-2} \text{s}^{-1}$: micromoles per metre per second
%	: per cent
$^{\circ}\text{C}$: degrees Celsius
$^{\circ}\text{C s}^{-1}$: degrees Celsius per second
d	: day(s)
DW	: dry weight
g	: gram(s)
g g^{-1}	: g H ₂ O g ⁻¹ dry matter
g/L	: gram per litre
h	: hour(s)
$\text{J g}^{-1} \text{DW}$: joules per gram dry weight
L	: litre
min	: minute(s)
M	: molar
mM	: millimolar
$\text{mol m}^{-2} \text{s}^{-1}$: moles per square metre per second
Pa s	: Pascals
pH	: hydrogen ion concentration
w/v	: weight (of solute) per volume (of solvent)

Chapter 1: Introduction

1.1 Exigency for preservation of plant biodiversity

The Red Data List of South African plants published in 2010 (through partnership between South African National Biodiversity Institute [SANBI] and International Union for Conservation of Nature [IUCN]), saw South Africa (SA) being recognised as the first megadiverse country with complete assessment of its conservation status of all 20456 of SA's described indigenous plant taxa, that make up 5% of world's total taxa (Raimondo *et al.*, 2013). Approximately 24% of South African plant taxa is threatened either with extinction (13%) or of conservation concern (11%), as per Red Data List (SANBI, 2010). Those statistics clearly indicate that there is an urgent need to preserve plant biodiversity, especially plant genetic resources (Food and Agriculture Organisation of the United Nations [FAO], 2010). Globally, more than fifteen million hectares of tropical forests are wiped out each year due to deforestation, developmental activities, urbanisation and introduction of new and uniform varieties (Rao, 2004), and so preservation using various plant breeding programmes is indispensable.

Conservation of plant germplasm for long-term storage of genetic resources and its re-introduction for rehabilitation is crucial, as the world is continually undergoing severe climatic changes and the consequences of other destructive human activities (Berjak *et al.*, 2011a), which has become critical in the last decade. In most cases, it is destructive human activities and emerging unfavourable climatic conditions that have led to the serious depression in plant diversity and distribution, and also the extinction of various plant species on earth. There are several plant species that have fallen into these categories of being "critically endangered/possibly extinct" or are "in danger of becoming extinct" (IUCN, 2010; Berjak *et al.*, 2011a) under the current conditions. Consequently, an immense pressure has been exerted on the propagation and survival of various plant species, especially those that produce short-lived and non-storable recalcitrant seeds (Engelmann, 2011a).

Conservation and subsequent sustainable use of genetic resources plays an essential role in supplying the demand for future food security and in agro-biodiversity (FAO, 1996 and 2010; Kaviani, 2011). In many countries, various methods or strategies (e.g. seed-banks and gene banks) for conservation of plants have been put into practice (Rao, 2004). Great progression in the areas of *in vitro* culture techniques and molecular biology have provided essential and powerful tools to ensure support and improvement of conservation and management of plant genetic resources (Ramanatha and Riley, 1994; Wiersum *et al.*, 2006). There are two broad approaches that are extensively used to conserve plant germplasm: *in situ* and *ex situ* conservation (Uyoh *et al.*, 2003; Rao, 2004).

1.2 In situ and ex situ conservation of plant genetic resources

Conservation of plant genetic resources can be conducted in the natural habitats (*in situ*) and outside the natural habitats (*ex situ*) (Convention on Biological Diversity [CBD], 1992; Uyoh *et al.*, 2003; Rao, 2004; Heywood and Dulloo, 2005; Kaviani, 2011). *In situ* conservation has received attention since the early 1980s. This strategy involves conserving entire communities in habitats where they have developed their distinctive properties. *In situ* conservation methods includes: conservation in natural reserves, biosphere reserves, national park, gene sanctuary etc. (Heywood and Dulloo, 2005; Kaviani, 2011). The challenge in using *in situ* conservation is that a rigid conservation approach is almost not feasible to implement, and its maintenance in long-term is almost impossible. Furthermore, there are also many problems associated with establishing these approaches to conservation: e.g. high cost, size of conservation area, the risk of genetic wipe out due to unpredictable natural disasters, etc. Heywood and Dulloo (2005) asserted that predicting the future effects of several “components of global change on *in situ* conservation programmes” is not easy. It is generally accepted the evolution of new characters within the species and natural selection may occur on species conserved in their natural habitat. Therefore, it is probably that the individual species and the ecosystems of their occurrence or conservation *in situ* will be put at risk by this approach. *In situ* conservation approach has been imperative in the conservation of number wild plants, tree crops and forest seeded species where *ex-situ* conservation approach is ineffective (King and Roberts, 1979; Hawkes, *et al* 2000; Hong *et al.*, 1996).

In contrast, it is generally accepted that *ex situ* conservation is the viable way to protect populations that are “critical threatened” or in danger of becoming “extinct” (Prescott-Allen and Prescott-Allen, 1981) in fact, sometimes it is the only possible strategy to conserve certain species. This approach includes methods like seed storage in seed banks, field gene banks, botanical gardens, *in vitro*, DNA and pollen storage etc. (Hawkes *et al.*, 2000; Paunescu, 2009; Gonzalez-Benito *et al.*, 2004; Rao, 2004; Reed *et al.*, 2011). It is however established that seed storage remains the most convenient method of long-term conservation for plant genetic resources (Engelmann and Engels, 2002; Paunescu, 2009), which involves desiccation of seeds to low moisture contents and storage at low temperatures (Robert, 1973; Hong *et al.*, 1996; Engelmann and Engels, 2002). Where seed banking is not possible as in recalcitrant seeds which cannot be stored successfully in conventional seed banks, *in vitro* culture then becomes a feasible alternative approach of conserving of plants.

There are also several *in vitro* techniques that have been established for storage of vegetatively propagated and recalcitrant seed producing species (Withers, 2001; Engelmann and Engels, 2002; Engels and Visser, 2003; Kaviani, 2011). According to those authors, these *in vitro* techniques can be categorised into two: (1) low growth methods, where germplasm materials are kept as sterile plant tissues or plantlets on suitable nutrient media (such as woody plant medium [WPM] or Murashige & Skoog [MS] medium etc.) which provides short to medium-term storage options, and (2) cryopreservation methods, where plant samples are stored at ultra-low temperatures (in liquid nitrogen [LN] or vapour phase of LN) for long-term storage and then transferred to suitable medium for recovery.

1.3 Storage through seeds

Over the past decades, many agencies and institutions such as Millenium Seed Bank (Royal Botanic Gardens Kew) have developed extensive expertise in storage of plant genetic resources through a seed. Conventional seed storage approaches involve maintenance at low relative humidities and temperatures, and have been known to be efficient in desiccation tolerant (‘orthodox’ seeds) (Roberts, 1973). Seeds are generally dried to low water contents (WC) (~3-7%, fresh mass basis [fmb]), and then

stored at low relative humidity (RH) and low temperatures (ranging from +4 to -18 °C) (Engelmann and Engels, 2002). This approach is not easy to apply in the conservation of species which produce desiccation intolerant seeds because of the low water contents required for conventional storage (Engelmann, 2004; Paunescu, 2009). Such seeds cannot be stored for extended periods. Conservation of genetic diversity through seed storage is one of the most convenient, widespread and valuable *ex situ* approach (Chin *et al.*, 1988; Pritchard, 1995). Those authors have documented that seed storage has considerable advantages over other methods of *ex situ* conservation, as the seeds require low maintenance under relatively low labour demands, and millions of seeds can be stored in a little space (e.g. small freezer, seed banks etc.) and frequently remain viable for extended periods. It's generally accepted that the most efficient and economical approach for *ex situ* germplasm storage is through seeds (Pritchard, 1995).

1.3.1 Storage behaviour of seeds

Based on longevity in storage, seeds can exhibit orthodox and non-orthodox seed storage behaviour. Orthodox seeds are those seeds that are usually shed dry from the mother plant after post maturation drying (Roberts, 1973; Berjak and Pammenter, 2001a), can tolerate relatively extreme dehydration (King and Robert 1980; Ellis *et al.*, 1989; Walters, 1998), and survive in the desiccated state below zero temperatures for extended periods (Roberts, 1973; Berjak, 2006). Seeds that deviate from such behaviour are considered as non-orthodox (Grout *et al.*, 1983; Farrant *et al.*, 1988; Berjak *et al.*, 1989; Finch-Savage and Blake, 1994), a group of seeds that include the non-storable and desiccation sensitive, recalcitrant (Roberts, 1973) and intermediate seeds (Hong and Ellis, 1996). According to Berjak (2006) and Pammenter and Berjak (1999), recalcitrant seeds are shed at high water content (WC) from the parent plant and they are metabolically active as they do not undergo maturation drying (Farrant *et al.*, 1988; Berjak *et al.*, 1989; Farrant *et al.*, 1992; Lin and Chen, 1995; Pammenter and Berjak, 1999), which is the final phase in orthodox seed development. Seeds of *Avicennia marina* (Farrant *et al.*, 1992; 1993) and *Trichilia emetica* (Kioko, 2003) are examples of species falling into this category and cannot be stored for extended period in hydrated storage. Over the past decades, there has been accumulative knowledge about storage behaviour of orthodox seeds, and also technology is well developed

(Chin, 1995), whilst, some essential phenomena (i.e. properties of water investigated in the present study) remain poorly understood in recalcitrant seeds, especially with regards to their long-term storage and survival from ultra-low temperatures.

1.3.2 Desiccation tolerance is crucial for successful storage

Information about seed WC is essential as it affects successful storage of seeds; however, mechanisms that avoid damage that can result from removal of water are important. A functional repair mechanism during rehydration and also protection of membrane and cellular components that preclude damage during removal of water plays a significant role in desiccation tolerance (Leprince *et al.*, 1993; Oliver and Bewly, 1997; Oliver *et al.*, 2000). In orthodox seeds, protective mechanisms which allow desiccation tolerance are suggested to be more prevalent and significant (Pammenter and Berjak, 1999), whilst recalcitrant seeds are believed to lack at least one or possibly more of these essential components (Pammenter *et al.*, 1991; Pammenter *et al.*, 1999; Berjak and Pammenter, 2013). Several mechanisms have been suggested to contribute to the desiccation tolerance of plant material from orthodox seeds, and listed below are some important mechanisms:

1. Intracellular de-differentiation that limits surface areas of membrane and cytoskeleton (Pammenter and Berjak, 1999; Berjak and Pammenter, 2001a; Ambika, 2006). Mature orthodox seeds can display intracellular de-differentiation, which entails simplifying and minimising intracellular structures (Vertucci and Farrant, 1995). According to Pammenter and Berjak (1999), de-differentiation in an organised manner is considered a requirement for survival in a dehydrated state.

2. “Temporal halt” of metabolism (Vertucci and Leopold, 1986; Pammenter and Berjak, 1999; Berjak and Pammenter, 2001a, b). When metabolism continues during dehydration, free radicals that cause desiccation damage will be produced. This damage can be limited only if metabolism is switched off. Work on *Phaseolus vulgaris* seeds by Rogerson and Matthews, 1977 and Farrant *et al.*, 1997 showed that a decline in respiration rate correlated with mitochondrial de-differentiation accompanied maturation drying. Those events are important as they were suggested to allow seeds to tolerate substantial removal of water. It is suggested that recalcitrant

seeds do not enter a state of quiescent despite evidence that respiration rates may decline to some extent some more tolerant, temperate recalcitrant species (Kermode and Finch-Savage, 2002). Even at intermediate WC, damage may occur in recalcitrant seed as metabolic activities continues.

3. Accumulation of protective molecules such as LEAs proteins and dehydrins (Kermode, 1990), and some sugars like sucrose and other oligosaccharides (Koster and Leopold, 1988). Accumulation of LEA proteins is important in the acquisition and maintenance of desiccation tolerance. The expression or absence of these proteins in association with the expression or absence of other factors may contribute to the degree of desiccation of tolerance. It generally accepted that the absence of LEAs can contribute to the degree of desiccation tolerance. In some recalcitrant seeds, processes that involve LEAs (dehydrins) (Kermode, 1990) and sugars (sucrose) (Koster and Leopold, 1988) do not occur in like manner as in orthodox seeds.

4. Reaction of the cytoskeleton to dehydration (Pammenter and Berjak, 1999; Mycock *et al.*, 2000). This type of mechanism offers an integrated intracellular support. During dehydration, the cytoskeleton network can disassemble (Mycock *et al.*, 2000; Gumede *et al.*, 2003) and also reassemble in an organised fashion during rehydration of orthodox seeds. Work by Mycock *et al.* (2000) on recalcitrant seeds showed that reassembling is not evident after dehydration levels that affects viability, which probably contribute in the loss of integral intracellular support and structural organisation (Pammenter and Berjak, 1999; Mycock *et al.*, 2000).

5. Reduction of vacuolation, increasing amount and nature of insoluble reserves (Farrant *et al.*, 1997). This process is believed to increase mechanical resilience of cells to dehydration. Work by Farrant *et al.* (1997) on *Phaseolus vulgaris* (orthodox) and *Avicennia marina* (recalcitrant) showed that different degrees of vacuolation and insoluble reserve deposition was associated with the degree of desiccation tolerance. In contrast to orthodox seeds, recalcitrant seeds do not undergo extensive reduction in vacuolation, or de-differentiation of organelles, or the temporal halt of metabolism all which essential for desiccation tolerance (Pammenter and Berjak, 1999).

1.3.3 Short-term storage of recalcitrant seeds

Drew *et al.* (2000) asserted that one of the problems encountered in the storage of recalcitrant seeds (desiccation sensitive) is that, unlike orthodox seeds, the WC cannot be lowered to levels that would allow conventional methods (under low temperature and reduced relative humidity [RH]) to be used. Many recalcitrant seeds, especially those originating in the tropical regions, are also chilling sensitive (Hong and Ellis, 1996; Varghese and Naithani, 2000; Tweddle *et al.*, 2003) being susceptible to damage at storage temperatures lower than 15 °C. The attribute of being desiccation sensitive allows them to be stored only under hydrated conditions (Motete *et al.*, 1997; Goveia *et al.*, 2004; Eggers, 2007; Sershen *et al.*, 2008), where the seeds are held at WC at which they are shed from parent plant. Storage of recalcitrant seeds under humid conditions can be carried out only for limited periods as a consequence of onset of germination (Engelmann and Engels, 2002). Moreover, Berjak *et al.* (1996) suggested that fungal contamination is another factor, which contribute to rapid deterioration especially when the seeds are maintained at higher WC and high temperatures. Seeds become more susceptible to fungal attack with increased time in hydrated storage. Moreover, because of prolonged hydrated storage, a mild, but sustained stress is imposed (Pammenter *et al.*, 1994; Motete *et al.*, 1997). Therefore, storage lifespan in hydrated storage can vary from 2-3 weeks (e.g. *Avicennia marina*; Pammenter *et al.*, 1984) to a few months for some species of tropical origin (e.g. *Syzgium cuminii* [Rawat and Nautiyal, 1997; Srimathi *et al.*, 1999]; *T. emetica* [Kioko, 2003]; *T. dregeana* and *Podocarpus henkelli* [Eggers, 2007]). However, some chilling-tolerant recalcitrant seeds from temperate habitats can be successfully stored for 2-3 years (*Aesculus hippocastanum* [Pritchard *et al.*, 1996]) at low temperatures at about 15 °C (Farrant *et al.*, 1989; Prichard *et al.*, 1996).

The fundamental understanding of loss of viability of hydrated recalcitrant seeds in storage has not been well established. One of the early proposed opinions for loss of viability was that metabolic processes were to be attributed to this loss, as these processes can be associated with free radical mediated damage (Berjak *et al.*, 1989; Hendry *et al.*, 1992; Chaitanya and Naithani, 1994; Finch-Savage, 1996; Varghese and Naithani, 2002; Varghese *et al.*, 2011; Farrant *et al.*, 1997). Earlier work by Farrant *et al.* (1986) and Berjak *et al.* (1989) suggested that recalcitrant seeds continue

developing even after shedding, and this leads to species germinating in storage (see in the previous page), therefore becoming more sensitive because of the need for more water to complete the process of germination. In a study by Farrant *et al.* (1993), development of seeds of *Avicennia marina* revealed that it was difficult to identify the switch from reserve accumulation to germinative metabolism. So, desiccation sensitivity of hydrated recalcitrant seeds increases during storage, particularly with continual germination related events, i.e. the phase of extensive vacuolation following the onset of mitosis (Farrant *et al.*, 1986). Studies have shown that under hydrated storage conditions, embryonic axes of recalcitrant seeds undergo germinative development and if additional water is not supplied to complete this process the seeds lose viability as a consequence of a mild desiccation stress (Pammenter *et al.*, 1984; Farrant *et al.*, 1986; Berjak and Pammenter, 2000) with some more rapidly and others slowly depending on the species as demonstrated by Chin and Roberts (1980). This leads to a loss of control for metabolism, causing inefficiency of antioxidant metabolism and resulting in free-radical mediated degradative events (Pammenter and Berjak, 1999; Berjak and Pammenter, 2001a, b).

1.3.4 Cryopreservation for long-term storage of germplasm from recalcitrant-seeded species

The first studies on plant cryopreservation began with investigating the freezing of mulberry twigs (Sakai, 1965). Over the past decades, advances in cryopreservation technology has enabled development of methods that allow successful ultra-low temperature maintenance of a number of plants for conservation which includes agricultural (Stanwood, 1985) and horticulture genotypes (Berjak *et al.*, 1996; Varghese *et al.*, 2009; Gebashe, 2015) as well as many critically endangered (Walters *et al.*, 2002a; Mandal and Dixit-Sharma, 2007) and threatened plant species (Mycock *et al.*, 1995; Kaczmarczyk *et al.*, 2011). Over the years, successful cryopreservation has been achieved with a variety of explants that includes: cell suspension and callus culture (Kantha and Engelmann, 1994), somatic embryos (Berjak *et al.*, 1999; Mycock *et al.*, 1995; Sershen *et al.*, 2012a, b), shoot tips (Volk and Walters, 2006; Varghese *et al.*, 2009; Teixeira *et al.*, 2013), pollen (Buitink *et al.*, 1998b; Towill and Walters, 2000; Ballesteros and Walters, 2007) or plant buds (Panis and Lambardi, 2005), from both recalcitrant and orthodox seeds (Reed, 2008).

Kioko *et al.*, 1998) and Kaviani (2011) described that cryopreservation protocols varies considerable depending on the species and selected explants. Recalcitrant seeds are shed at high WC which varies with species (Ballesteros *et al.*, 2014) and can also vary within one species from one harvest to the next (Berjak and Pammenter, 2004). It must be noted that unlike orthodox seeds (Grout *et al.*, 1983; Vertucci, 1989; Buitink *et al.*, 1998a, b), little success has been documented with the long-term storage of recalcitrant seeds since they are metabolically active and desiccation sensitive both during development and storage, dying as they slowly lose water (Pammenter and Berjak, 1999; Berjak, 2006; Berjak and Pammenter, 2008). Generally, whole seeds cannot be dried to WC content ranges suitable for successful cryopreservation as they are relatively large and can be affected by dehydration and cooling which are two essential steps of cryopreservation. As alternatives, embryonic axes, somatic embryos, meristems and shoot tips are preferred as the small size permit rapid dehydration and rapid cooling (Pammenter and Berjak, 1999; Varghese *et al.*, 2009). Cryopreservation protocols such as slow-cooling, vitrification, droplet vitrification, encapsulation or dehydration and encapsulation or vitrification have been developed and used for cryostorage of plant germplasm (Reed, 2008).

In principle, cryopreservation involves the storage of biological material at ultra-low temperatures usually in LN (at -196 °C) or in the vapour phase of LN (approximately -130 °C to -140 °C) (Kartha, 1981; Muldrew *et al.*, 2004; Panis and Lambardi, 2005; Berjak, 2006; Day *et al.*, 2008; Engelmann, 2011b). This type of storage theoretically results in the indefinite arrest of cellular metabolic functions, thus, enabling extended storage of plant germplasm with no possible alteration and modification (Kartha, 1981, 1985; Engelmann, 1991, 2004; Mycock *et al.*, 2004) as at LN temperature there is insufficient thermal energy for chemical reactions (McGee and Martin, 1962). At LN temperatures or below -40 °C liquid water does not exist, only a crystalline or glassy state exist. Contrary to this opinion, Walters *et al.* (2009) suggested that there is evidence that even dry orthodox seeds can possible deteriorate in cryostorage. Successful cryopreservation of plant germplasm involves many essential steps (Figure 1.1) that can be categorised into three phases: (1) pre-conditioning of samples; (2) exposure to cryogenic conditions including cooling and warming rates; and (3) recovery process (Zhao *et al.*, 2005). The success or failure of any protocol can be

influenced by various manipulations such as use the of cryoprotective agents, cooling rates (Wesley-Smith *et al.*, 1999), the extent and rate of dehydration (Kioko *et al.*, 1998; Berjak *et al.*, 1999) and the medium for rehydration (Berjak and Mycock, 2004). Most of the steps of cryopreservation protocol, right from the excision of explants to the post cryo recovery, induce stress and the stress accumulated during these steps may compromise survival after cryostorage (Berjak *et al.*, 2011b, Naidoo *et al.*, 2011). Over the years, there has been vast research on these stages of cryopreservation (Engelmann., 1997, 2000; Walters *et al.*, 2002b; Goveia *et al.*, 2004; Lane, 2004; Volk *et al.*, 2006; Volk and Walters, 2006; Ding *et al.*, 2008; Sershen *et al.*, 2007; Naidoo *et al.*, 2011; Ngobese, 2013), however they are still not fully understood; for example, processes such as cooling involves complex phenomena. It is popular belief that many of the lethal events in cryopreserved biological samples occurs during cooling and warming of samples (Mycock *et al.*, 1995; Volk and Walters, 2006; Mullen and Critser, 2007). Differential scanning calorimetry has great potential for providing possible explanation of what unfolds in step such as cooling and rewarming (Volk and Walters; 2006; Volk *et al.*, 2006; Nadarajan *et al.*, 2008; Teixeira *et al.*, 2013 Nadarajan and Prichard, 2014).

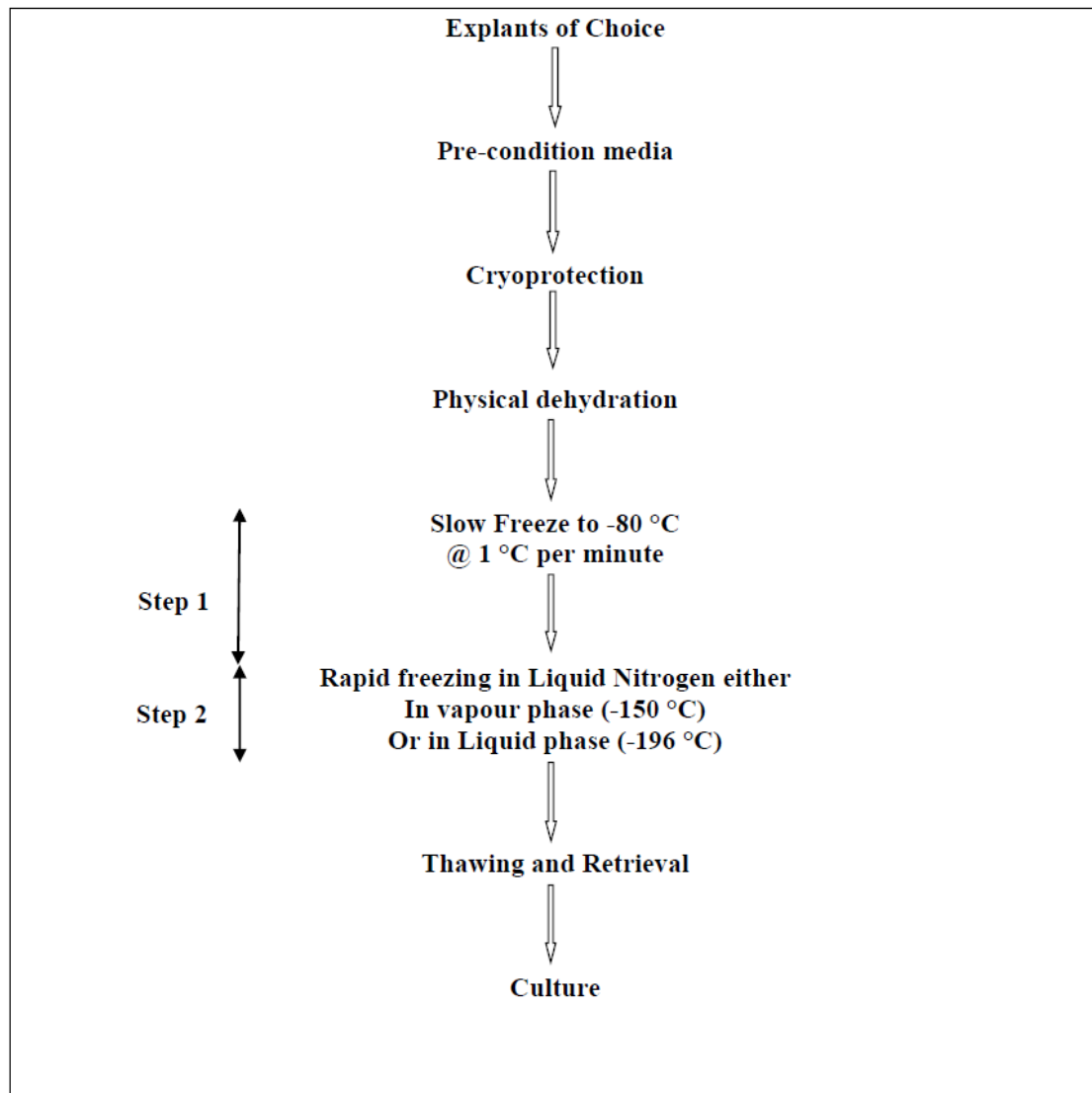


Figure 1.1: A simplified schematic representation of two-step cryopreservation for plant material.

1.3.4.1 Cryoinjury as a consequence of physical and biochemical events

There are two major difficulties, which are encountered during cryopreservation of fleshy cotyledonous seeds, especially the desiccation and chilling sensitive recalcitrant seeds. **Firstly**, the failure of biological material to recover from cryogenic conditions can be linked with the formation of ice crystals that cause damage to cell membrane and other organelles (Steponkus, 1984; Volk and Walters, 2006; Sershen *et al.*, 2012a, b; Wesley-Smith *et al.*, 2014). It is crucial that intracellular ice formation and expansion during cooling, storage, and thawing is prevented or minimized (Engelmann, 2004; Ballesteros and Walters, 2007). Intracellular water present at WCs below $0.28 \text{ g H}_2\text{O g}^{-1}$ dry weight does not show a melting transition in differential

scanning calorimetry (DSC) (Pammenter *et al.*, 1991), whilst, water present at WCs in excess of 0.28 g H₂O g dry weight does show melting transitions and is referred to as freezable water. The point at which non-freezable is first observed can be manipulated by application different dehydration rates and osmo-protective treatments. Ballesteros and Walters (2007) showed that there is a narrow range of WCs that preclude freezing injury whilst limiting desiccation damage, and these moisture levels vary among species.

Secondly, recent works have linked the failure of successful cryopreservation and/or the ability to produce shoots from the embryonic axes of various recalcitrant seeds following retrieval from cryogenic condition (Goveia, 2007; Naidoo *et al.*, 2011) with the production of free radicals or reactive oxygen species (ROS). These ROS are associated with free radical mediated damage at the various stages of cryopreservation (Benson, 1990; Hendry, 1993; Hoekstra *et al.*, 2001; Berjak and Pammenter, 2008; Varghese *et al.*, 2011). Berjak *et al.*, 2011b; Sershen *et al.*, 2012c). Previous studies have shown that at intermediate water contents (generally considered to be 0.25-0.45 g g⁻¹) metabolism induced damage can occur (Pammenter *et al.*, 1998; Leprince *et al.*, 2000; Walters *et al.*, 2001). It has been suggested that this failure of co-ordinated metabolic functions in cells results in imbalanced metabolism and consequent in aqueous-based degradative processes that are associated with free radicals (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999; Walters *et al.*, 2001, 2002a). The natural protective antioxidant systems can fail when WCs are considerably lowered and as a consequence sustained damage may be highly detrimental (Finch-Savage *et al.*, 1994). Varghese *et al.* (2011) reported that failure of the antioxidant systems in in axes of *T. dregeana* resulted from slow removal of water. Those authors asserted that such failure is a major factor in metabolism-linked damage. It has been said that it remains important that the accumulation of free radicals resulting from uncontrolled metabolism is neutralized by suitable antioxidant systems in desiccation-tolerant tissues and also in orthodox seeds where there is a switch in physiology (from tolerance to sensitivity) that is experienced upon rehydration and germination (Senaratna and McKersie, 1986; Vertucci and Farrant, 1995).

In attempt to ameliorate the oxidative stress, cathodic protection has been used. Cathodic protection is a method that is based on principles of electrode kinetics, and is

used to quench unregulated ROS (Pammenter *et al.*, 1974; Berjak *et al.*, 2011b). According to Srinivasan *et al.* (1996) cathodic protection is used industrially to limit oxidative damage, such as the corrosion of metals in a wide range of large structures. In 1974, Pammenter and colleagues were the first to investigate cathodic protection in plant tissues. Those authors placed seeds of *Zea mays* on the cathode in a static electric field, and this extended their life span when kept under accelerated ageing conditions. Moreover, those authors hypothesised that such effect was enabled by cathodic protection, which provide a source of electrons and therefore reducing free radicals in the seed tissues. In 2011, Berjak and colleagues extended the principle of cathodic protection as they used the cathodic fraction of the electrolysed salt solution known as cathodic water. This fraction was used as a solvent in the procedural steps of cryopreservation. In recalcitrant embryos that have been subjected to cathodic treatment during drying, rehydration in cathodic water had some limitations due to the fact that cathodic water was not applied during other procedural steps that have been shown to induce ROS production, such as explant excision (Berjak *et al.*, 2011b) and cryoprotection [Naidoo, 2012]). Application of calcium magnesium (detailed in section 2.9) (Mycock, 1999; Berjak and Mycock, 2004) and cathodic water (detailed in section 2.9) (Naidoo, 2012) to recalcitrant explants at other procedural steps that induce ROS production can be regarded as major improvement, as cathodic water has the properties of quenching free radical at various stages of cryopreservation and facilitating survival that has been previously unachievable, thus, optimising general cryopreservation protocol of recalcitrant seed species (*T. emetica*, *T. dregeana*, *Protorhus longifolia* [Naidoo, 2012]). Regardless of numerous challenges, cryopreservation at ultra-low temperatures in LN or in the vapour phase above LN remain as the most efficient (Fahy *et al.*, 1984; Berjak *et al.*, 2000; Engelmann, 2000; Wesley-Smith, 2003) and cost effective strategy for long-term storage of plant germplasm (Kaviani, 2011).

1.4 Water: a critical factor affecting the ability of the germplasm to be cryostored

Water is an essential constituent of living plant cells and it has been suggested that cells contain about 95% of water (Clegg *et al.*, 1982; Watterson, 1987; Reinhoud *et al.*, 2000; Sharp, 2001; Muldrew *et al.*, 2004). As it is known, recalcitrant seeds are shed from parent plant at high WC and are sensitive to freeze-dehydration, therefore

WC is a critical factor affecting the potential of germplasm to be successfully dehydrated and stored in cryogenic conditions (Stanwood, 1985). In 1995, Wesley-Smith and colleagues reported that the WC of plant material at time of exposure to cryogenic condition is important as it can determine chances of survival from cryogenic conditions. Water has properties of being an excellent solvent and also plays a crucial role in controlling metabolism (Vertucci and Farrant, 1995). Moreover, water is known to affect the structural attributes of macromolecules and macromolecules also affect the properties of water (Vertucci, 1990; Vertucci and Farrant, 1995). This interactive phenomenon is most apparent when solutions or emulsions of macromolecules are concentrated or when biological tissues are dehydrated (Benson, 2008). Ntuli and Pammenter (2009) reported in addition to providing turgor and filling intermolecular spaces, water is key in partitioning molecules within organelles and restricting reactivity among metal ions. As the WC continues to drop, the interaction between water and the solute becomes more intense, therefore causing the system to shift from the ideal behavior (Sharp, 2001; Ballesteros and Walters, 2007, 2011). Berjak and Pammenter (2013) expressed that when optimizing cryopreservation protocol, it is important to consider the rate (Pammenter *et al.*, 1998) and temperature (Berjak *et al.*, 1994; Ntuli *et al.*, 1997) at which water is removed from explants to avoid damage. Upon drying, the viscosity of cytoplasm constituents increases and the cytoplasm develops properties of a vitrified structure such as glass (Leopold and Vertucci, 1986; Koster, 1991). Benson (2008) suggests that the knowledge of regulating the phase transitions (e.g. glass, ice formation and nucleation) of water and stabilising the glassy state is imperative for development of successful cryopreservation protocols, as attempted also with the current study. An insight to the properties of intracellular water is crucial to cryopreservation since its unusual molecular properties that control the formation of frozen and vitrified state in cryopreserved germplasm (Teixeira *et al.*, 2013, 2014).

1.4.1 Properties of water

To date, the extent to which the properties of water change and their relevance to the function of macromolecules remains poorly understood, especially with respect to cryopreservation procedures and successful plant tissue cryopreservation (Ballesteros *et al.*, 2014). When the water molecule changes from one physical phase to another

(e.g. liquid to solid), its molecular motion and energy also change and molecules arrange themselves in distinctly different patterns (Sharp 2001; Benson, 2008). As temperature of pure water is reduced below 0 °C, the water molecules lock together forming lattice like matrix (stable crystalline nucleus [Hobbs, 1974]) and ice crystal will expand provided that temperature is below the equilibrium melting point. The structure of frozen water is complex as it possesses “open spaces” interspersed between more tightly associated parts of the structural matrix, which explains why liquid is denser than ice (Buitink *et al.*, 1998a; Benson, 2008).

It has also been generally established that the molecular arrangement taken by ice consequently leads to an increase in volume and a decrease in density (Buitink *et al.*, 1998a; Walters, 1998), and this increase in volume of solid form of water has been asserted as lethal to biological tissues stored under low temperatures (Ballesteros and Walters, 2007; Wesley-Smith, 2003; Engelmann, 2004; Berjak and Pammenter, 2013; Teixeira *et al.*, 2014). Vertucci (1990) asserted that when extensive dehydration is possible, WC of explants can be reduced to levels where remaining water has reduced mobility and is non-freezable and bound to macromolecular surfaces. This limits freezing injury that may occur in cryopreservation of many desiccation-sensitive axes. However, damage to cryopreserved material can occur under two circumstances: (1) at high WCs which results in an increase in cell volume this probably exerts mechanical stresses on the cell content (Levitt, 1980; Vertucci and Farrant, 1995), and (2), at low WC whereby maintenance of the integrity of intracellular structures is compromised as water is lost (Pammenter *et al.*, 1991; Wolfe and Bryant, 1999; Walters *et al.*, 2001).

1.5 Influence of dehydration rate on recalcitrant material

The ability of many explants (such as embryonic axes) to survive cryopreservation can partially depend on their degree of sensitivity to dehydration and tolerance to extreme removal of freezable water, and this understanding is essential for optimising cryopreservation protocols (Rajasekharan, 2006). In general, recalcitrant seeds are shed from the parent plant at a high WC and they can tolerate only partial removal of bulk water as they are desiccation sensitive. The current held view is that rapid dehydration is an imperative step for preparing explants from recalcitrant plants for

cryopreservation (Berjak *et al.*, 1992; Pammenter *et al.*, 1998; Berjak *et al.*, 2001b; Wesley-Smith *et al.*, 2001). A rapid dehydration rate allows tolerance of a far more substantial water loss with seeds still retaining viability (Farrant *et al.*, 1986; Berjak *et al.*, 1989; Berjak *et al.*, 1990; Varghese *et al.*, 2011). It has been established that for embryonic axes of recalcitrant seeds, the faster the drying rate, the lower the WC that can be tolerated before viability is compromised (Farrant *et al.*, 1986; Pammenter *et al.*, 1998; Liang and Sun, 2000; Varghese *et al.*, 2011). Rapid physical drying biological material such as embryonic axes can be attained through use of a flash drier (Berjak *et al.*, 1989; Pammenter *et al.*, 2002), where an air stream over activated silica gel decreases the WC to a level ideal for freezing (Pammenter *et al.*, 2002; Ballesteros *et al.*, 2014).

It is held that a rapid drying rate minimises the time of intermediate WC, and limiting the accumulation of damage associated with dehydration of metabolically active tissues (Pammenter and Berjak, 1999; Walters *et al.*, 2001). Whilst, slow drying is suggested to allow sufficient time for metabolic processes to continue, including unbalanced aqueous-based reactions that result in membrane degradation and loss of viability (Berjak *et al.*, 1989; Pammenter *et al.*, 1991; Berjak *et al.*, 1993; Pammenter *et al.*, 1998; Walters *et al.*, 2001; 2002a). It has been suggested that removal of water from biological tissues is even under a slow cooling rate, but uneven under a rapid drying rate (Pammenter *et al.*, 1998; Wesley-Smith *et al.*, 2001). Those authors further suggested that this uneven removal enable seeds to rapidly reach lower WCs with viability still retained in dried explants. Although rapidly dried recalcitrant material can tolerate lower WCs than if slowly dried, drying beyond a lower limit, which is asserted to be above the WC to which orthodox seeds can be dried, can be damaging (Pammenter and Berjak, 1999). At this lower limit, water remaining in the tissues is suggested to be structure-associated and unfreezable and is associated with lethal effect if removed (Pammenter *et al.*, 1991; 1993). Work by Ballesteros and Walters (2007) showed that the amount of unfreezable water can differ with different type of germplasm, e.g. fern spores ($0.09 \text{ g H}_2\text{O g}^{-1}$) had lower unfrozen water content compared to that of non-lipid dry matter for most plant ($0.25 - 3.0 \text{ g H}_2\text{O g}^{-1}$). It is important to note that rapid physical drying does not impart desiccation tolerance to material from recalcitrant species as they are intrinsically desiccation sensitive. However, it limits damage associated with deleterious metabolic activities, therefore

enabling the possibility of storage of recalcitrant tissues at cryogenic conditions with minimum damage/stress.

Similarly, cooling rates that successfully avoid lethal ice crystallisation and damage are indispensable for successful cryopreservation (Wesley-Smith *et al.*, 1992; Sershen *et al.*, 2007). Cryopreservation approaches that combines the rapid drying of embryonic axes (Pammenter *et al.*, 1991; Varghese *et al.*, 2011) and rapid cooling of these axes (Wesley-Smith *et al.*, 1992; Mycock *et al.*, 1995; Sershen *et al.*, 2008; Sershen *et al.*, 2012b) are desirable, since both these rates plays a significant role in minimising both desiccation damage and freeze damage of cells and tissues.

1.6 Theoretical basis of cooling

It is essential that cells/tissues stored at ultra-low temperatures should possess an ability to tolerate sub-zero temperatures, as it faces a challenge of having to traverse twice the lethality of intermediate zone of temperature (-15 to – 60 °C) once during cooling and once during warming (Mazur, 1984). This intermediate zone is known to promote ice formation and growth (Moor, 1973) which is often lethal to biological materials stored at this zone and below. Ozkavukcu and Erdemli (2002) suggested that as a consequence of increased viscosity and insufficient thermal energy for chemical reaction, there are no thermally driven reactions in aqueous systems at liquid nitrogen temperatures (-196 °C) which is the usually used refrigerant for low temperature storage. The report also indicated that only photophysical events such as formation of free radicals and production of breaks in macromolecules that results from “hits” by background ionization radiation or cosmic rays can occur. Moreover, these can produce adequate breaks or other damage to DNA to become deleterious after rewarming to physiological temperatures and no enzymatic repair can occur at these cryogenic temperatures. The cooling process of any biological material will always lead to ice crystallisation regardless of the cooling rate (Karow, 1969), unless the water remaining in the material is non-freezable (Vertucci, 1990).

The process of ice formation is rapid, whilst transportation of water through cell membrane is relatively slow, due to cell membrane serving as a resistant barrier (Mazur, 1970). The type of water (or crystal water) of any explants is important, as it

determines survival of biological material following exposure to cryogenic conditions (Wesley-Smith *et al.*, 1991, 2004, 2014). It is for this reason that different cooling procedures have been developed and they can be regulated. Plant material can either be cooled rapidly (Sakai and Yoshida, 1967; Sakai, 1997; Wesley-Smith *et al.*, 2004, 2015, Nuc *et al.*, 2016), or in a slow or stepwise manner (Kantha *et al.*, 1982; Sun., 1999; Varghese *et al.*, 2009; Gebashe, 2015).

1.6.1 Ice formation and solution effects injury during slow cooling

The major cause of intracellular damage in cryopreservation has been suggested to be ice crystallisation (Moor, 1973; Sakai, 2004; Volk and Walters, 2006). Previous studies (Withers and King, 1979; Acker and McGann, 2001; Kaviani, 2011; Prickett *et al.*, 2015) have revealed that both ice formation and solution effects injury play a significant role in cell damage, and restriction of each effect (with fewer exceptions) can be attained only at an optimum cooling rate. It is accepted that in explants and some from recalcitrant seeds, a slow cooling rate achieved through cooling 0.5 to 2 °C min⁻¹ to ~ -40 °C (Kantha, 1985) enables the process of protective dehydration to occur (Karow, 1969; Withers, 1991; Wesley-Smith *et al.*, 2004, 2014). According to Mazur (1990) and Simione (1992), slow cooling results in freezing external to the cell before intracellular ice begins to form. Those authors further suggested that if ice forms external to the cell, a deficiency of water in the extracellular environment causes an imbalance across the cell membrane, which then promotes movement of water out of the cell (Karow, 1969), thus leading to an increase in solute concentration outside the cell that can be detrimental to the cell survival (Pritchard *et al.*, 1995).

In a case where there is excessive water inside the cell, damage as a consequence of ice formation and recrystallization during warming can occur and is often lethal. Therefore, slow cooling leads to a greater loss of intracellular water (influenced by cell permeability) and minimal ice formation, however it increases solution effects injury. Previous studies (Pegg and Dieper, 1988; Mazur *et al.*, 1981) have suggested that biological cells cooled slowly can be killed by the summative effects of excessive shrinkage, exposure to hypertonic solution and the decline of temperature, and when cooled rapidly, death is a result of ice formation rather than shrinkage. Though a rapid cooling procedure is usually the preferred rate, there are instances that shows that

slow cooling procedure is just as effective in some cases (Varghese *et al.*, 2009; Gebashe, 2015). The cooling rate is important and in many cases (shoot tips of strawberry [Sakai, 1993]; shoot tips of *T. emetica* [Varghese *et al.*, 2009; Gebashe, 2015]) a two-step cooling process (Figure 1.1) is used where biological materials are held at about -30 °C to -140 °C for some time prior cooling to liquid nitrogen temperatures, is beneficial and is the ideal cooling process. The two-step method enhances dehydration of the cytoplasm prior to cooling.

1.6.2 Ice formation and solution effects injury during rapid cooling

Rapid cooling procedure can limit the growth of intracellular ice crystals to non-lethal levels (Engelmann, 2004), and is currently favoured in some cryopreservation protocols for plant germplasm (Walters *et al.*, 2002b; Wesley-Smith *et al.*, 1992; 2004). In 2007, Sershen and colleagues suggested that cooling rates that successfully minimise lethal ice crystals in plant material still requires empirical determination. A rapid cooling process of partially hydrated or cryoprotected plant material placed in cryovials can be attained through direct plunge into LN, and this is technically simple and easy to perform. This procedure has been successfully used for plant material at higher WCs, where deleterious intracellular ice formation is precluded (Luyet *et al.*, 1962; Wesley-Smith *et al.*, 1991; Wesley-Smith *et al.*, 2004; Volk and Walter, 2006).

One notable behaviour of water and aqueous solutions is that they have a tendency to cool below their melting point prior to occurrence of ice nucleation. For example, ice has a melting point of 0 °C, however the temperature can be reduced significantly below 0 °C prior to ice formation, and in fact water can be super cooled to -40 °C prior to ice nucleation becoming inevitable (Franks, 1985). It has been established that rapid cooling restricts intracellular ice damage below lethal levels (i.e. cooling at hundreds of °C s⁻¹) by minimizing the time spent by the tissue at temperatures (generally considered to be 0 to -80 °C) favouring ice formation and growth (Moor, 1973; Mazur, 1984). Intracellular ice crystals become very small and more numerous as the cooling rate increases (Carrington *et al.*, 1996) and probably evenly distributed intra- and extracellularly. Even though being very small, the crystals still possess the ability cause damage to cellular ultrastructure and possibly lead to cellular death

(Karow, 1969). The effect of these intracellular ice crystals on survival from cryopreservation may be associated to their localisation (Wesley-Smith, 2003).

Cell permeability affects the rate of water loss, with more permeable cells being more tolerant to rapid cooling than less permeable cells (Dumont *et al.*, 2004). The ideal cooling rate is that which is rapid enough to minimise the time of exposure to solution effects injury and avoid shrinkage, but not so much that intracellular ice crystallisation occurs (Mazur, 1970; Farrant *et al.*, 1977). Dehydration of recalcitrant embryonic axes to sufficiently low WC increases intracellular viscosity that can slow ice formation and this makes survival to be independent of cooling rate (Wesley-Smith, 2004). However, if the WCs are high, viscosity is reduced which requires rapid cooling in order to preclude damage caused by ice crystals, if heat is dissipated rapidly enough to preclude growth and severe damage. It must be noted that the optimum cooling rate which promote cryo-survival also depends on the species and tissue/cell type (Mazur (1963; 2004; Varghese *et al.*, 2009) as a consequence of differences in sensitivity to shrinkages, differences in permeability to cell water, and their surface area to volume ratios (Farrant *et al.*, 1977).

1.7. Application of cryoprotectants to plant germplasm

The discovery of natural cryoprotection in cold-hardy plants (Hirsh *et al.*, 1985) was a huge breakthrough in cryobiology (Polge *et al.*, 1949; Fahy *et al.*, 1984). This provided evidence that cryoprotection by biological material requires more than adjusting water content. As stated before (section 1.5), desiccation of embryos and embryonic axes through ultra-rapid drying conditions increases their chances of surviving to very low WCs (0.25-0.45 g H₂O g⁻¹ dry weight), a range of only non-freezable water remaining in biological tissues (Berjak *et al.*, 1993; Pammenter *et al.*, 1993). It is generally accepted that plant explants (for example, embryonic axes) that have a WC in the intermediate range of 0.25-0.45 g H₂O g dry weight or less (Pammenter *et al.*, 1991) usually survive preservation at ultra-low temperatures. This is thought to be the window of acceptable WCs for desiccation sensitive tissue (Sershen *et al.*, 2007) and this WC can be widened by increasing cooling rate to over 100 °C s⁻¹ (Wesley-Smith *et al.*, 1992; Volk and Walters, 2006) and possibly inducing a vitrified state. Furthermore, Volk and Walters (2006) showed, using shoot tips from

Mentha and *Allium sativum*, that cryoprotection via plant vitrification solutions can increase the WC.

Prior to cooling, explants such as embryonic axes and shoot tips are often immersed in concentrated solutions called cryoprotectants, (McGann, 1978; Sakai *et al.*, 1991; Varghese *et al.*, 2009; Engelmann, 2011b). These osmotically remove water from cells which makes the remaining cellular water more viscous and aids in the formation of vitrified state of cellular water (Fuller, 2004; Muldrew *et al.*, 2004; Volk and Walters, 2006). There are variations on solution composition that have been developed for biological tissues (Uragami *et al.*, 1989; Sakai *et al.*, 1990; Nishizawa *et al.*, 1993); this follows the knowledge that biological material treated with specific cryoprotective solutions can survive exposure to cryogenic conditions. Generally, these solutions are of two types: penetrative and non-penetrative cryoprotectants. Penetrating cryoprotectants are able to protect against the harmful effects of extensive cell volume changes and injurious toxic solution effects during cooling, and lowering the freezing point so that remaining water is no longer injurious (Pegg, 1995; Fuller, 2004; Panis and Lambardi, 2005; Benson, 2008). Those authors asserted that there are three requirements for such protection: (1) cryoprotectants must be highly soluble in water at low temperatures to enable them to lower the point at which freezable water is first observed, (2) possess the ability to penetrate the cell membranes, (3) and be non-toxic at their useful concentrations. Contrary to penetrative cryoprotectants, non-penetrative solutions have no ability to penetrate the cell membranes; however, they have been successful applied to dehydrate cells osmotically through removal of freezable water.

Improved survival of various biological materials following exposure and retrieval from cryogenic conditions has been achieved through penetrative cryoprotection, which has been suggested to more beneficial than non-penetrative (Sakai *et al.*, 1990). In some instances, the combination of penetrative and non-penetrative cryoprotectants such as that of plant vitrification solutions (PVS) contained in solid media and/or liquid solutions is preferred (McGann, 1978; Engelmann, 2011b). These solutions dehydrate biological material and thus reduce the amount of water that is available to form lethal ice crystals (Sakai *et al.*, 1991), providing protection towards low temperature effects. There are studies that have shown that these solutions play a

significant role in stabilising cell structures during dehydration and cooling of dry biological systems that exhibit glassy state (Crowe *et al.*, 1998; Bryant *et al.*, 2001).

1.7.1 Commonly applied cryoprotectants in plant cryopreservation protocols

The cryoprotective agents provided prior to cooling to cryogenic temperatures are believed to aid in yielding a higher post-thaw survival than can be obtained in its absence (Fuller, 2004; Volk and Walters, 2006; Varghese *et al.*, 2009; Nadarajan and Pritchard, 2014). Some cryoprotectant solutions, especially those containing dimethyl sulfoxide (DMSO), are chemically toxic and can cause osmotic swelling to cells since they are penetrative (Farrant *et al.*, 1977; Naidoo *et al.*, 2011). Glycerol is a penetrative cryoprotectant known to be highly soluble in water and that forms hydrogen bonds with water molecules, thus, disrupting the crystal lattice formation of ice (Fuller, 2004). Moreover, glycerol can stabilise lipid membranes through hydrogen bonding with the membrane lipids (Crowe *et al.*, 1987; De Leew *et al.*, 1993), usually in extreme dehydrated conditions. Glycerol has relatively low toxicity in short-term exposure to living cells and moves across the across the selectively permeable plasma membrane of different cell types at a slow rate (Fuller, 2004). That author further asserted that many cells can tolerate exposure to low concentrations of 1 to 5 mol/l; however, tolerance is dependent on the cell type and conditions of exposure. Work by Sershen *et al.* (2012a, b) on zygotic embryos from *Haemanthus montanus* and *Amaryllis belladonna* revealed that glycerol offers cryoprotection by maintaining integrity of cellular ultrastructure which result in survival following retrieval from LN. Generally, the increasingly high viscosity of glycerol at lower temperatures depresses the freezing point and possibly precludes or delays ice crystal expansion. Additionally, work by Sershen *et al.* (2012c) showed that intracellular H₂O₂ (ROS) was not detected in glycerol-cryoprotected explants. Therefore, it can be suggested that glycerol either quenched the ROS produced (Lee *et al.*, 2006) or enhanced the activity of endogenous antioxidants in explants (Hanaoka, 2001).

Prolonged exposure to other solutions like PVS can be problematic as they probably allow extreme dehydration (Fahy, 1986; Fahy *et al.*, 1990; Volk *et al.*, 2006; Nadarajan and Pritchard, 2014). Therefore, exposure to cryoprotectants must be controlled to enable sufficient cellular dehydration without causing an injury from

chemical toxicity. Moreover, quantifying the dehydration tolerance of material to be cryopreserved is crucial. Although toxicity is a problem, many previous studies (Mycock *et al.*, 1995; Volk and Walters, 2006; Varghese *et al.*, 2009; Naidoo *et al.*, 2011; Sershen *et al.*, 2011; Teixeira *et al.*, 2013) have demonstrated that pre-treatment of plant germplasm with cryoprotectant solutions (optimised exposure time and conditions) such as glycerol, sucrose, DMSO, PVS2 can lead to better recovery from cryogenic conditions. Volk and Walters (2006) asserted that shoot tips from *Mentha* and *Allium sativum* cannot survive cryogenic conditions without pre-conditioning and loading with cryoprotectants (PVS2). Once the acquired dehydration tolerance of biological material to cryoprotectants (i.e. PVS2) has been established, cryopreserved material can now withstand subsequent rapid cooling in LN with minimal or no additional loss in survival (Volk and Walter, 2006; Benson, 2008). Despite many successful cryopreservation studies via application of cryoprotectants, work by Kioko (2003) and Kistnasamy *et al.* (2011) on embryonic axes from recalcitrant seeds suggested that cryoprotection does not guarantee survival. This is because the application of cryoprotectants either alone, or when followed by rapid dehydration or exposure to ultra-low temperatures, can be lethal.

1.7.2 Vitrification circumvents injurious problems associated with ice formation

Vitrification is a physical process by which a highly concentrated aqueous solution solidifies into a metastable amorphous glass without undergoing ice crystallisation when the temperature is decreased (Fahy *et al.*, 1984; Sakai, 2004, Teixeira *et al.*, 2013). This helps the tissue to circumvent the injurious problems linked with ice crystal formation (Benson, 2004) when exposed to LN temperatures. This is an easy method to perform and often has a high post cryo survival and recovery (Volk and Walters, 2006; Varghese *et al.*, 2009; Nadarajan and Pritchard, 2014; Gebashe, 2015). This technique is widely applicable particularly in various plant species sensitive to low temperature (Takagi *et al.*, 1997; Thinh *et al.*, 1999), including explants such as embryos (Nadarajan and Pritchard, 2014) and shoot apices (Sakai, 1993; Takagi, 2000; Wang *et al.*, 2005) with relatively high WC. Vitrification of plant material can be achieved in many ways including removal of most or all freezable water by partial dehydration of explants (Grout, 1983). Additionally, vitrification can also be achieved through the use of highly concentrated plant vitrification solution (PVS), followed by

ultra-rapid freezing of explants which results in vitrification of intracellular solutes, thus forming an amorphous glassy structure, inhibiting crystallisation which is detrimental to cellular structure integrity (Sakai, 2004; Sershen *et al.*, 2012a, b; Wesley-Smith *et al.*, 2015).

1.7.3 Vitrification solutions

Many research groups globally have made attempts to develop different vitrification solutions. Glycerol based vitrification solutions are the most widely used in plant germplasm cryopreservation and designated as PVS2 (Sakai *et al.*, 1990; Sakai, 2004) and PVS3 (Nishizawa *et al.*, 1993). However, PVS2 is probably used more than PVS3 for plant cells, tissues and embryos. Recently, many studies have reported successful cryopreservation using PVS2 for a range of plant germplasm such as seeds (Rathwell *et al.*, 2016), embryonic axes (Cho *et al.*, 2002), pollens (Buitink *et al.*, 1998b) polyembryoids (Suranathan *et al.*, 2012), protocorm-like-bodies (Rajasegar *et al.*, 2015) and shoot tips (Varghese *et al.*, 2009; Chua and Normah, 2011) and Gebashe (2015) also reported success in cryopreservation of *in vitro* grown shoot-tips from *T. emetica*, a tropical species with recalcitrant seeds, the species used in the present study. Similarly, successful cryopreservation using PVS2 was also reported in the shoot tips of many other species such as *Solanum tuberosum* (Sarkar and Naik, 1998), *Musa* spp (Helliot *et al.*, 2003), *Mentha* (Volk *et al.*, 2006) *Allium sativum* and *Mentha* (Volk and Walters, 2006), *Candidatus Liberobacter asiaticus* (Ding *et al.*, 2008), *Nephelium ramboutan-ake* (Chua and Normah, 2011), *Fragaria x ananassa* Duch (Yamamoto *et al.*, 2012), *Paraisometrum mileense* (Lin *et al.*, 2014) etc. PVS2 solution contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose (pH 5.8), whereas PVS3 comprises of 40% (w/v) glycerol and 40% (w/v) sucrose in basal culture medium, and all these constituents vary in effects on permeability and alter the freezing behavior of intracellular water. It has been established that when temperature is decreased below -100 °C, PVS2 solution easily supercools upon rapid cooling and finally solidifies into a metastable glass at about -115 °C (Volk and Walters, 2006). It has been noted using differential scanning calorimetry (DSC) that the vitrified PVS2 solution displays a glass transition (T_g) at about -115 °C (Volk and Walters, 2006; current study), with an exothermic devitrification (crystallization) at

about -60 to -75 °C and an endothermic melting at about -30 to -40 °C (Volk and Walters, 2006; current study).

1.8. A glassy state exploited in nature

Leprince and Walters (1995) asserted that the glassy state exploited by dried plant and animal systems in nature to withstand unfavorable conditions of the surrounding environment could represent a defense mechanism for promoting desiccation tolerance, quiescence, and storage stability. This concept of glassy states in developing storage protocols has been utilised for cryopreservation of hydrated explants (Wesley-Smith *et al.*, 1992; Pritchard, 1995; Benson, 2008) and also in dried and frozen food material (Noel *et al.*, 1990; Slade and Levine, 1994). Generally, a glass is exceedingly viscous (typically in the order of 10^{14} Pa s) and limits all chemical reactions that require molecular diffusion; its formation stops all metabolic activities which lead to stability over time (Burke, 1986). This amorphous metastable state resembles a solid, brittle material however, it retains the disorder and physical properties of the liquid state. Through application of various vitrification procedures a high viscosity is achieved without any major molecular reorganization (Angell, 1988; 1991; Slade and Levine, 1991; Yonezawa, 1991; Angell *et al.*, 1994; Perez, 1994) and thus, preventing any major changes in the cellular structures (Burke, 1986). As a result, a high intracellular viscosity is achieved through glass formation which precludes molecular diffusion and the chances of any chemical reactions. Ideally, when frozen and/or dried seed tissues are stored below the T_g in cryogenic conditions, the glassy state would ensure retention of viability for long periods, as deleterious changes in structure and chemical composition are minimised within tissues (Burke, 1986; Vertucci and Roos, 1990; Williams *et al.*, 1993). In practical, the presence of intracellular aqueous glasses is known in seeds, although their physiological role in storage stability is still speculative. Fundamental insight into the conditions at which a glass is induced in plants can aid in regulating the glassy status during cryopreservation of plant germplasm. Therefore, thermal analysis is a useful tool in the study of thermal behaviour of water during cooling and warming, particularly determination of glass transition.

1.8.1 Molecular properties of glass

In principle, water in a solid state can exist in two forms: (1) crystalline form that has defined structure, stoichiometric composition and melting points and (2) amorphous form (i.e. glass) which does not have a defined structure (Walters, 1998). The three-dimensional long-range order observed in a crystalline form does not exist in the glassy state. The molecules of a glass are randomly positioned and are interconnected as opposed to those in the liquid state (Atkins, 1982). Therefore, this arrangement results in a matrix that imposes restricted mobility compared with a liquid. Despite such organisation as in liquids, glasses still lack a specific molecular orientation which results in free volume (“space”) within the matrix. Such free volume permits diffusion of small molecules (Walters, 1998) or other chemical species, i.e. free radicals (Benson, 1990; Benson and Bremner, 2004), through the matrix. Where there is sufficient water, the amorphous matrix has the loose character of a rubber which has a high free volume and lower viscosity and increasing diffusivity (Walters, 1998). Findings by Buitink *et al.* (1998a) using EPR revealed that molecular mobility of the cytoplasm is inversely related with storage stability of cattail (*Typha latifolia*) pollen and pea (*Pisum sativum* L.) embryonic axes. Those authors indicated that minimum mobility and increased storage stability occurred at a similarly low WC, and a sharp increase in mobility at certain temperature was correlated with the melting of the glassy matrix.

According to Buitink and Leprince (2004), glasses are not in an equilibrium phase, which means they will eventually move to an equilibrium phase, i.e. they will crystallise. Those authors also mentioned that as a consequence of high viscosity of the glassy state, this transformation to equilibrium occurs at a slow rate that is referred to as “semi-equilibrium” state. Additionally, the transition from equilibrium to equilibrium (i.e. crystal-to-liquid) is a first order transition, a phase transition. Whereas, glass-to-liquid is referred to as second order transition, a transition from non-equilibrium state to an equilibrium phase.

1.8.2 Formation of intracellular glasses

It has been generally accepted that desiccation tolerant biological systems possess the ability to form glasses. Walters (1998) asserted that in seeds this can be viewed as a natural consequence of drying. Glass is formed by the complex mixtures of molecules making up the cytoplasm of cells and as a consequence of increased drying; this is likely to preclude crystallisation when cells are exposed to ultra-low temperatures. Naturally occurring glasses have been reported in a number of tissues such as seeds (Leprince and Walters-Vertucci, 1995), pollen (Buitink *et al.*, 1996), prokaryotes (Potts, 1994) and “resurrection” plants (Wolkers *et al.*, 1998). Those tissues were reported to possess the ability to resist extreme dehydration and allowing metabolic activity to resume once water becomes available. A large accumulation of mixtures of sugars and some protective proteins (i.e. Late Embryogenesis Abundant [LEA] proteins) have been reported in these tissues, which led Hirsh (1987) and Koster (1991) to propose that those sugars play a significant role in the formation of glass, since these molecules are known to be excellent glass formers in biological systems (Williams and Leopold, 1989; Koster, 1991; Slade and Levine, 1991; Buitink and Leprince, 2004; 2008). The earliest opinions were that mixture of sugars (especially sucrose) are effective in replacing water at the hydrophilic sites of the cellular membrane of desiccation tolerant systems (Leopold and Vertucci, 1986). Furthermore, in some biological systems glasses are formed by these sugars which provide protection from denaturation of large molecules and formation of molecular aggregates. Therefore, sugars reportedly possess ability to fill spaces in the tissue, and during dehydration may serve to preclude excessive increases in tissues collapse, solute concentration, and pH alteration (Burke, 1986).

1.8.3 Function of intracellular glasses

Intracellular glasses were initially proposed to have a significant role in both desiccation tolerance and storage stability of seeds, as they contribute to the stability of the seed components during storage (William and Leopold, 1989; Sun and Leopold, 1994; Buitink *et al.*, 1998a; Buitink and Leprince, 2004). It is assumed that the high viscosity of intracellular glasses decreases molecular mobility and impede diffusion within the cytoplasm, thus slowing down deleterious reactions and changes

in structure and chemical composition during ageing (Buitink and Leprince, 2004). Generally, variation in the physiological activity among seeds suggests that there are differences in mobility between seed glasses, and only a few tools are available to measure these differences in mobility. Using axes of bean (*Phaseolus vulgaris* L.), Leprince and Walters-Vertucci (1995) showed that the interaction between water and the other glass-forming components is complex, and therefore suggesting that water did not behave as a typical solvent in intracellular glasses. Despite numerous assumptions that molecular mobility might play an important role in storage stability (Sun and Leopold, 1993; Leopold *et al.*, 1994; Buitink *et al.*, 1998a), not much is known the translational, rotational or vibrational motion of molecules in the cytoplasm of recalcitrant seed tissues. In addition, findings by Ballesteros and Walters (2011) show that pea seeds display intramolecular motion whose nature and extent differs with moisture and temperature. Hence, some recent views suggest that glasses may appear to be less relevant to processes such as ageing during storage than previously believed, as a consequence of intramolecular motion exhibited at ultra-dry conditions. Nowadays, there are many reports that have emerged on the detection of glass transition (second order transition) and other first order transitions using different techniques, e.g. Differential Scanning Calorimetry (DSC), Electron Paramagnetic Resonance (EPR), Thermally Stimulated Depolarization Currents (TSDC), and other mechanical thermal analysis.

1.9 Modern application of Differential Scanning Calorimeter (DSC) in cryopreservation protocols

Differential Scanning Calorimetry is a thermal analysis technique that can be used to measure the temperature and heat flow associated with phase transition of water and lipids in plant samples as a function of time and temperature in a controlled atmosphere (Williams and Leopold, 1989; Kaczmarczyk *et al.*, 2011, 2012). This technique can be very useful in cryopreservation and has been used previously (Pammenter *et al.*, 1991; Martinez *et al.*, 1998, 2000; Block, 2003; Volk and Walters, 2006; Teixeira *et al.*, 2013; Ballesteros *et al.*, 2014). These measurements provide quantitative and qualitative information about physical changes that involve endothermic or exothermic processes, or changes in heat capacity. This is a powerful tool to be applied for determination of glass transition, temperature of ice nucleation,

melting, boiling, crystallization time and kinetic reaction, the most crucial features useful for cryopreservation (Zámečník and Faltus, 2009; Zámečník *et al.*, 2009). It has been asserted that the danger of nucleation and subsequent intracellular ice crystallisation that is consequent to freeze damage during cooling and warming of the samples is regarded as a critical point of plant survival at ultra-low temperatures (Zámečník *et al.*, 2012). Therefore, these thermal profiles can give important information about the cryoprotectants required to obviate lethal ice formation and stabilise glasses (Benson *et al.*, 2007). In this current study, thermal analysis was applied to embryonic axes and shoot tips of *T. emetica* prior to and after pre-culture, and cryoprotection using PVS2 and glycerol. One of the major problem encountered is that DSC measurements of water in biological materials may be confounded by interfering signals from lipids and by drying, cooling and warming protocols. Many cryo-researchers including the author of the current study, are of the view that precise interpretation of thermal analysis can lead to improved understanding of fundamental processes of cryopreservation and support the enhancement of the method.

1.10 Plant species investigated: Trichilia emetica

Trichilia emetica (Meliaceae), commonly known as Natal mahogany, is a forest tree of immense horticultural importance known to produce recalcitrant seeds (Kioko *et al.*, 2006; Goveia, 2007). It is indigenous to most countries in tropical and sub-tropical east Africa, usually trees occur within the same area. The genus name as described in Greek “tricho” means in “three parts”, which refers to the three-celled fruits. The epithel, *emetic*, is derived from *emetic* properties of the powdered bark that is traditionally used as a medicine (Pooley, 1993; Schmidt *et al.*, 2002). This is an evergreen, medium to large tree, up to 5-10 m high (Pooley, 2003). The leaves can be up to 50cm long, unevenly compound with 3-5 pairs of leaflets plus a terminal one, dark green and glossy above, covered with short brownish hairs below, margins entire, veins prominent on lower surface. The trees usually start flowering from August to November and the flowers are creamy to green and velvet. The fruits are round, velvet capsules, ~3 cm in diameter, which splits usually into 3 valves, and on splitting the capsule reveals bright red (mature) seeds (Figure 1.2) that are rich in oil (Adinew, 2014). Additionally, the fruit capsule has a distinct neck that joins it to the

stalk. The fruiting season usually occurs from January to late May (Allaby, 1998; Coates-Palgrave, 2000), with availability differing with different seasons.



Figure 1.2: Mature fruits and seeds of *T. emetica* (photo: Thabiso Thabethe)

Trichilia emetica is reported to have a wide variety of uses. They are known to be a food source as usually they are edible to monkeys and baboons. Additionally, some southern African tribes find these seeds edible, whereby seed coats are removed and then the arils are cooked as vegetables or they can be crushed to make a milky juice that becomes flavour when added in spinach (van Wyk *et al.*, 2000). The oil extracted from the seeds can be used for making soap, candle and furniture oil (von Breitenbach, 1965), and also widely used in cosmetics. The powdered bark is used an emetic and is effective in treatment of rheumatism. The traditional use of *T. emetica* in pharmacology has prompted many researchers to screen for a wider range of biological activities such as antibacterial, anticandidal, anti-inflammatory, anticancer and hepatoprotective properties etc. (Komane *et al.*, 2011). Lastly, reddish brown wood from the trees is often used for furniture, musical instruments dugout, canoes, fish floats and tradition carvings such as *izibhenge* (bowls for eating), *uGqoko* (trays for eating meat) and *izinkezo* (spoons) (Grant and Thomas, 1998).

1.10.1 T. emetica shoot tips: explants alternate to embryonic axes for cryopreservation

The seed of this species are usually shed from the parent plant at a WC of about 2.8 g g⁻¹, and viability retention is compromised when stored at 6 °C and upon dehydration (Kioko *et al.*, 2006). They follow the similar behaviour observed in majority recalcitrant seeds (Berjak *et al.*, 1989; Chaitanya and Naithani, 1994; Pammenter *et al.*, 1998; Varghese and Naithani, 2002; Sershen *et al.*, 2008) and it is difficult to cryopreserve them as seeds (Varghese *et al.*, 2004; Berjak and Pammenter, 2008; 2013) due to their large size and high WC. However, cryopreserving the embryonic and meristematic tissues is highly advantageous and plausible (Sershen *et al.*, 2007; Goveia *et al.*, 2004; Naidoo *et al.*, 2011; Naidoo *et al.*, 2016) because the zygotic embryos or embryonic axes are small and easy to dehydrate rapidly to amenable WCs for cryopreservation (Berjak *et al.*, 1992; Pammenter *et al.*, 1998; Wesley-Smith *et al.*, 2001; Pammenter *et al.*, 2002; Ballesteros *et al.*, 2014). There are a number of factors that need to be taken into consideration with cryopreservation protocol of any species. For example, use of embryonic axes of *T. dregeana* showed that the developmental stage of the explant plays an essential role in promoting retention of viability after exposure to cryogenic conditions (Goveia *et al.*, 2004). Those authors suggested that with development of embryos in hydrated storage, the physical and biochemical stress on the shoot meristems is reduced thereby increasing the chances of cryo-survival in these seeds.

A number of attempts made in our laboratory to cryopreserve the embryonic axes of *T. emetica* were not successful (Kioko, 2003; Kioko *et al.*, 2006, Goveia, 2007, Naidoo, 2012). Survival of embryonic axes after retrieval from liquid nitrogen was limited to root development; cryopreserved explants showed no shoot development (Goveia, 2007). Kioko (2003) asserted that that explants (especially embryonic axes) will respond differently to various cryogenic stresses. Other reports by Kioko *et al.* (1998, 2006) also revealed that viability and ultrastructure of the explant can be affected by dehydration rate and storage conditions. Therefore, the physiological state of the cells and tissues to be cryopreserved must be suitable for the attaining required, maximum possible dehydration tolerance and retention of viability.

Due to limited success with cryopreservation of embryonic axes of this species, a protocol for an alternate form of explant had to be developed. *In vitro* grown shoot tips (with shoot meristems and the apical dome with one or two pairs of leaves) are regarded as ideal explants for cryopreservation (Karthi *et al.*, 1980; Varghese *et al.*, 2009; Gebashe, 2015) as they possess a make-up of undifferentiated cells that remain genetically stable during the regeneration process (Mroginski *et al.*, 1991). The possibility of using shoot tips of *T. emetica* for long-term cryopreservation was previously explored in this laboratory (Varghese *et al.*, 2009, Gebashe, 2015). The shoot tips in these studies were obtained from *in vitro* sub-culturing of axillary buds that were excised from *in vitro* grown shoot cultures. In both studies shoot tips showed successful post cryo-survival, therefore prompting the basis of the current studies on behaviour of water in these two types explants (embryonic axes and shoot tips). The hydrated state and sensitivity of these explants to dehydration had to be accommodated by the newly developed protocol, where such limitations were partially overcome by introducing explants to pre-culture and cryoprotection treatment (Varghese *et al.*, 2009). Those authors reported that pre-culture of shoot tips on MS medium with 0.7 M sucrose or with 0.3 M sucrose + 0.5 M glycerol followed by cryoprotection with a mixture of glycerol and DMSO or with PVS2 was crucial for successful recovery following cryostorage. Slow cooling of the shoot tips (WC 1.24 g g⁻¹ DW) pre-cultured on medium with 0.3 M sucrose + 0.5 M glycerol and cryoprotected with PVS2 resulted in high shoot production (71%).

1.11 Prospects of the current study

A study by Volk and Walters (2006) on *Mentha* spp. and *Allium sativum* shoot tips showed that immersion in cryoprotective solution reduces the magnitude and in some rare cases the temperature of the 1st order transition. However, it remains important to understand the process of reducing the magnitude of freezing transitions (onset temperature and the enthalpy), whether it occurs as a consequence of cells drying out in response to cryoprotectants or whether the thermodynamic properties of the water are altered by cryoprotectants. According to Teixeira *et al.* (2013) and, Volk and Walters (2006), thermal analyses of plant materials with the aid of DSC suggests a strong relationship between the presence of water, freezing or melting transitions and survival or damage at sub-freezing temperatures. To date, there has been no report on

the physical properties of water (or effect of cryoprotectants) using DSC on the embryonic axes and shoot tips of *T. emetica*.

Aim and Rationale:

The aim of this study was to gain fundamental understanding of the changes in physical characteristics of water within (1) the shoot of embryonic axes, (2) the shoot tips grown from seedling culture, and (3) the shoot of embryonic axes pre-conditioned in same manner as shoot tips. A further aim was to assess the impact of cryoprotectants on these characteristics (e.g. crystallisation) through use of DSC, and relate these to resultant survival or damage. The information gained in this study could form basis of better understanding of fundamental processes of cryopreservation and aid in the advancement of the method, especially in the context of recalcitrant seeded species.

Objectives:

- To use DSC to measure the size and temperature of first order (exothermic and endothermic events) and second order transitions (glass transitions) within cryoprotected shoot tips and embryonic axes of *T. emetica* at three steps (pre-culture, cooling and warming) of a cryopreservation procedure that uses PVS 2 and glycerol as cryoprotectants.
- To assess the effect of cooling rate where cryoprotected explants were subjected to either rapid cooling (directly plunged in liquid nitrogen) or slow cooling (10 °C min⁻¹).
- To assess viability after subjecting embryonic axes and shoot tips to pre-conditioning solutions, followed by subjection to either rapid cooling or slow cooling.

Chapter 2: Materials and methods

2.1 Seed collection and processing

Mature fruits of *Trichilia emetica* (Figure 1.2) were harvested from late January to mid-March (2014-2016) directly from trees growing in or around uMtunzini (28° 56' 59, 99" S, 31° 44' 59, 99" E; google earth) and St. Lucia (28° 22' 59, 88" S, 32° 25' 0, 12; google earth), North of KwaZulu-Natal, South Africa. The fruits were transported by road in open plastic buckets within 3 to 4 h to the laboratory (Durban). Upon arrival, only mature seeds with no signs of bacterial/fungal infection were extracted from open (mature) fruits for these experiments. The arils from the seeds were removed and cleaned seeds were surface decontaminated and temporarily hydrated stored as described below (section 2.2 and 2.3).

2.2 Surface decontamination of whole seeds

Freshly harvested seeds were decontaminated in 1% (w/v) aqueous solution of sodium hypochlorite (3:1 dilution of commercial bleach) containing a few drops of wetting agent Tween 20[®] (Sigma, Germany), for 20 min. The seeds were then soaked in deionised water with 500 μ L L⁻¹ early Impact (active ingredient, triazole and benzimidazole; Zeneca Agrochemicals, South Africa) and 2.5 μ L L⁻¹ Previcur N (active ingredient, propamocarb-HC, AgrEvo, South Africa) a 'cocktail' of fungicides that curtail fungal contamination in storage (Calistru *et al.*, 2000; Goveia *et al.*, 2004; Mayeza, 2005), for a further 1 h on a shaker at 120 rpm at ambient conditions. Seeds were retrieved from the shaker, rinsed thrice with deionised water and then dried back to their original WC on a laboratory bench (lined with 2 layers of paper towel) at ambient temperature. They were either used immediately or treated and stored in the hydrated state at 16 °C as described in section 2.3.

2.3 Seed storage

Following the initial decontamination, seeds were then dusted with Benomyl 500 WP (active ingredient, benzimidazole; Villa Protection, South Africa) and placed a molayer on plastic mesh 200 mm above water-saturated paper towel (Pammenter *et al.*, 1994; Eggers, 2007, Sershen *et al.*, 2007) in white, translucent 5 L buckets that were disinfected by soaking in a 1% (w/v) sodium hypochlorite aqueous solution for 24 h and allowed to dry prior to use. Sealed buckets were stored in dark cold-room at 16 °C and were opened weekly for aeration and checking for microbial contamination.

2.4 Selection of explant type

2.4.1 In vitro establishment of seedlings

Whole cleaned and hydrated stored seeds of *T. emetica* (n = 1000) were decontaminated by immersion in 1% (w/v) sodium hypochlorite with a drop of Tween 20[®] for 5 min followed by 0.2% (w/v) mercuric chloride for 10 min in 0.02% (w/v) Cicatrin (w/v) (10 min) and 500 ppm sodium dichloroisocyanurate (NaDCC) in deionised water for 60 min. The seeds were rinsed with sterile deionised water in between treatment with different sterilising agents and finally they were rinsed thrice with sterile deionised water.

Sterilised seeds were blotted dry on sterile filter paper and cultured on germination medium that consisted of half strength (2.2 g L⁻¹) Murashige & Skoog (1962) medium salts with vitamins, 30 g L⁻¹ sucrose, 8 g L⁻¹ agar (Agar Bacteriological; Merck, South Africa) and 0.5 mg L⁻¹ benzylaminopurine (BAP) at pH of 5.6. After six weeks in diffused light with a 16 h photoperiod (light intensity approximately 50 µmol m⁻² s⁻¹), shoot tips (apical dome) from established *in vitro* seedlings were excised by hand under aseptic conditions in a laminar flow using a dissecting microscope. The excised shoot tips were pre-cultured for 3 d and cryoprotected (as described in section 2.6) before being used for various experiments.

2.4.2 Explant type used for all experiments

Three types of explants were used in these experiments: 1. shoot tips were excised from *in vitro* cultured seedlings and used for viability and DSC assessments; 2. embryonic axes with 1 to 2 mm basal cotyledon segments attached and used for only viability assessment. Basal cotyledon segment was left attached to the axis in order to avoid excision induced oxidative stress (Berjak *et al.*, 2011b); 3. embryonic axis shoot meristem segment excised from embryonic axes and used for only DSC assessments.

2.5 In vitro germination to standardise shoot development

Twenty-five freshly excised shoot tips (control) and shoot tips excised from *in vitro* grown plants and pre-cultured as discussed in 2.6.1 were used to determine shoot development prior to cooling. The shoot tips were cultured under aseptic conditions in sterile 65 mm Petri dishes (5 shoot tips per dish) with MS medium consisting of 0.05 mg L⁻¹ BAP and 0.1 mg L⁻¹ gibberellic (GA₃). These dishes were sealed with water proof Parafilm[®] and then kept in the dark (at 25 ± 2 °C) for eight weeks, after which they were transferred to diffused light with a 16 h photoperiod (light intensity approximately 50 μmol m⁻² s⁻¹). Shoot production was scored as percentage of total number of shoot tips cultured which expressed visible leaf expansion and organised regrowth after 8 weeks in culture (Varghese *et al.*, 2009).

Similarly, 15 whole embryonic axis with cotyledonary segments attached from freshly excised (control) and pre-cultured for three days (see section 2.6.1) were used to determine shoot development prior to cooling. Prior shoot determination explants were dried as described in section 2.7. Then explants were cultured in MS medium containing 0.05 mg L⁻¹ BAP and 0.1 mg L⁻¹ GA₃, in sterile 65 mm Petri dishes (5 axes per dish), sealed with water proof Parafilm[®] and kept in the dark (at 25 ± 2 °C) for eight weeks, after which they were transferred to diffused light with a 16 h photoperiod (light intensity approximately 50 μmol m⁻² s⁻¹). Shoot production was scored as 5 mm protrusion of shoot after 12 weeks in culture.

2.6 Pre-conditioning by pre-culture and cryoprotection

2.6.1 Pre-culture medium

Excised shoot tips from *in vitro* grown seedlings, embryonic axes with cotyledonary segments attached, and embryonic axes shoot meristem segments excised from seeds were pre-cultured on a pre-conditioning medium (MS medium containing 0.3 M sucrose + 0.5 M glycerol, pH 5.6; Matsumoto *et al.*, 1998; Varghese *et al.*, 2009) for 3 days. Explants were laid on Whatmann filter paper soaked in cathodic water (described in section 2.9), and then placed over the described medium for pre-culture. This was kept in the dark at 25 ± 2 °C for 3 d and then used for further experiments.

2.6.2 Cryoprotection

After pre-culture, explants were transferred into 2 ml polypropylene cryovials (Lasec, South Africa) and were treated with the following cryoprotectants:

(1) PVS2 - explants were initially osmo protected through immersion in a loading solution (2.0 M glycerol and 0.4 M sucrose) for 15 min and then exposed to chilled PVS2 on ice. Plant Vitrification Solution 2 (PVS 2) consists of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO in liquid MS medium (pH 5.6-5.8) containing 0.4 M sucrose (Sakai *et al.*, 1990), and in this study, was made in cathodic water. To minimise potential toxicity of PVS 2 on shoot tips, exposure to PVS2 was sequential (Benson *et al.*, 2007). Shoot tips were first immersed in 50% PVS2 solution for 5 min followed by immersion in 100% PVS2 for 15 min (Varghese *et al.*, 2009). For embryonic axes, before DSC experiments, cotyledonary attachments from the embryonic axis were removed and shoot meristem segments were excised.

(2) Glycerol - exposure of shoot tips and embryonic axes with cotyledonary segments attached to the glycerol was done sequentially (Sershen *et al.*, 2007) by treating with 5% glycerol solution for 30 min followed by 10% glycerol for a further 30 min and used for further experiments. Embryonic axes shoot segments were excised from embryonic axes with cotyledonary segments attached after cryoprotection and rapid drying (section 2.7) and used for further DSC experiments. None pre-cultured and

non-cryoprotected explants (both axes and shoot tips) served as the control (referred to as fresh in the results section).

2.7 Flash drying of embryonic axes

Freshly excised (non-cryoprotected control) and cryoprotected embryonic axes (cotyledonary segments removed) from *T. emetica* were rapidly dried by flash drying (Berjak *et al.*, 1990; Pammenter *et al.*, 1998; Walters *et al.*, 2001; Pammenter *et al.*, 2002). This procedure involved placement of explants on a mesh in a glass jar containing activated silica gel and a computer fan to circulate air in the jar. Explants were excised in batches and cryoprotected (section 2.6.2) before flash drying. Drying was done up to 2 h with sampling intervals of 30 min, and drying curve was repeated twice. Embryonic axis shoot segments were excised from embryonic axes with cotyledonary segments removed after rapid drying and used for further DSC experiments as described in the next sections. No flash drying was performed on shoot tips excised from cultured seedlings.

2.8 Water content determination

The WC for freshly excised or cryoprotected embryonic axes with cotyledonary segments removed, and those rapidly dried for various intervals (section 2.7) was estimated gravimetrically for individual embryonic axis (n=10), for each drying time in two independent drying curves. Embryonic axes were weighed using a six place electronic balance (Mettler MT5, Germany) before and after drying at 80 °C for 48 h. Water content was expressed on dry mass basis (g H₂O per g dry matter, represented hereafter as g g⁻¹). A similar WC determination procedure was repeated for embryonic axis shoot segments that were excised from embryonic axes with cotyledonary segments removed after rapid drying. Lastly, a similar procedure was repeated for shoot tips excised from cultured seedling, this followed a 3 d pre-culture and cryoprotection. Shoot tips placed in batches and WC was determined immediately after pre-culture, immediately pre-culture plus cryoprotection. Shoot tips were blotted with filter paper before WC determination.

2.9 Generation of cathodic water

Embryonic axes with cotyledonary segments attached were rehydrated in cathodic water (the fraction obtained after electrolysis of the CaMg solution (1:1 solution of 0.5 μ M CaCl₂·2H₂O and 0.5 mM MgCl₂·6H₂O as described by Berjak and Mycock, 2004; Berjak *et al.*, 2011b). The CaMg solution was electrolysed using a Bio-Rad™ Powerpac (BioRad, Hercules, California, USA) that has two platinum electrodes, anode and cathode. The electrodes were placed in in two separate 250 ml glass beaker that each contained 200 ml of the CaMg solution. Completion of the circuit was achieved via use of an agar-based salt bridge, made from 3 g KCl and 0.3 g bacteriological agar in 10 ml of distilled water. The CaMg solution was electrolysed for 60 minutes, at 60 V potential difference. The beaker with cathodic was used for further experiments.

2.10 Rehydration

Explants were treated with cathodic water at different stages of cryopreservation in attempt to avoid stress from free radicals. During pre-culture, explants were cultured on filter paper initial soaked in CW and placed over the pre-culture medium. For cryoprotection, embryonic axes and shoot tips treated with loading solution and PVS2 were made up in cathodic water. Additionally, explants were treated with unloading solution made in CW. Lastly, after flash drying, embryonic axes with cotyledonary segments attached were rehydrated in cathodic water within closed 90 mm Petri dishes in dark (at 25 \pm 2 °C) for 30 min and then decontaminated as described in section 2.11 and regenerated *in vitro* (see section 2.12.2).

2.11 Surface decontamination of embryonic axis explants

Immediately after cryoprotection, freshly excised (control) and flash dried embryonic axes with cotyledonary segments attached were decontaminated by immersion in 1% sodium hypochlorite (w/v) with a drop of Tween 20® for 5 min followed by 0.01% Cicatrin (w/v) or 500 ppm NaDCC (dissolved in deionised water) (10 min), for 5 min,

before three rinses with sterile CaMg solution. There was one rinse with CaMg solution in between the sterilizing agents. Thereafter, at each drying time in two independent curves 15 embryonic axes were transferred to MS germination medium in a 65 mm Petri dish sealed with water proof Parafilm[®] and kept in a growth room in the dark (at 25 ± 2 °C) conditions.

2.12 Cryopreservation of explants

2.12.1 Shoot tips from in vitro generated seedlings (methodology developed and described by Varghese et al., 2009)

Shoot tips that were pre-cultured and cryoprotected were placed in sterile 2 ml cryovials (10 shoot tips in each cryovial) and the cryovials were subjected to two cooling rates: (1) slow cooling, where cryovials were cooled in isopropanol in a -80 °C ultra-freezer at 1 °C min^{-1} using Mr. Frosty[®] (Nalgene[™], USA) from room temperature to an intermediate temperature of -40 °C in 65 minutes. Thereafter, cryovials were plunged into LN at -196 °C, and held there for at least 24 h; (2) rapid cooling, where cryovials with explants were mounted on cryocanes and directly plunged into liquid nitrogen. This facilitated cooling at approximately 200 °C min^{-1} .

Following storage in LN, shoot tips were retrieved, warmed rapidly by adding cathodic water solution at 40 °C to the cryovial (for approximately a minute). Thereafter, samples were rehydrated for 15 min by placing them on filter paper moistened with cathodic water solution at 25 °C in dark. In samples treated with PVS2, samples were placed in 1.2 M sucrose (unloading solution) for 20 min at 25 °C, which promotes replacement of PVS2 from the sample. The shoot tips were then transferred to a sterile filter paper placed upon MS medium (Gebashe, 2015) supplemented with 0.05 mg L^{-1} BAP and 0.1 mg L^{-1} GA₃. Thereafter, the retrieved shoot tips were cultured in the same medium in 65 mm Petri dishes. Those Petri dishes were sealed with water proof Parafilm[®] and then kept in the dark (at 25 ± 2 °C) for eight weeks, after which they were transferred to diffused light with a 16 h photoperiod (light intensity approximately 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Shoot production was

scored as percentage of total number of shoot tips cultured which expressed visible leaf expansion and organised regrowth after 8 weeks in culture.

2.12.2 Embryonic axes with small piece of basal segment attached

Freshly excised embryonic axes with cotyledon basal segments attached were immersed in the following cryoprotectants for a total duration of 1 h in the dark, with the first 30 min at the lower concentration followed by 30 min at the higher concentration: 0.5 M and 1.0 M sucrose, and 5% and 10% glycerol. When PVS2 was used, embryonic axes with cotyledons were immersed for 30 min in dark and then used for cryopreservation experiments. After cryoprotection, the explants were blotted dry using filter paper and then flash dried to a water content of approximately 0.4 g g^{-1} .

A set of ten cryoprotected embryonic axes with cotyledonary segments attached were also enclosed in a sterile cryovial and then subjected to slow cooling, where cryovials were cooled in isopropanol in a $-80 \text{ }^{\circ}\text{C}$ ultra-freezer at $1 \text{ }^{\circ}\text{C min}^{-1}$ using Mr. Frosty[®] (Nalgene[™], USA) from room temperature to an intermediate temperature of $-40 \text{ }^{\circ}\text{C}$ in 65 minutes. Thereafter, cryovials were plunged into LN at $-196 \text{ }^{\circ}\text{C}$, and held there for at least 24 h. Another set of pre-cultured and cryoprotected embryonic axes with cotyledonary segments attached were subjected to rapid cooling procedure, where cryovial with explants were mounted on cryocanes and directly plunged into liquid nitrogen. This facilitated cooling at approximately $200 \text{ }^{\circ}\text{C min}^{-1}$.

The embryonic axes with cotyledonary segments attached were warmed by rapid immersion in cathodic water ($40 \text{ }^{\circ}\text{C}$) for two minutes, and then rehydrated in cathodic water in dark for 30 min. Explants were then subsequently sterilised (as described in section 2.11) and cultured on solid MS medium as described above (section 2.12.1). Petri dishes were sealed with water proof Parafilm[®] and then kept in the dark (at $25 \pm 2 \text{ }^{\circ}\text{C}$) conditions for eight weeks, after which they were transferred to diffused light with a 16 h photoperiod (light intensity approximately $50 \text{ mol m}^{-2} \text{ s}^{-1}$). All

experiments were repeated twice. Shoot production was scored as 5 mm of shoot protrusion.

2.13 Differential Scanning Calorimetry

Differential scanning calorimetry of explants with computer-aided data analysis software was used to determine how individual treatment or combination of cryoprotectants, dehydration and cooling rates during the procedural steps of cryopreservation affect the thermal behaviour of water in embryonic axes and in shoot tips excised from cultured seedlings. These studies were carried out on fresh, dehydrated and/or cryoprotected embryonic axes, pre-cultured embryonic axes, and pre-cultured shoot tips. The embryonic axis shoot meristem segment and shoot tip from *in vitro* seedlings were carefully excised using a sharp blade. They were then dehydrated (with the exception of shoot tips) to various water contents in a flash drier (section 2.7 and 2.8), hermetically sealed in aluminium DSC pan (TA, T150420, Lot 605376, T_{zero} pan 901683.901, T_{zero} Hermetic Lid 901684901, Switzerland), weighed, and immediately subjected to calorimetric measurements. Thermal behaviour of the sample was measured using a differential scanning calorimeter (DSC Q2000, TA Instruments, USA). Before starting the measurements, one heating scan (from ambient to 200 °C at 10 °C min⁻¹ followed by an isotherm for 2 minutes and cooled back to ambient temperatures) before start of experiments was performed to ensure that moisture in the flange, if any, does not interfere with the thermograms resulting in erroneous peaks.

Two cooling methods were applied to simulate cooling rates used during cryopreservation procedures in this study:

(1) samples were equilibrated at 25 °C, cooled slowly using DSC from 40 °C to -150 °C at 10 °C min⁻¹ and thereafter re-warmed to 40 °C at 10 °C min⁻¹ and (2) samples were sealed in pans and weighed after which the pans were directly plunged in LN for approximately a minute. Thereafter, the pans were loaded into a pre-chilled flange inside the sample holder of DSC to -150 °C. Thereafter, the pans were rewarmed to 40 °C at 10 °C min⁻¹. After DSC measurements, pans were punctured with fine forceps and dry weights were determined after placing them in an oven at 80 °C for 48 h.

During rapid cooling procedures relative humidity inside the box covering the DSC was kept below 15%, which was attained by flushing the plastic box built around the DSC with nitrogen gas, placing 2 trays of activated silica gel, and anti-humidity power tabs (Rubson, South Africa). The purge gas that was used for both cooling procedures was helium. An empty pan served as the reference during all the runs. Four shoot tips were used for analyses for selected treatments of cryopreservation

Thermograms generated using DSC TA 2000 were evaluated and analysed using Universal Analysis software 2000 for Windows (TA Instruments, USA). The endothermic characteristics and glass transition were evaluated in detail using heating thermal profile, as they reveal more information than cooling thermal profiles (Marikkar *et al.*, 2002), where crystallization temperatures were recorded. Onset temperatures of first and second order (T_g) crystallization and melting were calculated from intersection of the baseline and tangent to the steepest part of the transition peak. The enthalpy of melt was calculated (1st order transition) as the energy of transition of dry sample divided by the water content of the sample and was expressed as $J g^{-1} DW$. For each cooling rate and cryoprotectant treatment, the enthalpy was regressed against the water content. Freezable water was calculated from the equation of a linear regression ($y = mx + c$, where X-intercepts and slopes were used to quantify the amount of water that was non-freezable, and the energy of the melting transition (on a per g H_2O basis) for the water that was frozen (Vertucci, 1990). In addition, in very wet samples sharp peaks were observed in the melting endotherms at about 0 °C, and only sharp temperatures were able to be determine and no other characteristic of the sharp peak was determined. The occurrence of this sharp peak signifying presence of pure water in samples has been reported in previous studies (Vertucci *et al.*, 1991 and Berjak *et al.*, 1992)

2.14 Statistical analysis

2.14.1 Viability assessment

All Data were analysed using the statistical software programme SPSS (SPSS Inc., Chicago, Illinois, USA) version 23 for Windows.

Viability of both embryonic axes with cotyledonary segments attached and shoot tips established *in vitro* were tested for inter treatment differences using ANOVA test. H_0 (null hypothesis): There is no difference in shoot survival among the three treatments (fresh, glycerol and PVS2). Thereafter, assumptions of normality and equality of variance was tested using 1-sample Kolmogorov-Smirnov test and a Levene's test respectively. These assumptions were satisfied ($P > 0.05$)

2.14.2 Comparing water properties

To determine if the three treatments had a magnitude effect on the enthalpies of melts, a linear regression was performed. H_0 : Cryoprotection has no effect on the enthalpies of melts. Assumptions of normality, Levene's test, independent of residuals, and no measure of error on the X variable (treatments) was tested. These assumptions were satisfied ($P > 0.05$). Furthermore, data were linear distributed ($R^2 > 0.8$, Figures 3.10; 3.14; 3.18 and 3.22) across all treatments.

Chapter 3: Results

This section describes the responses of embryonic axes from seeds and shoot tips excised from *in vitro* grown seedlings of *T. emetica* to pre-culture, cryoprotection, dehydration, cooling and rewarming in terms of viability assessments and calorimetric measurements. The assessments were performed at different hydration levels in an attempt to understand the behavior of water in these tissues and treatments during cryopreservation.

3.1 Response of embryonic axes with no cotyledonary segments attached, to cryoprotection and rapid drying, with or without pre-culture

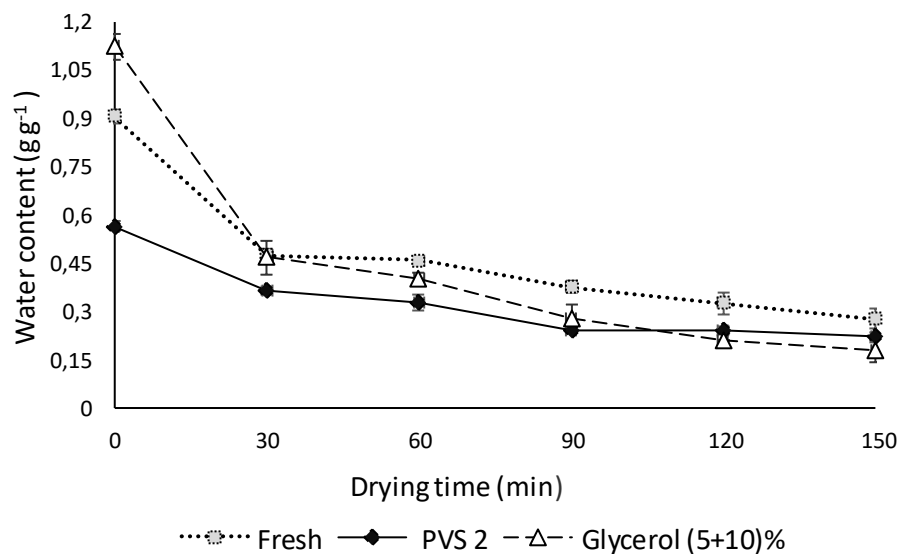


Figure 3.1: Water content (mean \pm SD) of rapidly dried, fresh (non-cryoprotected control) and cryoprotected *T. emetica* embryonic axes with no cotyledonary segments, without a 3 d pre-conditioning.

Water content is an important factor that affects successful cryopreservation of embryonic axes. Dehydration of embryonic axes (without cotyledonary segments attached) to a WC of approximately 0.45 g g⁻¹ DW is generally considered to be suitable for successful cryopreservation of embryonic axes from many recalcitrant

seeds. Prior to drying, results reveal that cryoprotectants had different effects in the embryonic axes with no cotyledonary segments attached (Figure 3.1). Embryonic axes with no cotyledonary segments attached that received no cryoprotective treatment (fresh) had an initial WC of $0.9 \pm 0.02 \text{ g g}^{-1}$, before rapid drying. Immersion of embryonic axes with no cotyledonary segments attached in 5% glycerol followed by 10% glycerol increased the WC to $1.1 \pm 0.02 \text{ g g}^{-1}$ prior to drying, while treatment with PVS2 significantly lowered the WC to $0.57 \pm 0.04 \text{ g g}^{-1}$). Furthermore, Figure 3.1. reveals that for all three treatments, rapid drying had a marked effect on the WC as a decline occurred with increasing drying time, and WCs of approximately 0.45 g g^{-1} was achieved within 90 min where WCs levelled out. This decline in WC corresponded to the loss of viability in embryonic axes with cotyledonary segments attached as observed in Figure 3.3.

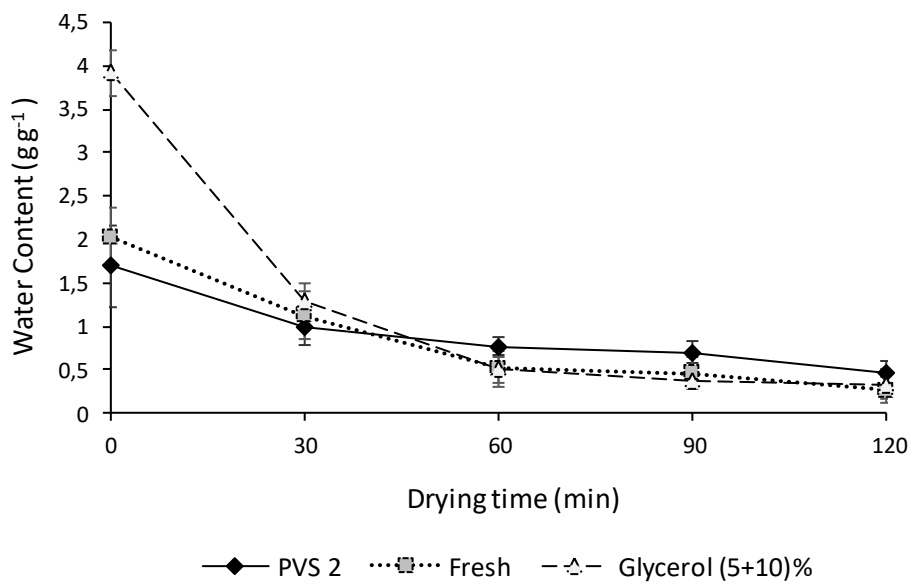


Figure 3.2: Water content (mean \pm SD) of rapidly dried, fresh (non-cryoprotected control) and cryoprotected *T. emetica* embryonic axes with no cotyledonary segments, where embryonic axes were pre-conditioned for 3 d in Murashige & Skoog medium (with 0.3 M sucrose + 0.5 M glycerol) prior to exposure to cryoprotectants and drying.

Pre-conditioning in MS medium with supplementary sucrose and glycerol has been shown to substantially improve cryo survival of germplasm, especially shoot tips. Pre-conditioning of embryonic axes with no cotyledonary segments for 3 d in culture medium increased WCs across all treatments prior to drying (Figure 3.2) far above that were observed in the absence a 3 d pre-conditioning treatment of embryonic axes with no cotyledonary segments attached (Figure 3.2). The lowest initial WC was observed for PVS2 treated axes (Figure 3.2) which still was higher than the highest WC observed for glycerol treated axes where there was no pre-conditioning (Figure 3.2). Similarly, as in the absence a 3 d pre-conditioning treatment (Figure 3.1), in these axes also, it was evident that treatment with cryoprotectants had a differing effect on the embryonic axes (with no cotyledonary segments), prior to drying (Figure 3.2). Across all treatments, 3 d pre-culture followed by cryoprotection resulted in WCs almost twice of those of non-precultured axes, probably due to the penetrative constituents of pre-conditioning medium such a glycerol. The initial WC of embryonic axes with no cotyledonary segments treated with no cryoprotectants was $2.03 \pm 0.73 \text{ g g}^{-1}$. Prior to drying, treatment with 5% glycerol followed by 10% glycerol significantly increased the WC within embryonic axes to $3.9 \pm 0.86 \text{ g g}^{-1}$), whereas immersion in PVS2 resulted in lower in embryonic axes to $1.7 \pm 0.47 \text{ g g}^{-1}$). A rapid dehydration pattern similar to that observed in the absence a 3 d pre-conditioning treatment (Figure 3.1), was also observed in pre-cultured and cryoprotected axes (Figure 3.2). Rapid drying had a marked effect on the WC as a decline was observed with increasing drying time, and WCs of approximately $< 0.5 \text{ g g}^{-1}$ DW was achieved within 120 minutes where WCs had levelled out across all treatments. Here also, a decline in WCs was accompanied by the loss of viability in embryonic axes with 1-2 mm cotyledonary segment attached (Figure 3.4).

3.2.1 Viability assessment of embryonic axes with 1-2mm cotyledonary segments attached, with or without pre-culture followed by cryoprotection, rapid drying and cooling

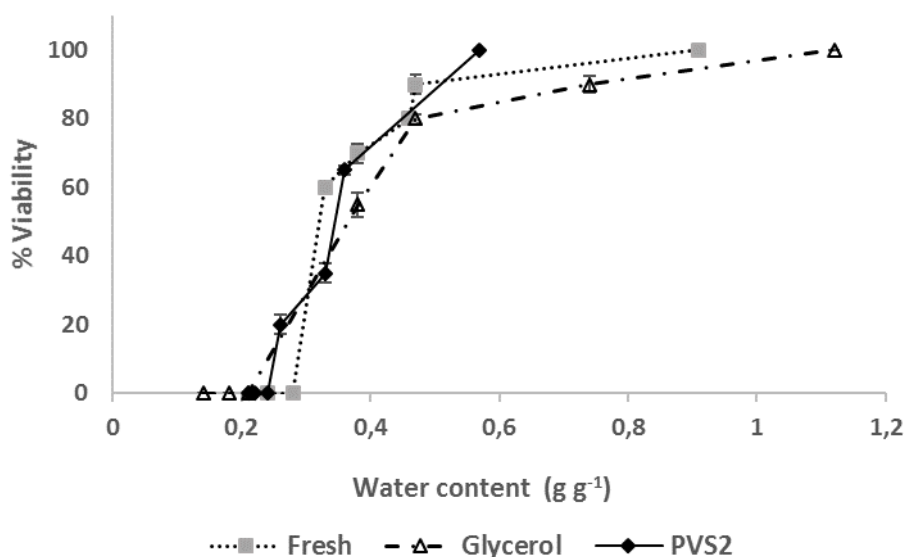


Figure 3.3: Viability (mean \pm SD) of rapidly dried, fresh (non-cryoprotected control) and cryoprotected *T. emetica* embryonic axes with 1-2 mm cotyledonary segments attached, in the absence of the pre-culture treatment.

Viability of cryoprotected embryonic axes with cotyledonary segments attached was monitored after each step of the dehydration process (Figure 3.3 and Figure 3.4). Viability data present here was based on shoot production (1 cm protrusion of shoot tip only (Figure 3.5)). In the absence of a 3 d pre-conditioning of embryonic axes, the shoot production prior to rapid drying remained high (100%) across all cryoprotection treatments (Figure 3.3). Dehydration down to WCs *c.* of 0.4 g g⁻¹ DW which is the suitable for successful cryopreservation of embryonic axes of many species, resulted in a sharp decrease in the viability of embryonic axes all treatments (Figure 3.3). There was no considerable variation in viability across the three treatments. Therefore, below this suitable WC, only PVS2 promoted retention of viability by embryonic axes with cotyledonary segments attached. High shoot production can be attributed to high WCs across all treatments. When embryonic axes were cryoprotected and dried to a water content of about 0.45 g g⁻¹, and then subjected to cryogenic conditions (rapidly cooled or slowly cooled), shoot production or survival was zero.

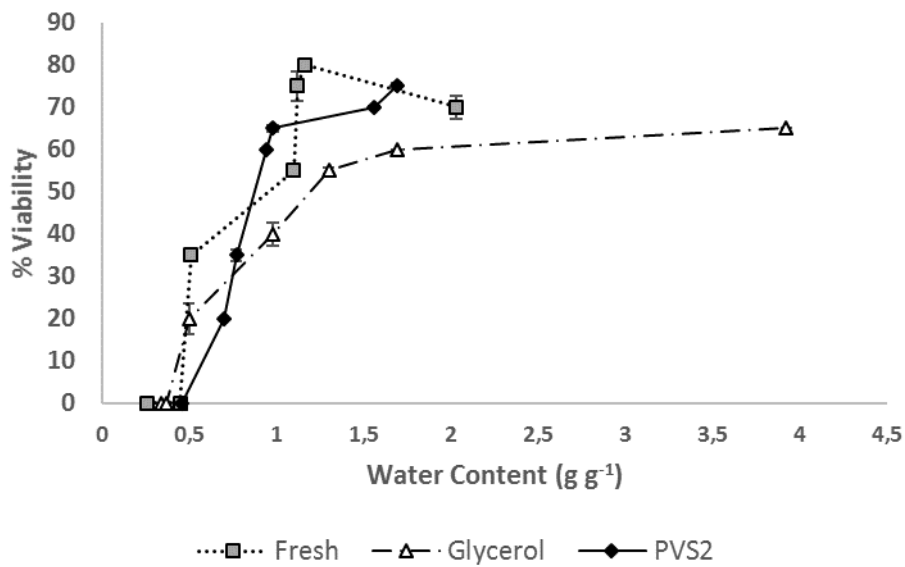


Figure 3.4: Viability (mean \pm SD) of rapidly dried, fresh (non-cryoprotected control) and cryoprotected *T. emetica* embryonic axes with 1-2 mm cotyledonary segments, pre-cultured for 3 d in Murashige & Skoog medium (0.3 M sucrose + 0.5 M glycerol).

Figure 3.4 shows that in embryonic axes with cotyledonary segments attached, which received that received a 3 d pre-conditioning, shoot production in cryoprotected axes prior to rapid drying remained high ($70 \pm 1.4\%$, $65 \pm 0.7\%$, $75 \pm 0.7\%$) in fresh, PVS2 and glycerol respectively. Similar to non-precultured axes (Figure 3.3), dehydration below $\pm 1 \text{ g g}^{-1}$ resulted in a decline in viability, and there was no marked variation in viability across the three treatments (Figure 3.4). Here too, cryoprotectants did not promote retention of viability of embryonic axes with cotyledonary segments attached when dried to lower WCs (approximately 0.4 g g^{-1} and below). Overall, pre-conditioning of embryonic axes (with cotyledonary segments attached) did not promote retention of viability, as survival across all treatments was lower than in axes where there was no pre-conditioning even prior to rapid drying. Moreover, a 3-day pre-conditioning, prior to cryoprotection and drying had no substantial improvement in cryopreservation of embryonic axes and irrespective of cooling rates, embryonic axes did not survive cryopreservation.

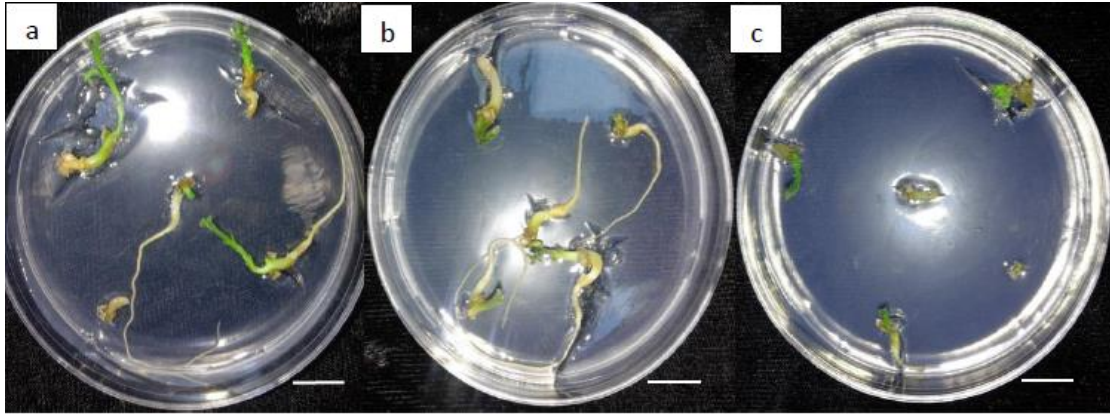


Figure 3.5: Viability of embryonic axes of *T. emetica* with cotyledonary segments attached: after 12 (a), 8 (b) and 4 (c) weeks before cryopreservation. Scale bar = 10 mm.

3.2.2 Viability assessment for shoot tips following cryoprotection and exposure to cryogenic conditions.



Figure 3.6: Survival of cryopreserved shoot tips of *T. emetica* following a 3 d pre-culture in, cryoprotection using PVS2 and cooled using slow cooling, 1 month (a), 2 months (b), and 4 months (c) into recovery. Scale bar = 2 mm

Table 3.1: Percentage survival of *in vitro* grown shoot tips of *T. emetica*, upon recovery on MS with 1 mg L⁻¹ BAP and 0.1 mg L⁻¹ IBA following retrieval from cryogenic conditions. SC = slow cooling, and RC = rapid cooling.

Treatments	Survival (%)					
	Fresh		Glycerol		PVS2	
<i>In vitro</i> grown shoot tips prior to cryopreservation	84		76		80	
	SC	RC	SC	RC	SC	RC
<i>In vitro</i> grown shoot tips after cryopreservation	0	0	0	0	68	0

Table 3.2: Water contents (mean \pm SD) of *in vitro* grown shoot tips of *T. emetica* exposed to pre-culture, cryoprotection, and subjected to two cooling rates. Water contents were measured after the cooling and rewarming on a DSC pan at 10 °C min⁻¹. WC = water content, SD = standard deviation

Rapid cooling	WC (Mean \pm SD)	Slow cooling	WC (Mean \pm SD)
3 d pre-culture	1.72 (\pm 0.93)	3 d pre-culture	1.62 (\pm 0.26)
glycerol (5%)	1.80 (\pm 0.21)	glycerol (5%)	1.87 (\pm 0.10)
glycerol (5+10)%	1.48 (\pm 0.25)	glycerol (5+10)%	2.10 (\pm 0.58)
PVS2 (50%)	2.70 (\pm 0.68)	PVS2 (50%)	2.04 (\pm 0.78)
PVS2 (50+100)%	2.03 (\pm 0.46)	PVS2 (50+100)%	1.40 (\pm 0.15)

Table 3.1 shows that viability of *in vitro* grown shoot tips of *T. emetica* remained high (\geq 76%) across all three treatments prior to cooling. Similar to the chemically protected embryonic axes mentioned above, *in vitro* grown shoot tips also did not survive cryopreservation with the exception of PVS2 treated shoot tips. A 3 d pre-conditioning of shoot tips, then immersion in 50% followed by 100% PVS2, and slowly cooled showed *c.* 68% of viable shoot tips upon their recovery. In contrast, rapid cooling of the same treatment showed no recovery from cryogenic conditions, regardless of treatment with PVS2. Both, fresh (control) and glycerol cryoprotected

shoot tips showed no recovery (Table 3.1), regardless of cooling rate, probably due to intracellular ice, as both pre-cultured and cryoprotected shoot tips were highly hydrated (Table 3.2). Therefore, survival from cryopreservation required both PVS2 treatment and a slow cooling method. Survival and development of PVS2 treated shoot tips (Figure 3.6) after retrieval from LN remained high when the WC was at $1.4 \pm 0.15 \text{ g g}^{-1}$ (Table 3.2).

3.3 Differential Scanning Calorimetry assessments of the properties of water in embryonic axis shoot segments and in vitro established shoots tips excised from in vitro cultured seedlings

Calorimetric measurements were performed for embryonic axis shoot segments and *in vitro* grown shoot tips of *T. emetica* at different hydration levels and at different stages of the cryopreservation protocol in an attempt to determine how dehydration and cryoprotectants influences the properties of water and subsequent survival.

3.3.1 Thermal analyses for assessing the effect of a slow cooling rate in embryonic axis shoot segments in the absence of a 3 d pre-conditioning.

Embryonic axis shoot segments of *T. emetica* were subjected to DSC measurements to determine the behavior of water, by looking at the melt enthalpy and melt temperature of water (Figures 3.7-3.22).

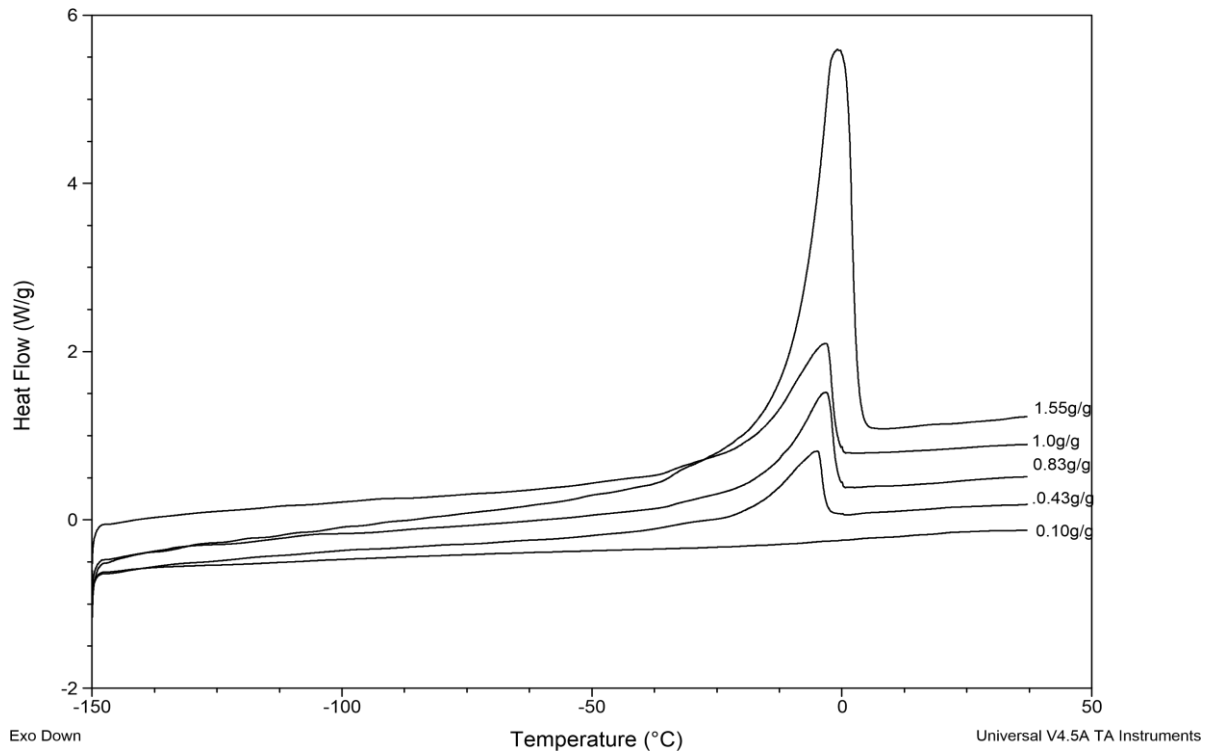


Figure 3.7: DSC endotherms of fresh embryonic axis shoot segments (no CPA) dried to various WCs (1.55 to 0.1 g g⁻¹), first, slowly cooled from 40 °C to -150 °C and warmed back to 40 °C at 10 °C min⁻¹, in absence of a 3 d pre-conditioning).

Figure 3.7 shows a composite of DSC warming thermograms (first order transitions) of embryonic axis shoot segments at the various WCs tested. The intake of heat by the embryonic axis shoot segments during heating can be unequivocally interpreted as a melting of some liquid component(s) of that had crystallised (-25 °C) during the cooling. Melt transitions were centered between approximately -35 °C (onset) and 10 °C, the prevalent event where the size and onset temperature of transition were dependent on the WC corresponded to the melting of ice (Vertucci, 1990). Melt transitions remained largest in undried embryonic axis shoot segment with a water content of 1.55 g g⁻¹. As the WC decreased due to drying, the magnitude of the transitions decreased signifying an increase in intracellular viscosity (Pammenter *et al.*, 1991). Additionally, as WC decreased, the onset of melt transitions slightly shifted to lower temperatures, which indicated that appearance of crystallisation transitions also shifted towards lower temperatures. Interesting, at a WC of 0.43 g g⁻¹ which falls within the range suitable for successful cryopreservation, embryonic axis shoot

segment of *T. emetica* still exhibited a broad melt transition ($31.25 \text{ J g}^{-1} \text{ DW}$). However, below this WC no melting transitions were observed, which indicated that there were no freezing events that had occurred during cooling.

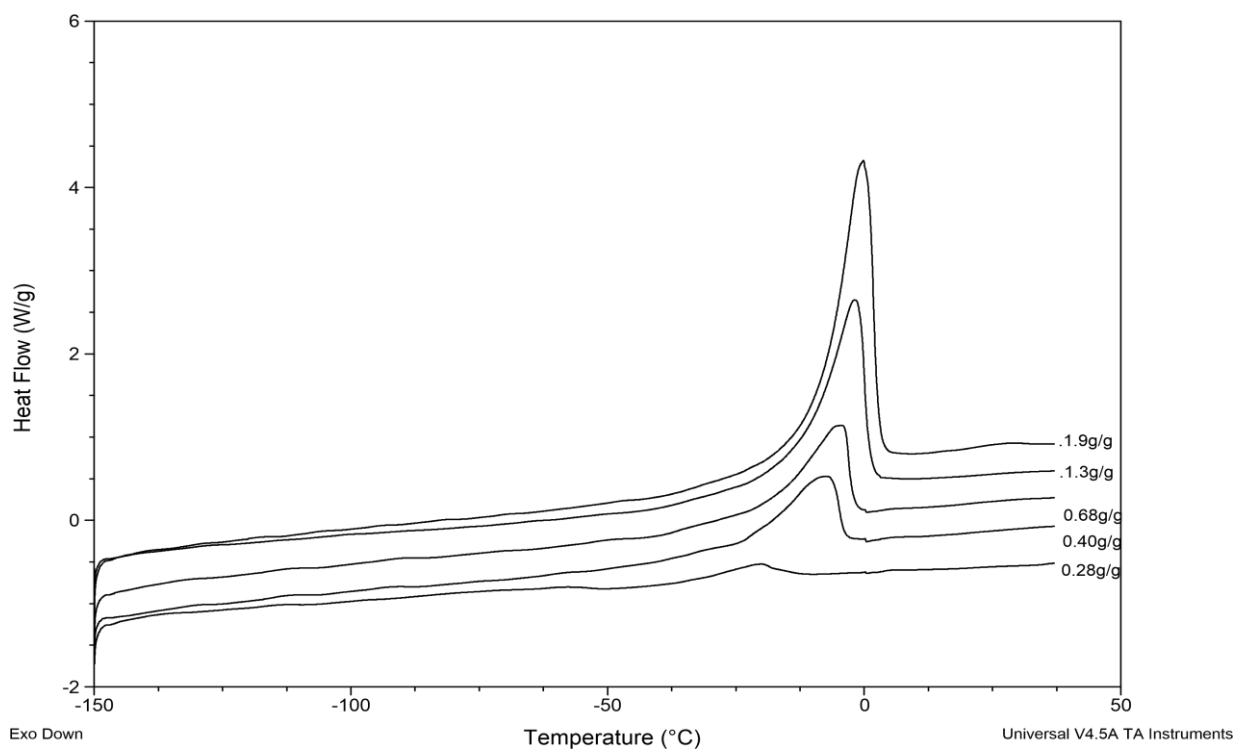


Figure 3.8: DSC endotherm scans of embryonic axis shoot segments, cryoprotected with glycerol (5% followed by 10%, for 1 h) and then dried to various WCs (1.9 to 0.28 g g^{-1}), slowly cooled from $40 \text{ }^\circ\text{C}$ to $-150 \text{ }^\circ\text{C}$ and then warmed back to $40 \text{ }^\circ\text{C}$ at $10 \text{ }^\circ\text{C min}^{-1}$, with no 3 d pre-conditioning).

Figure 3.8 shows an overlay of DSC scans (first order transitions) showing the warming thermograms for axes at the various WCs. A pattern similar to that of fresh embryonic axis shoot segments (Figure 3.7) was observed for melting endotherms in glycerol treated axes, except that changes were observable in onset temperatures. The intake of heat by the embryonic axis shoot segments during heating can be unequivocally interpreted as a melting of some liquid component(s) of that had crystallised (-35 to $-25 \text{ }^\circ\text{C}$) during cooling. With endothermic transitions centered between approximately $-45 \text{ }^\circ\text{C}$ (onset) and $10 \text{ }^\circ\text{C}$, the prevalent event where the size and onset temperature were dependent on the WC corresponded to the melting of ice. Here also, melt transition remained largest in undried embryonic axis shoot segment.

The magnitude of the transitions decreased with a decline in WC, probably increasing in intracellular viscosity with smaller peaks. A decline in WC of embryonic axis shoot segments, caused the melt transitions to slightly shift towards lower temperatures. Even at WC of 0.40 g g^{-1} ($15.41 \text{ J g}^{-1} \text{ DW}$) which falls within the suitable range for cryopreservation, a melting event is observed, simply signifying that intracellular water froze during cooling. There were no melting transitions observed at WC below 0.28 g g^{-1} , signifying that no freezing events were witnessed during cooling. Overall, when comparing with fresh (Figure 3.7), glycerol increased the WC of the embryonic axis shoot segments, thus resulting in the increase of the magnitude of melt transitions.

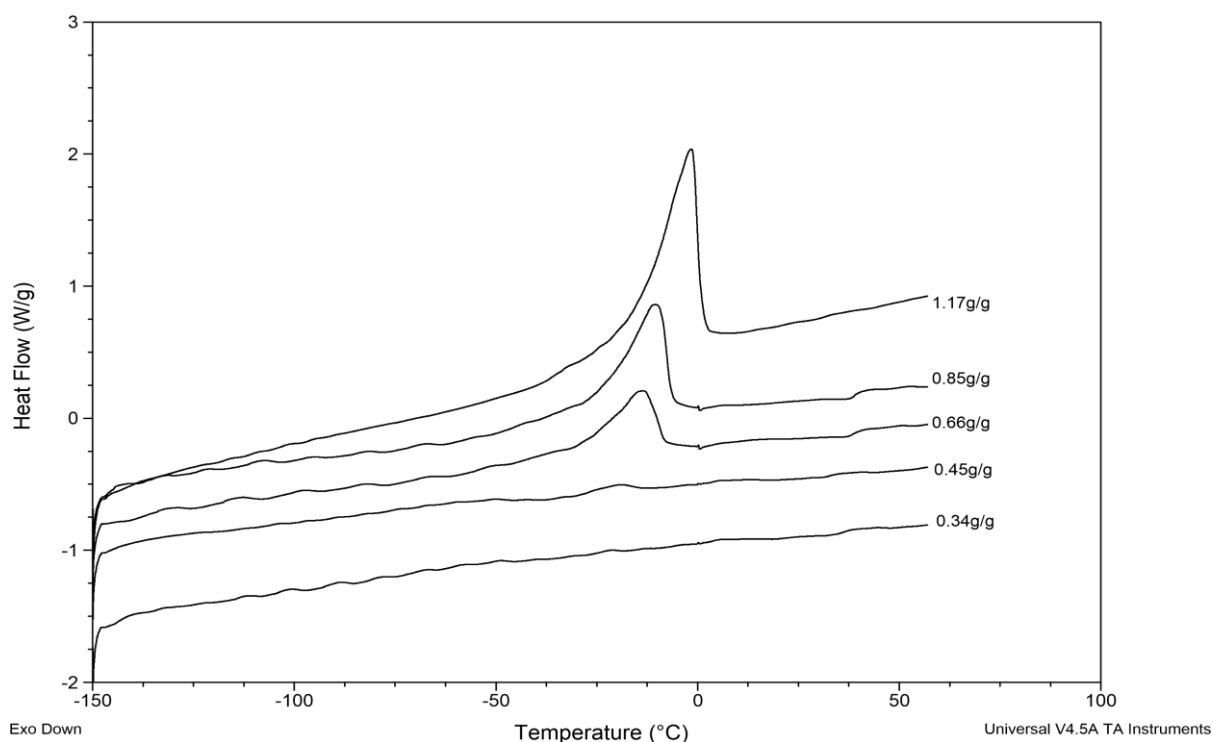


Figure 3.9: DSC endotherm scans of embryonic shoot axis segments cryoprotected with PVS 2 and then dried to various WCs (1.17 to 0.34 g g^{-1}). First, slowly cooled from $40 \text{ }^\circ\text{C}$ to $-150 \text{ }^\circ\text{C}$ and warmed back to $40 \text{ }^\circ\text{C}$ at $10 \text{ }^\circ\text{C min}^{-1}$, with no 3 d pre-conditioning).

Figure 3.9 shows an overview of DSC endotherms (first order transitions) for PVS2 treated embryonic axis shoot segments that were dried to the various WCs (1.17 to 0.34 g g^{-1}). A pattern similar to that of fresh and glycerol treated embryonic axis shoot

segments (Figures 3.7 and 3.8) was observed for melting endotherms in glycerol treated embryonic axis shoot segments, except that changes were observed in onset temperatures. Endothermic transitions centered between approximately -55 °C (onset) and 7.5 °C, which constituted the prevalent event where the size and onset temperature were dependent on the WC corresponded to the melting of ice. Here also, undried embryonic axis shoot segment exhibited the largest melt transition. Magnitude of the transitions decreased with a decline in WCs, tissue water content becoming more concentrated with smaller peaks. At a WC of 0.45 g g⁻¹ which falls within the suitable range for cryopreservation, a very small melting transition (8.54 J g⁻¹ DW) is observed (-10 °C to -40 °C) which indicated that a freezing event had occurred during cooling. Below this WC there were no melting transitions observed indicating that no crystallisation occurred during cooling. So, cryoprotection by PVS2 combined with dehydration promoted a shift of the melt transitions towards much lower temperatures compared with fresh and glycerol treated embryonic axis shoot segments. Overall, when comparing with fresh and glycerol treatment (Figures 3.7 and 3.8), PVS2 lowered the WC of the embryonic axis shoot segments, thus altering the properties of water that is able to undergo a freezing event.

To determine the point at which freezable water is no longer observed and the enthalpy associated with melting, changes in the energy of warming thermograms for embryonic axis shoot segments were measured as a function of WC (Figure 3.10). Estimates of the energy of the transition(s) were obtained through calculating the area of the thermogram transition as in Figures 3.7-3.9. Linear regressions of moisture content and enthalpy of melt were made to determine the best fit to the data.

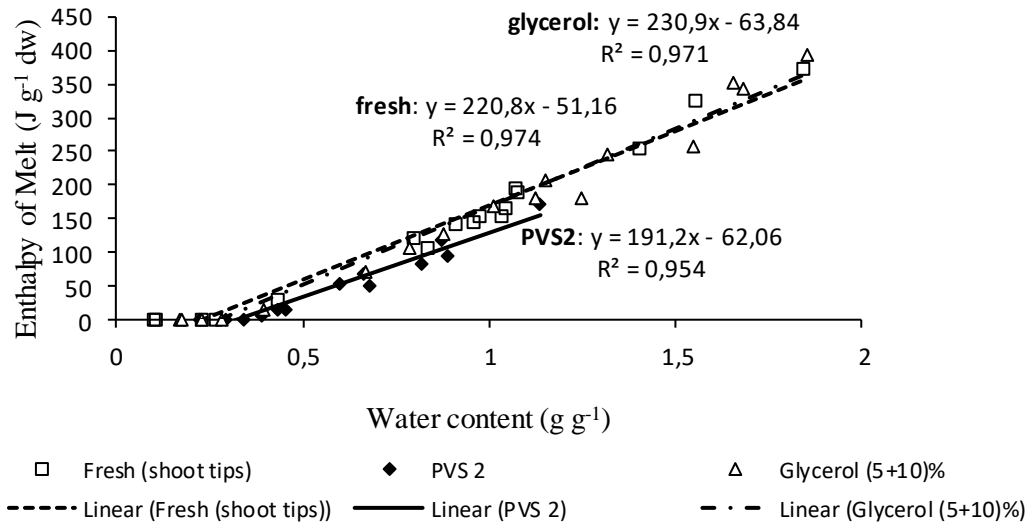


Figure 3.10: The relationship between WC and enthalpy of the melting transition of embryonic axis shoot segments following exposure to the different cryoprotectants and dehydration, and then slowly cooled from 40 °C to -150 °C and warmed back to 40 °C at 10 °C min⁻¹, in the absence of a 3 d pre-conditioning).

Figure 3.10 show the enthalpies of melt regressed against the water contents, to determine the amount of water that did not freeze and the heat fusion of the water that did freeze. Comparisons of the enthalpies as a function of WC for warming thermograms reveal linear relationships ($r^2 = 0.974, 0.971$ and 0.954) for fresh, glycerol and PVS2 treated embryonic axis shoot segments. Therefore, 97.4%, 97.1% and 95.4% (respectively), of all observed changes in the enthalpies of melt can be explained by the change in the WC of different treatments. The lines were distinguishable for the different treatments (enthalpies: 220.8, 230.9 and 191.2 J g⁻¹ DW, respectively for fresh, glycerol and PVS2). All enthalpies were below the enthalpy of pure water 333 J g⁻¹ H₂O, signifying that dehydration resulted in an increased intracellular viscosity in PVS2 treated embryonic axis shoot segments whilst glycerol slightly decreased intracellular viscosity. Fresh and glycerol treatments had almost similar slopes (and enthalpies), however, treatment with PVS2 caused a shift (increased) of the point at which freezable water is first observed. The WC at which water did not undergo a phase transition (unfreezable water) [x-intercept] was higher for PVS2 (0.34 g g⁻¹) followed by that of glycerol (0.21 g g⁻¹) and fresh (0.22 g g⁻¹) embryonic axis shoot segments.

3.3.2 Thermal analyses for assessing the effect of a direct plunge into LN in embryonic axis shoot segments in the absence of a 3 d pre-conditioning

The method of physical dehydration followed by cryoprotection and rapid cooling using LN has been successfully applied to cryopreserve of some recalcitrant seed tissues. This method is known to vitrify components of small tissues, therefore, limiting the formation of intracellular ice and dehydration damage.

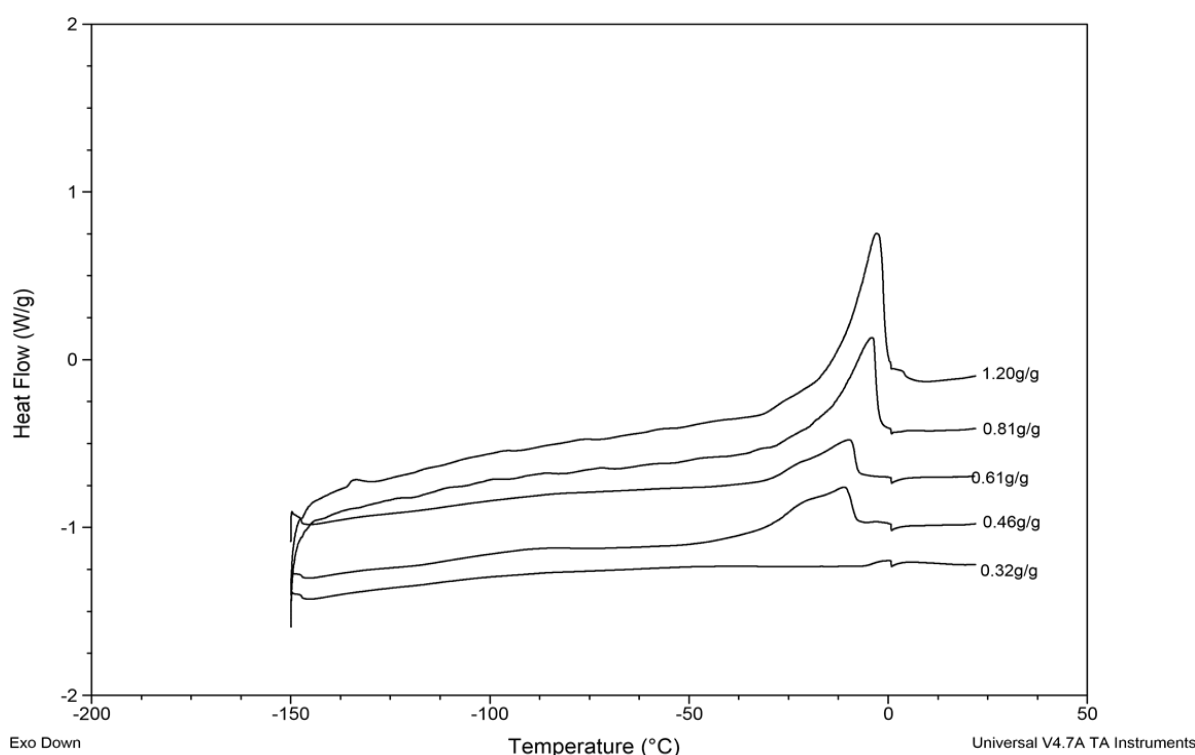


Figure 3.11: DSC endotherm scans of fresh embryonic axis shoot segments that have been dried to various WCs (1.20 to 0.32 g g⁻¹). Tissue was physically dehydrated and then rapidly cooled by direct plunge of into liquid nitrogen, and then loaded into the DSC sample holder pre-cooled to -150 °C, warmed to 25 °C at 10 °C min⁻¹, with no 3 d pre-conditioning).

The DSC thermograms obtained from the dehydrated and cryopreserved embryonic axis shoot segments also revealed one type of thermal transition (that of the melting of freezable water) when the frozen segments were heated from -150 °C to 25 °C (Figure 3.11). Generally, the melt onset decreased with increasingly dried embryonic axis

shoot segments. In addition, it was observed (Figure 3.11) that the enthalpy of melting of the frozen water was dependent upon the WC of the embryonic axis shoot segments. The non-dehydrated embryonic axis shoot segment gave the highest enthalpy ($287.3 \text{ J g}^{-1} \text{ DW}$) which decreased to $15.24 \text{ J g}^{-1} \text{ DW}$ when the WC decreased to 0.46 g g^{-1} . Additionally, the frozen water of both dehydrated and non-dehydrated embryonic axis shoot segments melted at temperatures lower than expected for pure water ($0 \text{ }^\circ\text{C}$). This may result probably due to possible effects of solutes known for their ability to depress melting point of frozen water. However, presence of solutes in non cryoprotected germplasm material is not expected to substantially change the enthalpy. Overall, rapid cooling of fresh axes slightly reduced the amount of water that can undergo a freezing transition.

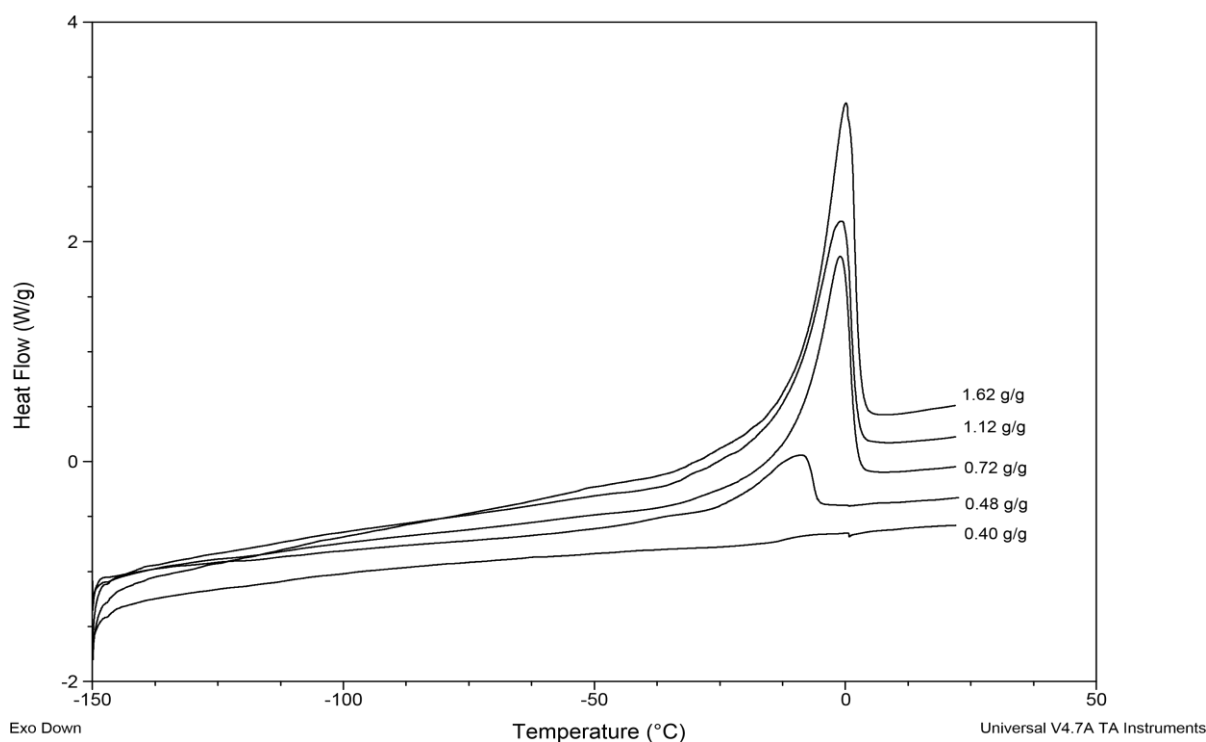


Figure 3.12: DSC endotherm scans of melting from embryonic axis shoot segments cryoprotected with glycerol, and then dried to various WCs (1.62 to 0.40 g g^{-1}), thereafter rapidly cooled by direct plunge into liquid nitrogen, and then loaded into the DSC sample holder pre-cooled to $-150 \text{ }^\circ\text{C}$, warmed to $25 \text{ }^\circ\text{C}$ at $10 \text{ }^\circ\text{C min}^{-1}$, with no 3 d pre-conditioning).

The DSC thermograms obtained from the dehydrated and cryopreserved embryonic axis shoot segments revealed one type of thermal transition (that of the melting of freezable water) when the frozen segments were heated from -150 °C to 25 °C (Figure 3.12). There was no considerable difference from embryonic axis shoot segments that received same treatment, and a slow cooling rate (Figure 3.8). In the like manner, the melt onset decreased with dried embryonic axis shoot segments, and the enthalpy melting of the frozen water was dependent upon the WC of the embryonic axes shoot segments. The non-dehydrated embryonic axis shoot segment gave the highest enthalpy (376.5 J g⁻¹ DW) which decreased to 98.8 J g⁻¹ DW when the WC decreased to 0.48 g g⁻¹. Additionally, the frozen water of both dehydrated and non-dehydrated embryonic axes shoot segments melted at temperatures lower than expected for pure water (0 °C). Overall, when comparing with non-cryoprotected (fresh) axis segments (Figure 3.11), glycerol increased the WC of the embryonic axis shoot segments, thus resulting in the increase of melt transitions. A rapid cooling rate had no significant effect in altering the properties of water in glycerol treated embryonic axis shoot segments.

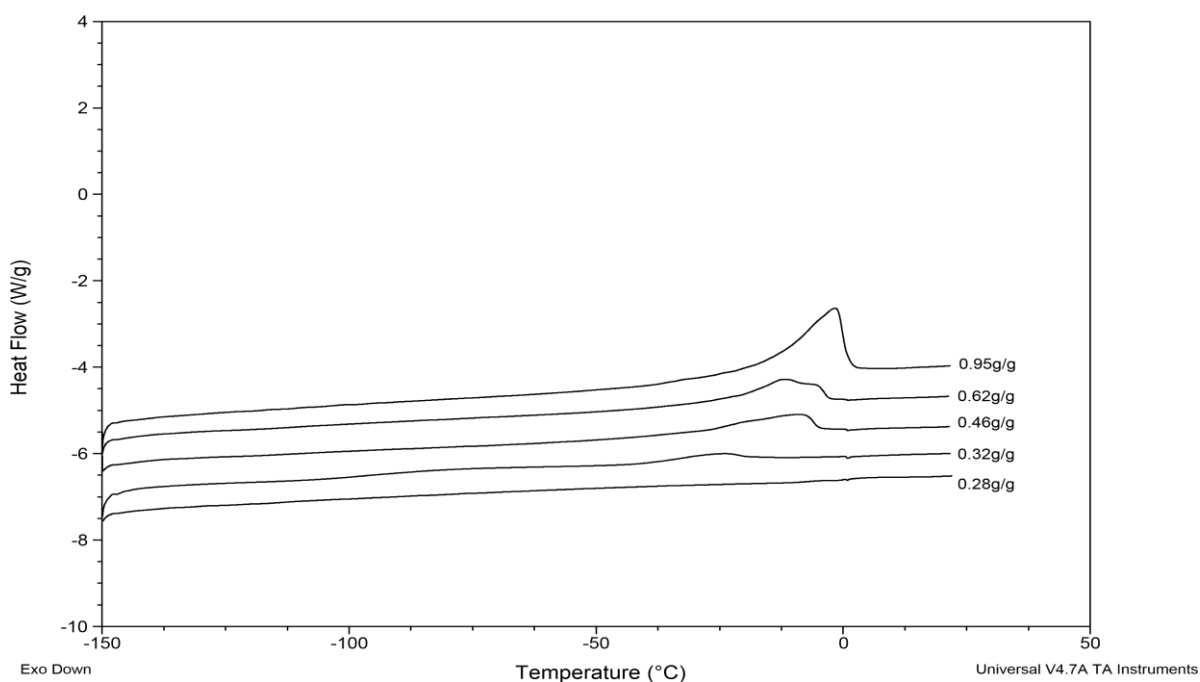


Figure 3.13: DSC endotherm scans of melting from embryonic axis shoot segments cryoprotected with PVS2, and then dried to various WCs (0.95 to 0.28 g g⁻¹). Thereafter rapidly cooled by direct plunge into liquid nitrogen, and then loaded into

the DSC sample holder pre-cooled to -150 °C, warmed to 25 °C at 10 °C min⁻¹, with no 3 d pre-conditioning).

Rapid cooling of embryonic axis shoot segments, considerably reduced the amount of water that is able to undergo a freezing transition. Figure 3.13 shows an overview of DSC endotherms (first order transitions) showing the warming thermograms for embryonic axis shoot segments at the various WCs. The melt onset decreased with dried embryonic axis shoot segments. Magnitude of the transition decreased with a decline in WC, and possibly increasing in intracellular viscosity with smaller peaks. At a WC of 0.32 g g⁻¹ which falls within the ideal range for cryopreservation, a very small melting event (8.45 J g⁻¹ DW) is observed (-45 °C to -30 °C) which indicated that a freezing event had occurred during cooling. Below this water content there were no melting transitions observed indicate no crystallisation occurred during cooling. So, cryoprotection by PVS2 coupled with rapid cooling promoted a shift of the melt transitions towards the lower temperatures. Overall, when comparing with fresh and glycerol treatments, PVS2 lowered the WC of the embryonic axis shoot segments, thus decreased the magnitude of melt transitions.

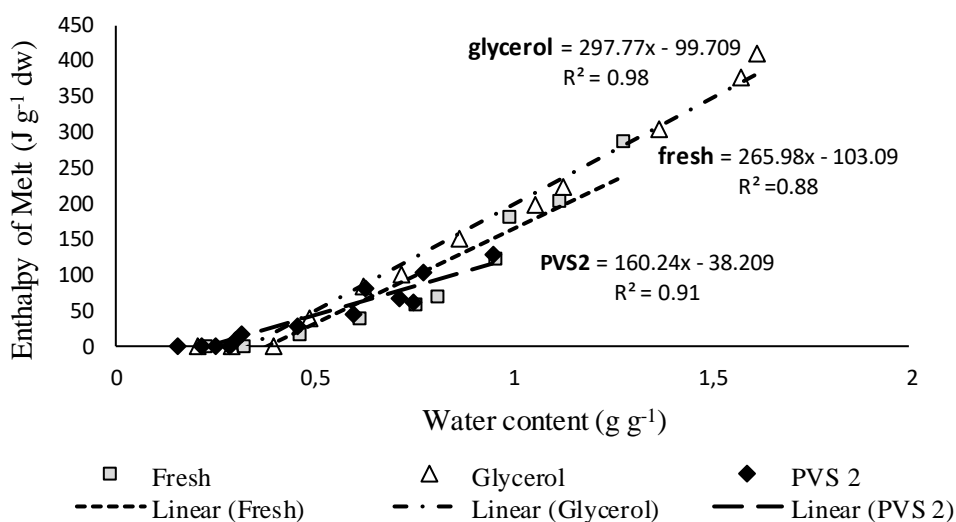


Figure 3.14: The relationship between WC and enthalpy of the melting transition of embryonic axis shoot segments following exposure to the different cryoprotectants and dehydration, rapidly cooled by direct plunge into liquid nitrogen, then loaded into

the DSC sample holder pre-cooled to -150 °C, warmed to 40 °C at 10 °C min⁻¹, with no pre-conditioning.

Figure 3.14 show the enthalpy of melt regressed against the WC, to determine the amount of water that did not freeze and the heat fusion of the water that did freeze. Comparisons of the enthalpies as a function of WC for warming thermograms display linear relationships ($r^2 = 0.88, 0.98$ and 0.91) for fresh treated, glycerol treated and PVS2 treated embryonic axis shoot segments. Therefore 88%, 98% and 91% (respectively), of all observed changes in the enthalpies of melt can be explained by the change in the WC of different treatments. The lines were distinguishable for the different treatments (enthalpies: 265.98, 297.77 and 160.24 J g⁻¹ DW, respectively for fresh, glycerol and PVS2). All enthalpies were below the enthalpy of pure water 333 J g⁻¹ H₂O, signifying that dehydration resulted in increased intracellular viscosity, especially in PVS2 treated axes. The WC at which water did not undergo a phase transition (unfreezable water) was higher for fresh embryonic axis shoot segment (0.40 g g⁻¹) followed by glycerol (0.32 g g⁻¹) and lowest for PVS2 (0.22 g g⁻¹). Therefore, lower point of unfreezable water especially in PVS2 treated embryonic axis shoot segments was a result a rapid cooling process.

3.3.3 Thermal analyses for assessing the effect of a slow cooling where embryonic axis shoot segments were pre-conditioned for 3 d

The method of pre-conditioning explants in Murashige & Skoog medium (0.3 M sucrose + 0.5 M glycerol) has been successfully applied to cryopreservation of some recalcitrant seed tissues such as shoot tips and embryonic axes. This method is known to have led to substantial improvement in survival following retrieval from cryogenic conditions.

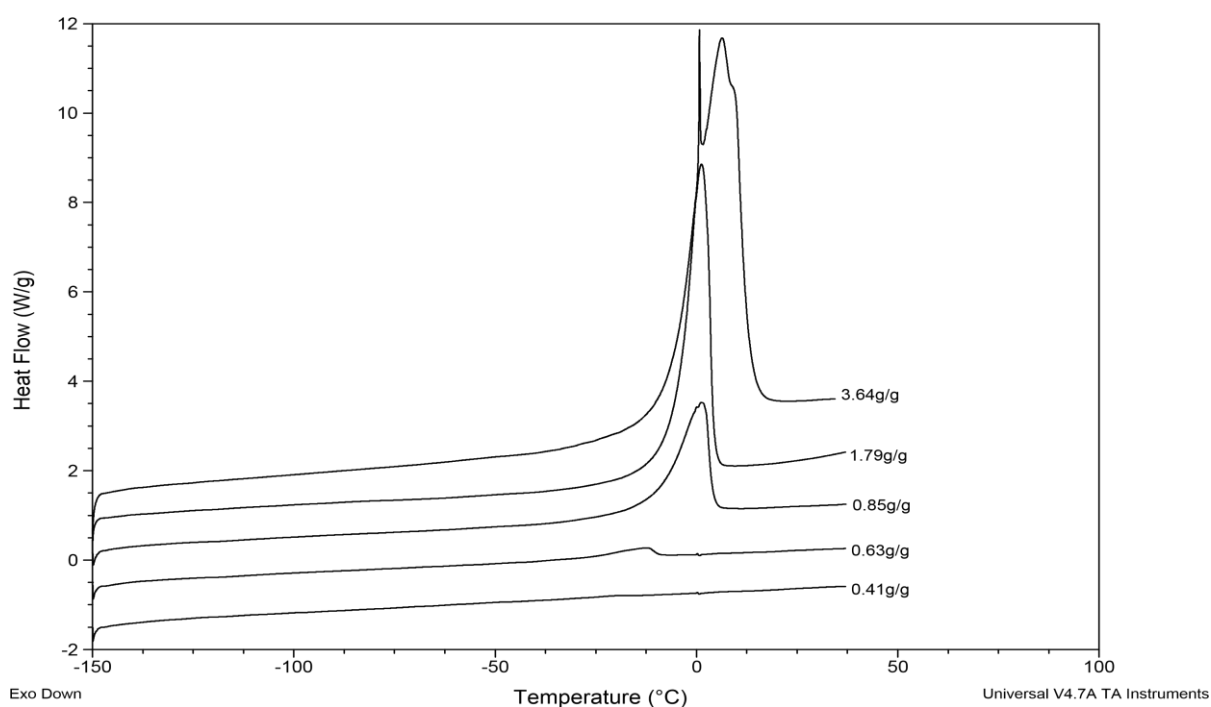


Figure 3.15: DSC endotherms of fresh embryonic axis shoot segments (no CPA) that have been dried to various WCs (3.64 to 0.41 g g⁻¹), first, slowly cooled from 40 °C to -150 °C and warmed back to 40 °C at 10 °C min⁻¹, with 3 d pre-conditioning).

The DSC thermograms obtained from the dehydrated and cryopreserved embryonic axis shoot segments revealed one type of thermal transition (that of the melting of freezable water) when the frozen segments were heated from -150 °C to 25 °C (Figure 3.15). Generally, the melt onset decreased with increasingly dried embryonic axis shoot segments. It was observed (Figure 3.15) that the enthalpy of melt for the frozen water was dependent upon the WC of the embryonic axis shoot segments. The non-dehydrated embryonic axis shoot segment gave the highest enthalpy (912.8 J g⁻¹ DW) which decreased to 58.8 J g⁻¹ DW when the WC decreased to 0.63 g g⁻¹. A different type of melting transition (sharp peak) was observed at about 0 °C, which signifies presence of pure water (Vertucci, 1990). Additionally, the frozen water of both non-dehydrated embryonic axis shoot segment melted at temperatures higher than expected for pure water (0 °C). Overall, pre-conditioning of fresh embryonic axis shoot segments increased considerably the amount of water that can undergo a freezing transition.

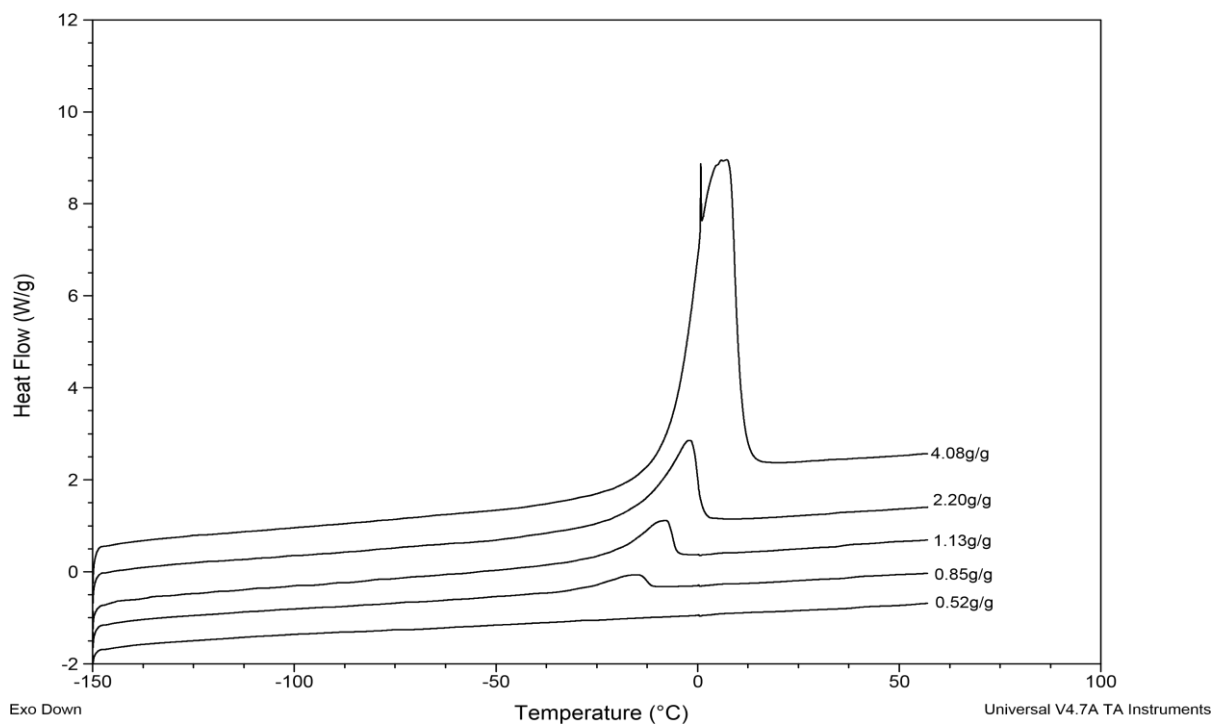


Figure 3.16: DSC endotherm scans of embryonic axis shoot segments, cryoprotected with glycerol (5% followed by 10%, for 1 h) and then dried to various WCs (4.08 to 0.52 g g⁻¹), slowly cooled from 40 °C to -150 °C and then warmed back to 40 °C at 10 °C min⁻¹, with 3 d pre-conditioning).

The DSC thermograms obtained from the dehydrated and cryopreserved embryonic axis shoot segments revealed two types of thermal transition (that of the melting of freezable water) when the frozen segments were heated from -150 °C to 25 °C (Figure 3.16). Generally, the melt onset decreased with increasingly dried embryonic axis shoot segments. It was observed (Figure 3.16) that the enthalpy of melt for the frozen water was dependent upon the WC of the embryonic axis shoot segments. The non-dehydrated embryonic axis shoot segment gave the highest enthalpy (1039 J g⁻¹ DW) which decreased to 73.5 J g⁻¹ DW when the WC decreased to 0.85 g g⁻¹. A sharp peak was observed at about 0 °C, which signifies presence of pure water. Additionally, the frozen water of dehydrated embryonic axis shoot segments melted at temperatures lower than expected for pure water (0 °C). Similarly, to the fresh treatment, pre-conditioning followed by glycerol treatment led to an increase in WC of undried embryonic axis shoot segment (Figure 3.16). The point at which unfreezable water is first observed was increased, above that observed in the absence of pre-conditioning

(Figures 3.10 and 3.14). Overall, pre-conditioning of glycerol treated embryonic axis shoot segments increased considerably the amount of water that can undergo a freezing transition.

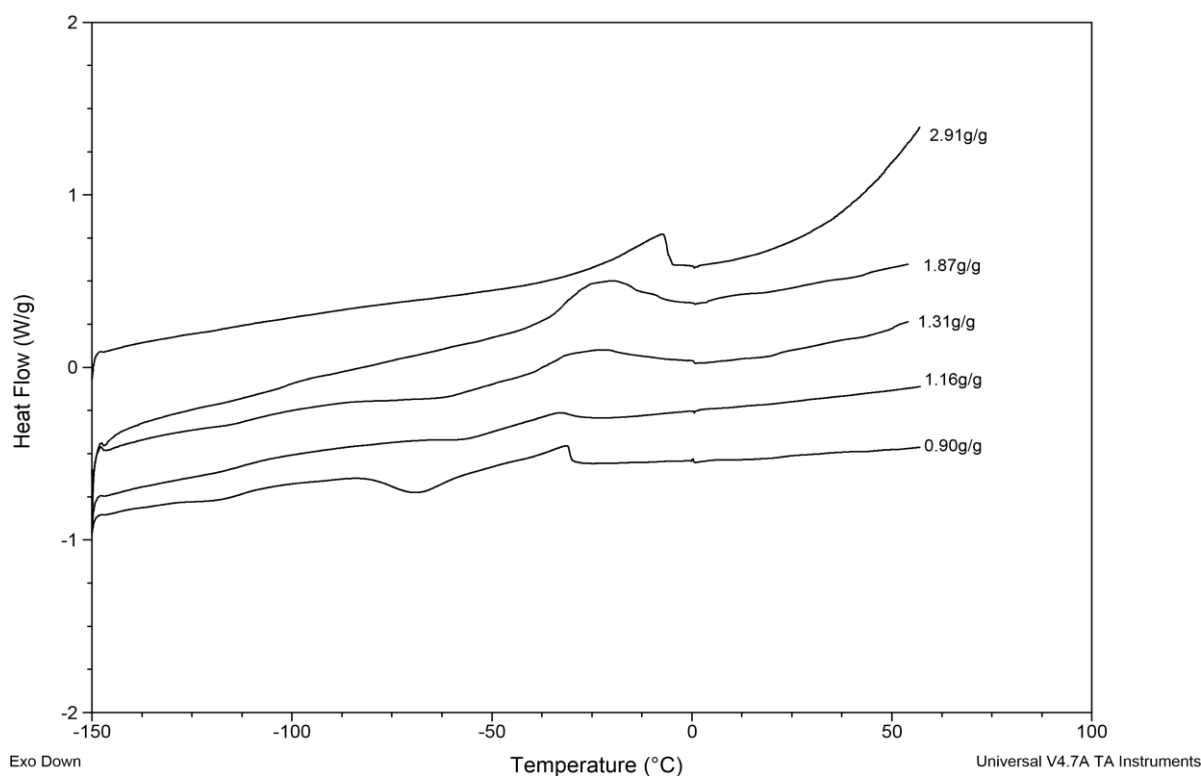


Figure 3.17: DSC endotherm scans of embryonic axis shoot segments, cryoprotected with PVS2 and then dried to various WCs (2.91 to 0.90 g g⁻¹), slowly cooled from 40 °C to -150 °C and then warmed back to 40 °C at 10 °C min⁻¹, with 3 d pre-conditioning).

The DSC thermograms obtained from the dehydrated and cryopreserved embryonic axis shoot segments treated with PVS2 revealed two types of thermal transitions: a glass transition and the melting of freezable water when the frozen segments were heated from -150 °C to 25 °C (Figure 3.17). A 3 d pre-conditioning coupled with slow cooling greatly reduced the freezing transition, though WC remained high in embryonic axis shoot segments. Generally, the melt onset decreased with increasingly dried embryonic axis shoot segments. It was observed (Figure 3.17) that the enthalpy of melt for the frozen water was dependent upon the WC of the embryonic axis shoot segments. The non-dehydrated embryonic axis shoot segment gave the highest enthalpy (540 J g⁻¹ DW) which decreased to 30.9 J g⁻¹ DW when the WC decreased to

1.16 g g⁻¹. A glass transition (step) was observed at about 100 °C signifies a devitrification event. Additionally, the frozen water of dehydrated embryonic axis shoot segments melted at temperatures lower than expected for pure water (0 °C). Plant vitrification solution 2 increased the point at which unfrozen water is first observed, above that observed in the absence of pre-conditioning (Figures 3.10 and 3.14), no any other treatment. Overall, pre-conditioning of PVS2 treated embryonic axis shoot segments considerably quenched the amount of water that can undergo a freezing transition.

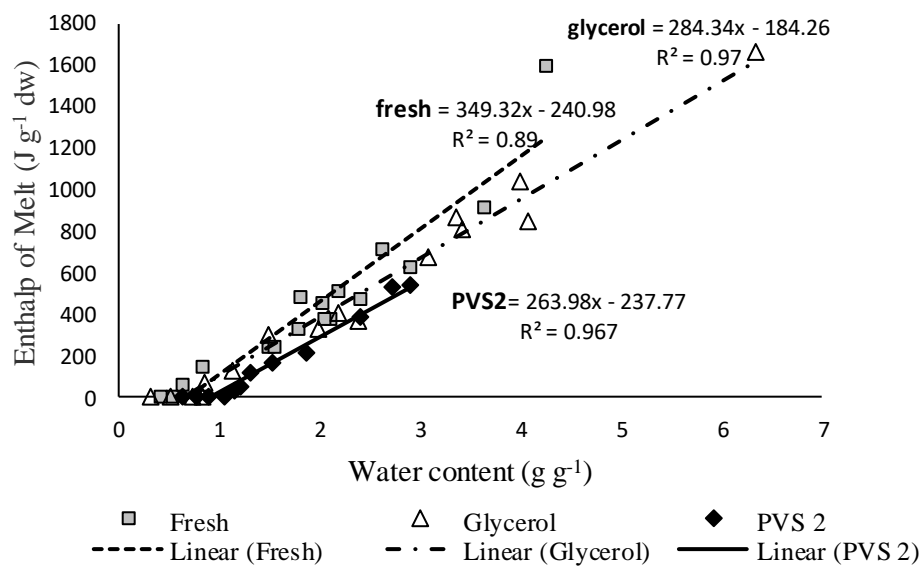


Figure 3.18: The relationship between WC and enthalpy of the melting transition of embryonic axis shoot segments following exposure to the different cryoprotectants and dehydration, and then slowly cooled from 40 °C to -150 °C and warmed back to 40 °C at 10 °C min⁻¹, pre-conditioned for 3d).

Figure 3.18 show the enthalpy of melt regressed against the WC, to determine the amount of water that did not freeze and the heat of fusion of the water that did freeze. Comparisons of the enthalpies as a function of WC for warming thermograms reveal linear relationships ($r^2 = 0.899, 0.979$ and 0.967 for fresh treated, glycerol treated and PVS2 treated embryonic axis shoot segments. Therefore 89.9%, 97.9% and 96.7% (respectively), of all observed changes in the enthalpies of melt can be explained by the change in the WC of different treatments. The lines were distinguishable for the different treatments (enthalpies: 349.32, 284.34 and 263.98 J g⁻¹ DW, respectively for

fresh, glycerol and PVS2). It was observed that the enthalpy of melt for fresh embryonic shoot segments ($349.32 \text{ J g}^{-1} \text{ DW}$) was above the enthalpy of pure water ($333 \text{ J g}^{-1} \text{ H}_2\text{O}$), which signified that pre-conditioning resulted in an increase of intracellular viscosity. The enthalpy of melt for glycerol treated embryonic axis shoot segments was below the enthalpy of pure water ($333 \text{ J g}^{-1} \text{ H}_2\text{O}$), signifying that pre-conditioning and dehydration resulted in a decreased intracellular viscosity, with PVS2 having the lowest enthalpy of melt. Therefore, all treatments had different slopes, however treatment with PVS2 caused a shift of the point at which non-freezable water is first observed, which was far higher than in the absence of pre-culture. The WC at which water did not undergo a phase transition (unfreezable water) was higher for PVS2 (1.06 g g^{-1}) followed by fresh and glycerol which had the same point of intercept (0.63 g g^{-1}).

3.3.4 Thermal analyses for assessing the effect of a direct plunge of embryonic axis shoot segments into LN after receiving a 3 d pre-conditioning

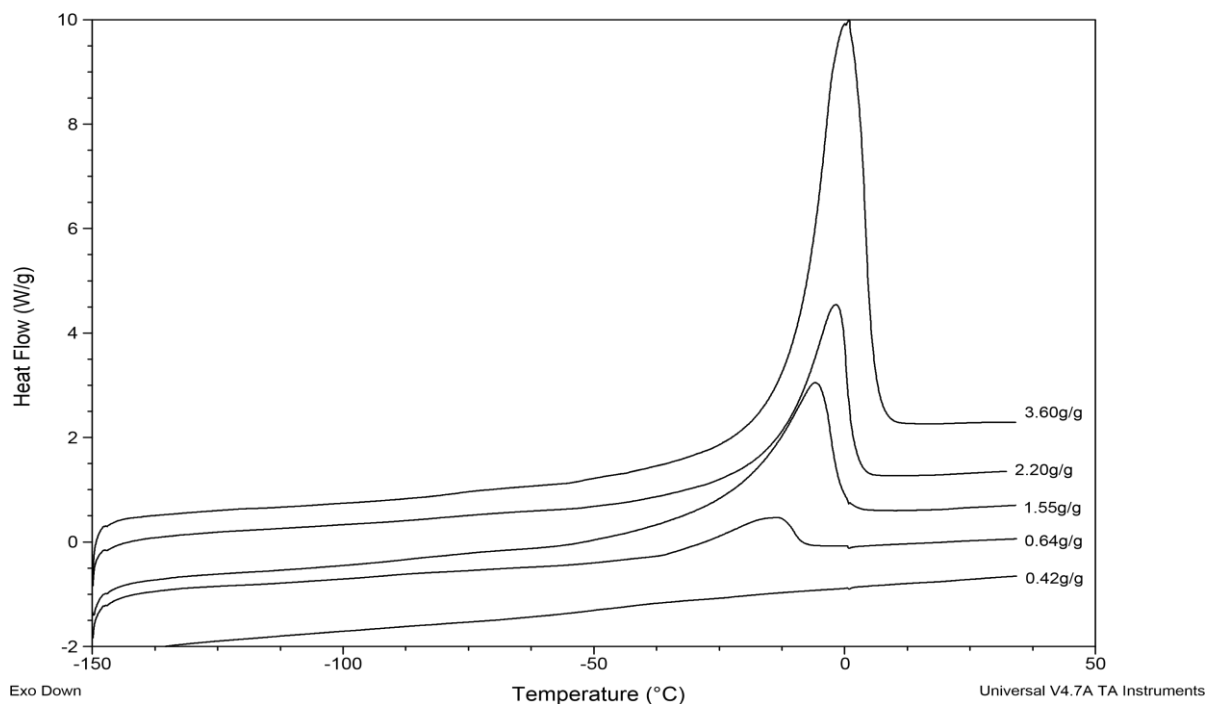


Figure 3.19: DSC endotherm scans of fresh embryonic axis shoot segments that had been dried to various WCs (3.60 to 0.42 g g^{-1}). Rapidly cooled by direct plunge into liquid nitrogen, and then loaded into the DSC sample holder pre-cooled to $-150 \text{ }^\circ\text{C}$, warmed to $25 \text{ }^\circ\text{C}$ at $10 \text{ }^\circ\text{C min}^{-1}$, after receiving a 3 d pre-conditioning).

A 3 d pre-conditioning followed by rapid cooling slightly reduced the magnitude of freezing transitions in dried embryonic axis shoot segments. The DSC thermograms obtained from the dehydrated and cryopreserved embryonic axis shoot segments revealed one type of thermal transition (that of the melting of freezable water) when the frozen embryonic axis shoot segments were heated from -150 °C to 25 °C (Figure 3.19). Generally, the melt onset decreased with increasingly dried embryonic axis shoot segments. It was observed (Figure 3.19) that the enthalpy of melt for the frozen water was dependent upon the WC of the embryonic axis shoot segments. The non-dehydrated embryonic axis shoot segment gave the highest enthalpy (823.6 J g⁻¹ DW) which decreased to 58 J g⁻¹ DW when the WC decreased to 0.64 g g⁻¹. No sharp peak was observed at about 0 °C, contrary to that observed in slow cooling procedure. Overall, rapid drying following pre-conditioning of fresh embryonic axis shoot segments did not substantially reduce the amount of water that can undergo a freezing transition, or cause an increase in the point at which unfreezable water is first observed in embryonic axis shoot segments in comparison with slow cooling.

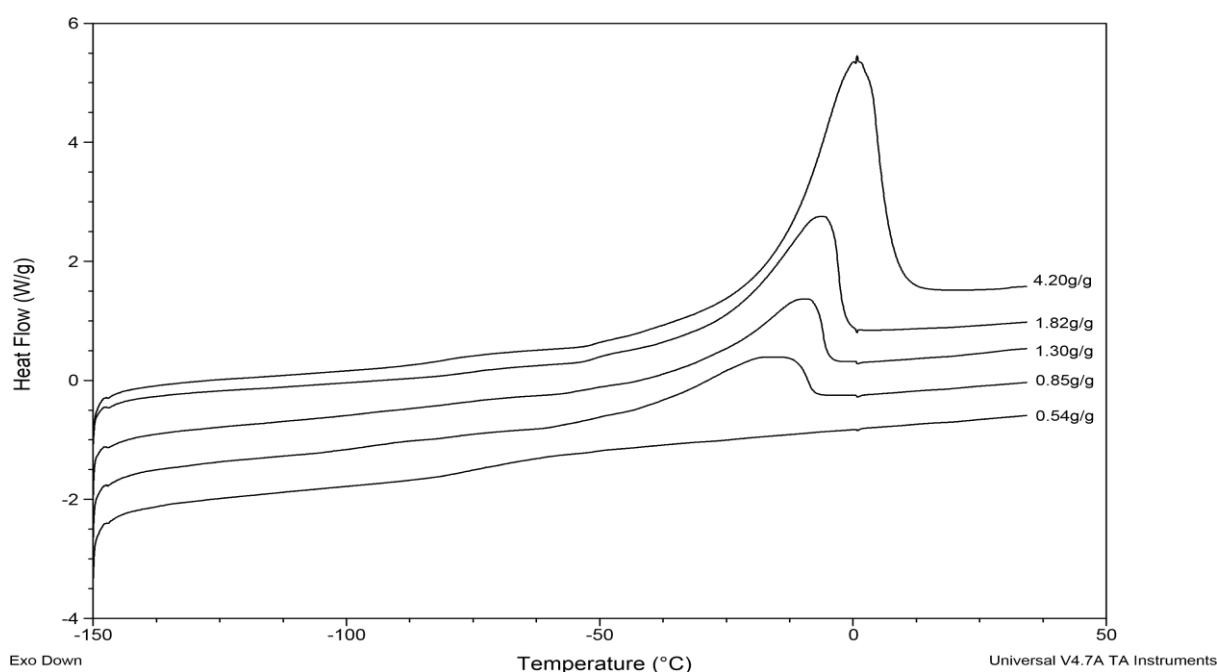


Figure 3.20: DSC endotherm scans of embryonic axis shoot segments cryoprotected with glycerol, and then dried to various water contents (4.2 to 0.52 g g⁻¹). Thereafter, rapidly cooled by direct plunge into liquid nitrogen, and then loaded into the DSC

sample holder pre-cooled to $-150\text{ }^{\circ}\text{C}$, warmed to $25\text{ }^{\circ}\text{C}$ at $10\text{ }^{\circ}\text{C min}^{-1}$, after receiving a 3 d pre-conditioning).

Here also, a 3 d pre-conditioning coupled with rapid cooling slight reduced the magnitude of the freezing transition in dried embryonic axis shoot segments. The DSC thermograms obtained from the dehydrated and cryopreserved embryonic axis shoot segments revealed one type of thermal transition (that of the melting of freezable water) when the frozen segments were heated from $-150\text{ }^{\circ}\text{C}$ to $25\text{ }^{\circ}\text{C}$ (Figure 3.20). Generally, the melt onset decreased with increasingly dried embryonic axis shoot segments. It was observed (Figure 3.20) that the enthalpy of melt for the frozen water was dependent upon the WC of the embryonic axis shoot segments. The non-dehydrated embryonic axis shoot segment gave the highest enthalpy ($1114\text{ J g}^{-1}\text{ DW}$) which decreased to $89.93\text{ J g}^{-1}\text{ DW}$ when the WC decreased to 0.85 g g^{-1} . Additionally, the frozen water of dehydrated embryonic axis shoot segments melted at temperatures lower than expected for pure water ($0\text{ }^{\circ}\text{C}$). Overall, rapid drying following pre-conditioning of glycerol treated embryonic axes shoot segments did not reduce significantly the amount of water that can undergo a freezing transition, or cause an increase in the point at which unfreezable water is first observed in embryonic axis shoot segments in comparison with slow cooling.

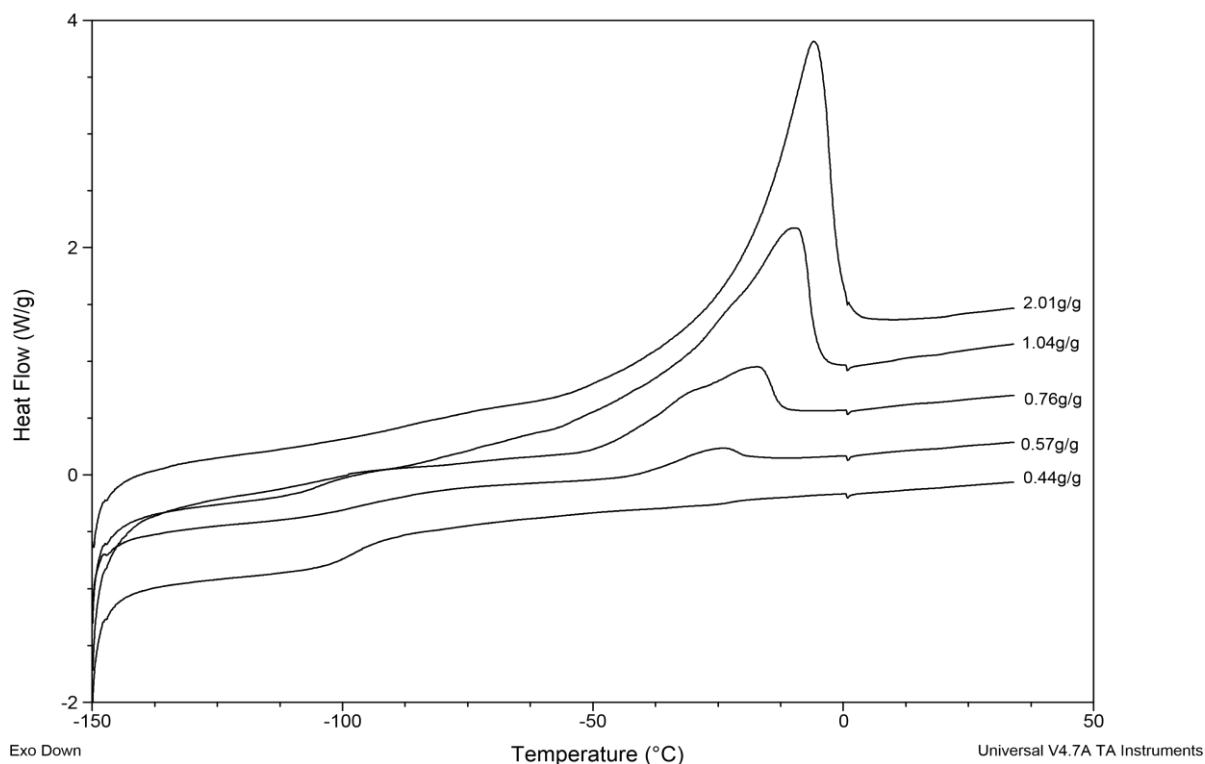


Figure 3.21: DSC endotherm scans of embryonic axis shoot segments cryoprotected with PVS2, and then dried to various WCs (2.01 to 0.44 g g⁻¹). Thereafter, rapidly cooled by direct plunge into liquid nitrogen, and then loaded into the DSC sample holder pre-cooled to -150 °C, warmed to 25 °C at 10 °C min⁻¹, with no 3 d pre-conditioning).

The DSC thermograms obtained from the dehydrated and cryopreserved embryonic axis shoot segments revealed two types of thermal transition (glass transition and that of the melting of freezable water) when the frozen segments were heated from -150 °C to 25 °C (Figure 3.21). A 3 d pre-conditioning coupled followed by rapid cooling greatly reduced the magnitude of the freezing transitions, though WC remained high in axes. It was observed (Figure 3.21) that the melt onset and enthalpy of melt for frozen water was dependent upon the WC of the embryonic axis shoot segment. The non-dehydrated embryonic axis shoot segment gave the highest enthalpy (84.34 J g⁻¹ DW) which decreased to 6.63 J g⁻¹ DW when the WC decreased to 0.57 g g⁻¹. A glass transition was observed at about -98 °C signifies a de-vitrification event in embryonic axis shoot segment at 0.44 g g⁻¹, which is less prominent than that observed for shoot tips of *T. emetica* (Figures 3.23-3.25). Additionally, the frozen water of dehydrated embryonic axis shoot segments melted at temperatures lower than expected for pure

water (0 °C). Overall, pre-conditioning of PVS2 treated embryonic axis shoot segment considerable reduced the amount of water that can undergo a freezing transition. Formation of a glass transition was promoted, contrary to slow cooling procedure.

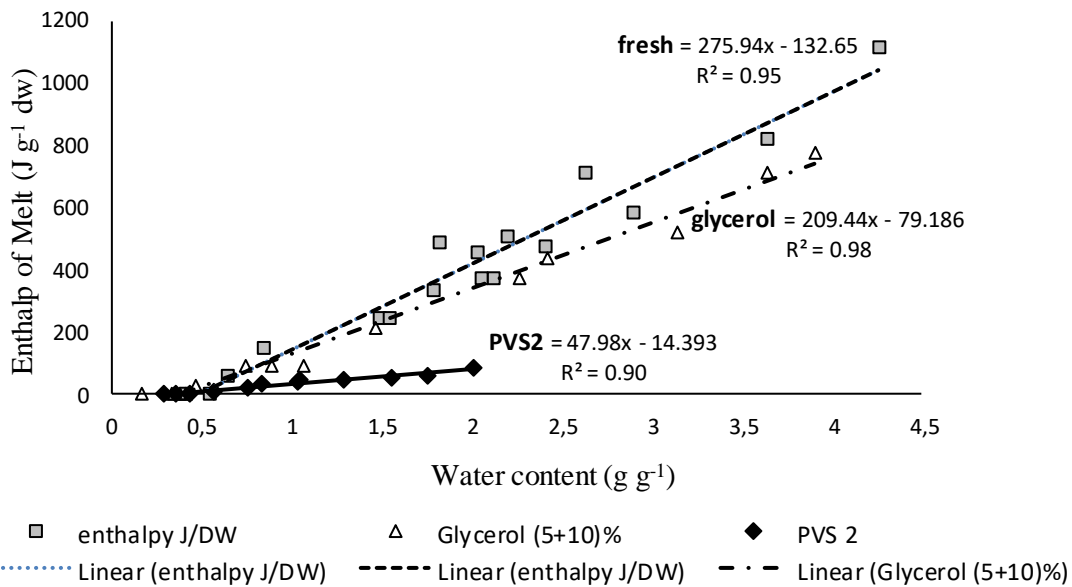


Figure 3.22: The relationship between WC and enthalpy of the melting transition of embryonic axis shoot segment following exposure to the different cryoprotectants and dehydration, rapidly cooled by direct plunge into liquid nitrogen, then loaded into the DSC sample holder pre-cooled to -150 °C, warmed to 40 °C at 10 °C min⁻¹, pre-conditioned for 3d).

Figure 3.22 show the enthalpy of melt regressed against the water content, to determine the amount of water that did not freeze and the heat of fusion of the water that did freeze. The relationships were linear ($r^2 = 0.95, 0.98$ and 0.90 for fresh treated, glycerol treated and PVS2 treated embryonic axis shoot segments. Therefore 95%, 98% and 90% (respectively for fresh, glycerol and PVS2), of all observed changes in the enthalpies of melt can be explained by the change in the WC of different treatments. The regression lines were distinguishable for the different treatments (enthalpies: 275.94, 209.44 and 47.98 J g⁻¹ DW, respectively for fresh, glycerol and PVS2). All enthalpies were below the enthalpy of pure water 333 J g⁻¹ H₂O, signifying that dehydration resulted in increased intracellular viscosity,

especially in PVS2 treated segments where a drastic change was observed. Fresh and glycerol had greater slopes, however treatment with PVS2 caused a shift of the point at which freezable water is first observed. The WC at which water did not undergo a phase transition (unfreezable water [x-intercept]) was higher for PVS2 treated embryonic axis shoot segments (0.57 g g^{-1}) followed by fresh (0.54 g g^{-1}) and lowest for glycerol (0.41 g g^{-1}), however slightly lower than those observed with slow cooling procedure (Figure 3.18). Therefore, across all treatment a slight reduction of point of unfreezable water was a consequence of rapid cooling.

3.3.5 DSC assessments of *in vitro* established shoot tips after various treatments of cryopreservation

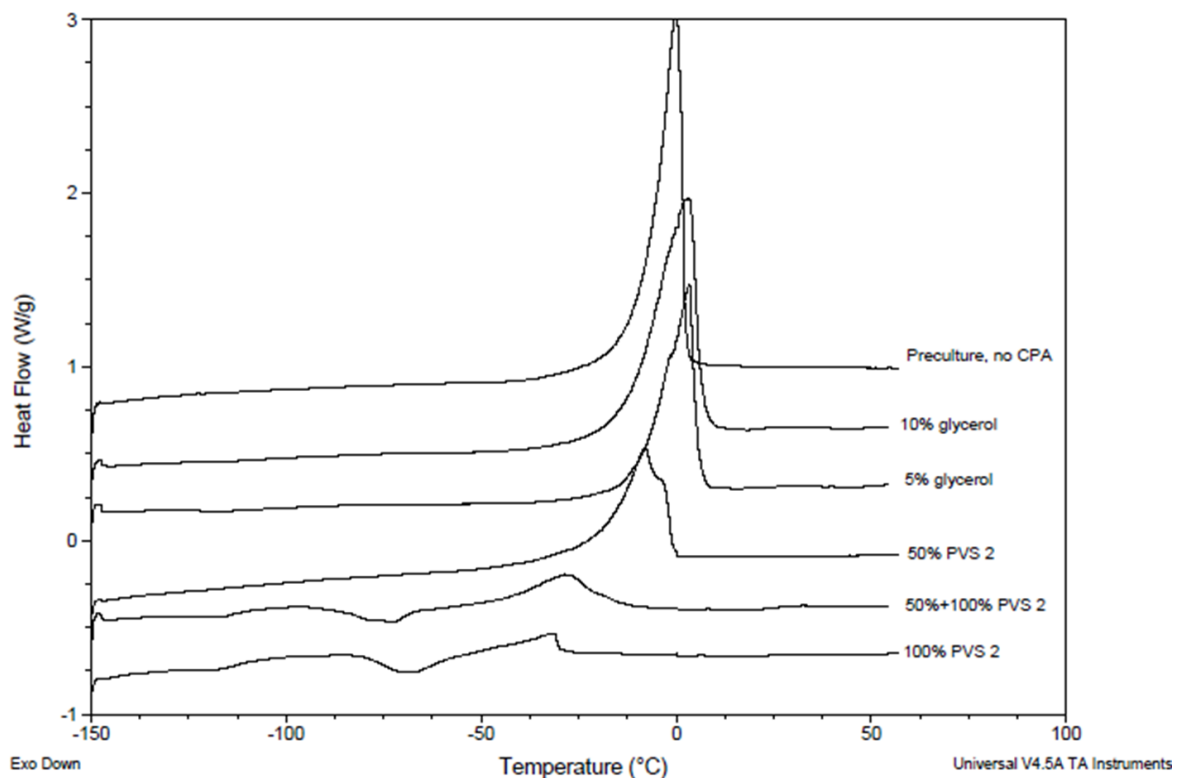


Figure 3.23: DSC endotherms of melting from *in vitro* grown shoot tips of fresh (no CPA), PVS2, glycerol (5% followed by 10%), pre-conditioned for 3 d, then slowly cooled from 40 °C to -150 °C and warmed back to 40 °C at 10 °C min^{-1} .

Figure 3.23 shows a composite of DSC endotherms (first and second order transitions) showing the warming thermograms for *in vitro* grown shoot tips. The intake of heat by the shoot tips during heating can be unequivocally interpreted as a melting of some liquid component(s) of that had crystallised (5% followed by 10% and PVS2 was exceptional) during cooling. Endothermic transitions across all treatment centered between approximately -65 °C (onset) and -7.5 °C. The size and onset temperature were dependent on the cryoprotectant corresponded to the melting of ice (Vertucci, 1990). The magnitude of the melt transition increased with glycerol (5% followed by 10%) treatment (-37 °C, onset temperature) above that of with shoot tips that have only receive 3 d pre-conditioning (-40 °C, onset temperature). The onset of melt transitions decreased with increasing concentration of PVS2, which indicated that tissue content became more concentrated with smaller peaks. Plant vitrification solution 2 (50% followed by 100%) shifted the melt transition to much lower temperature (- 65 °C, onset temperature). A deviation from the baseline that occurred around -115 °C in PVS2 (both [50+100] % and 100%) treated shoot tips, signified a glass transition (second order transition). This second order transition appeared before a melt transition, and a recrystallization event centered between -75 °C and -60 °C.

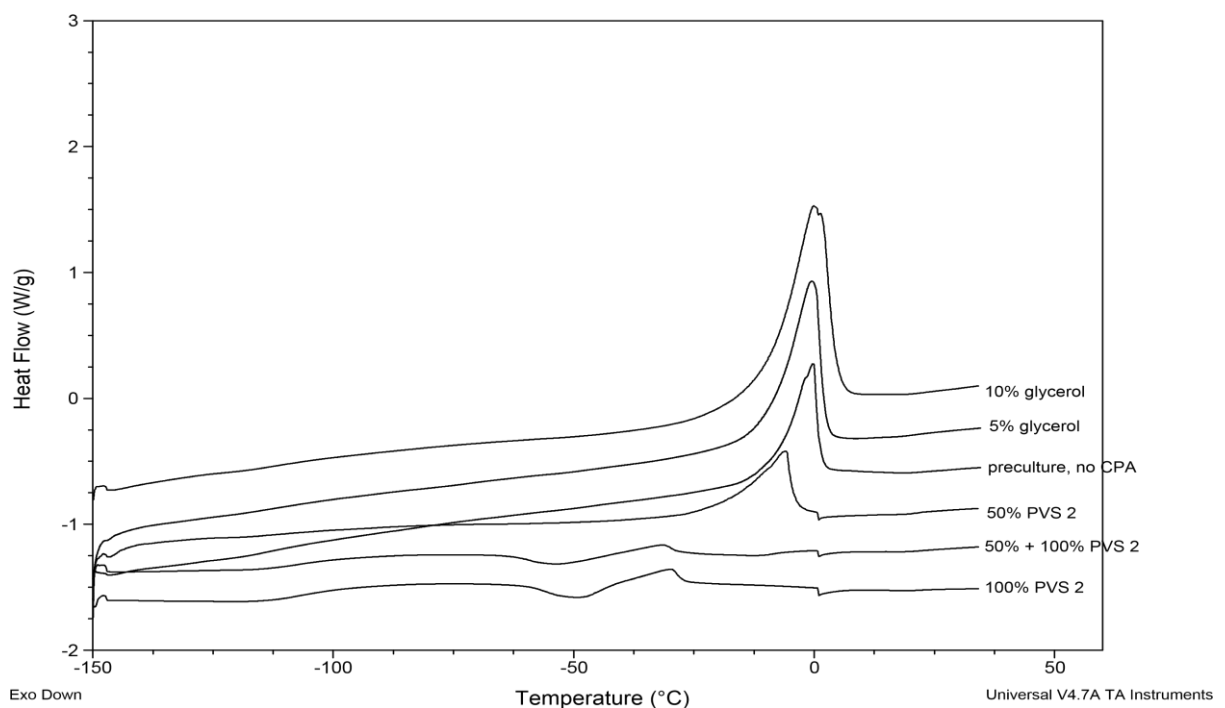


Figure 3.24: DSC endotherms of melting from *in vitro* grown shoot tips of fresh (no CPA), PVS2, glycerol (5% followed by 10%), pre-conditioned for 3 d, then rapidly

cooled by direct plunge into liquid nitrogen, then loaded into the DSC sample holder pre-cooled to $-150\text{ }^{\circ}\text{C}$, warmed back to $40\text{ }^{\circ}\text{C}$ at $10\text{ }^{\circ}\text{C min}^{-1}$. Relative humidity of the surrounding was reduced to about 10 to 15%.

Figure 3.24 shows an overlay of DSC endotherms (first order transitions) showing the warming thermograms for in vitro grown shoot tips. The intake of heat by the shoot tips during heating can be unequivocally interpreted as a melting of some liquid component(s) that had crystallised during cooling. With endothermic transitions centered at approximately $-55\text{ }^{\circ}\text{C}$ (onset) and $10\text{ }^{\circ}\text{C}$, which constituted the prevalent event where the size and onset temperature were dependent on the cryoprotectant corresponded to the melting of ice. Magnitude of the transition increased with a glycerol treatment ($-35\text{ }^{\circ}\text{C}$, onset) and was smaller in PVS2 treatment where tissue content become more concentrated in these smaller peaks. Plant vitrification solution 2 shifted the freezing event to much lower temperature ($-55\text{ }^{\circ}\text{C}$, onset). A deviation from the baseline that occurred around $-110\text{ }^{\circ}\text{C}$ in PVS 2 (50% followed by 100%) treated shoot tips, signified a glass transition (second order transition). This second order transition appeared before a melting transition. Additionally, a recrystallization event centered between $-75\text{ }^{\circ}\text{C}$ to $-60\text{ }^{\circ}\text{C}$.

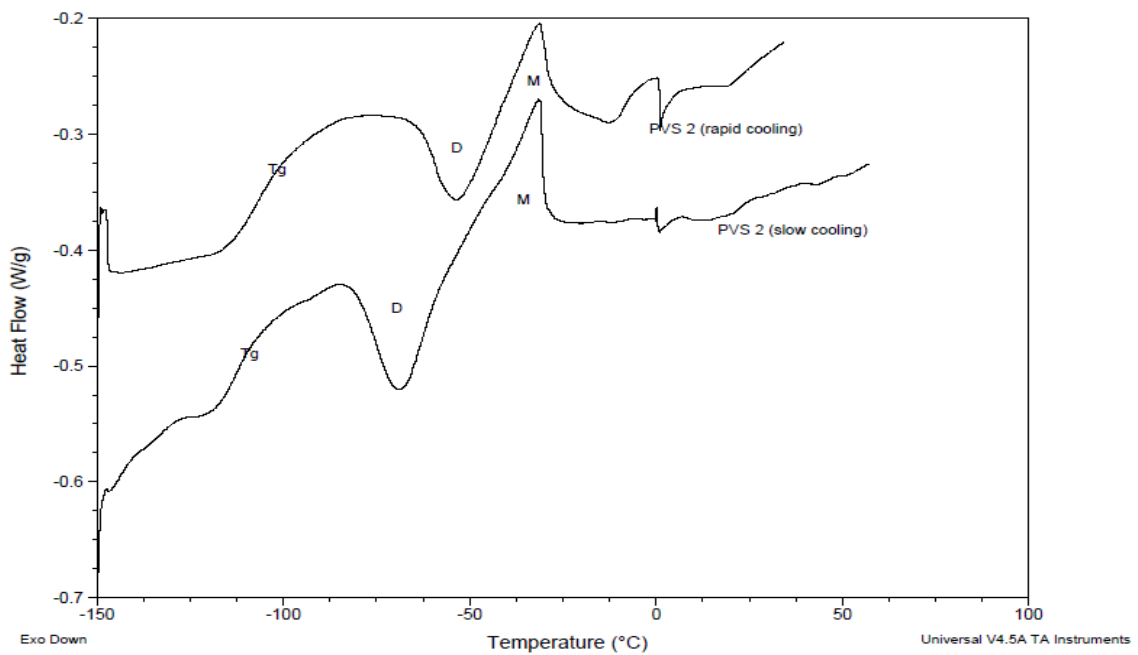


Figure 3.25: Glass transitions from vitrified pre-cultured shoot tips treated with PVS2, slow cooled (from $40\text{ }^{\circ}\text{C}$ to $-150\text{ }^{\circ}\text{C}$ and warmed back to $40\text{ }^{\circ}\text{C}$ at $10\text{ }^{\circ}\text{C min}^{-1}$),

and also rapidly cooled (by direct plunge into liquid nitrogen, then loaded into the DSC sample holder pre-cooled to $-150\text{ }^{\circ}\text{C}$, warmed back to $40\text{ }^{\circ}\text{C}$ at $10\text{ }^{\circ}\text{C min}^{-1}$). T_g represents glass transition, D is de-vitrification transition and M is the melting transition.

Examining shoot tips for evidence of glass signals by DSC characteristically yields the set of thermal properties such as shown in Figure 3.25. Deviation from the baseline was observed in PVS2 treated shoot tips, and rarely in rapidly cooled, PVS2 treated axes, such occurrence is a diagnostic of a glass transition. For both slow and rapid cooling, a glass transition temperature was observed at approximate -110 and $-120\text{ }^{\circ}\text{C}$. There was development of ice (de-vitrification) during heating that is characterised by an exotherm in the DSC thermogram which followed the transition. The glass transition temperature of PVS2 treated, slowly cooled shoot tips was $111\text{ }^{\circ}\text{C}$, slightly lower than that of rapidly cooled, which might be associated with the relatively higher survival from cryopreservation (or storage stability) of shoot tips (Table 3.1, Figure 3.6).

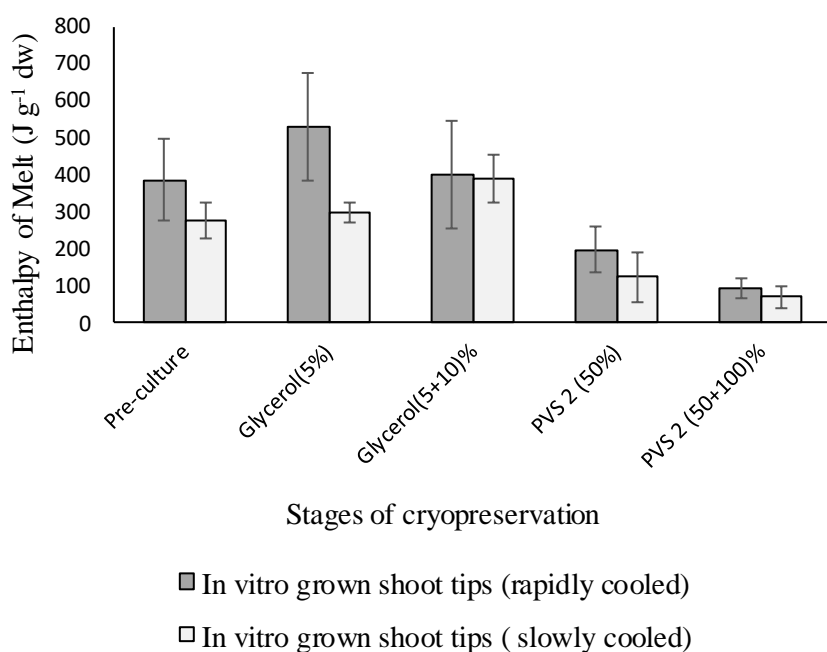


Figure 3.26: DSC melt endotherms of *in vitro* grown shoot tips after exposure to PVS2, glycerol (5% followed by 10%) or no exposure to CPA, slow cooled (from $40\text{ }^{\circ}\text{C}$ to $-150\text{ }^{\circ}\text{C}$ and warmed back to $40\text{ }^{\circ}\text{C}$ at $10\text{ }^{\circ}\text{C min}^{-1}$), and also rapidly cooled (by

direct plunge into liquid nitrogen, then loaded into the DSC sample holder pre-cooled to $-150\text{ }^{\circ}\text{C}$, warmed back to $40\text{ }^{\circ}\text{C}$ at $10\text{ }^{\circ}\text{C min}^{-1}$).

Figure 3.26 shows the relationship between the melt enthalpies of shoot tips at different stages of cryopreservation, which was dependent on the WC (Table 3.2). Enthalpies of melt for rapidly cooled were consistently higher than those of slow cooling. The treatments in both cooling rate were not significantly different ($P > 0.05$, ANOVA), with the exception of 5 % glycerol treatment. The initial enthalpies of melt were $276.43 \pm 50.3\text{ J g}^{-1}\text{ DW}$ and $386.5 \pm 110.5\text{ J g}^{-1}\text{ DW}$ for slow cooling and rapid cooling respectively. Treatment with glycerol (5% followed by 10%) increased the enthalpies of melt to $389.32 \pm 63.4\text{ J g}^{-1}\text{ DW}$ and $401.3 \pm 144.9\text{ J g}^{-1}\text{ DW}$ for slow cooling and rapid cooling respectively. These enthalpies were far above that of pure water is $333\text{ J g}^{-1}\text{ H}_2\text{O}$. PVS2 (50% followed by 100%) lowered the melt enthalpies to $68.64 \pm 28.2\text{ J g}^{-1}\text{ H}_2\text{O}$ and $94.8 \pm 27.6\text{ J g}^{-1}\text{ H}_2\text{O}$ for slow cooling and rapid cooling respectively. These low enthalpies signified a vitrified state of the cell content of shoot tips.

Chapter 4: Discussion

For successful cryopreservation of plant germplasm, the property of water is accepted to be of paramount importance as it plays a significant role in cryosurvival of explants especially those that are hydrated and cannot lose much water due to their inherent desiccation sensitivity, for example recalcitrant-seeded species. As mentioned earlier, this study was an attempt to understand the role of pre-conditioning of explants by pre-culture and cryoprotection and the effect of dehydration and cooling on embryonic axes and shoot tips of a recalcitrant-seeded species from South Africa, *Trichilia emetica*.

4.1 Effects of cryoprotectants and physical dehydration in relation to retention of viability by explants

This study showed that at different stages of the cryopreservation protocol had an effect on the water content and properties, and viability of embryonic axes and shoot tips from *T. emetica*. A study by Kioko *et al.* (1998) showed that cells from non-cryoprotected and cooled axes of *T. dregeana* displayed extensive subcellular damage, whereas cryoprotected axes retained a well-organised structure and developing organelles prominent with continuation of growth. In this study, there was considerable variation in the effects of both cryoprotectants (glycerol and PVS2) prior to drying of embryonic axes (with no cotyledonary segments attached) (Figures 3.1 and 3.2), where treatment with 5% followed by 10% glycerol significantly increased the WC in embryonic axes, whilst exposure to PVS2 decreased the WC in the axes. A similar osmotic behaviour was observed in shoot tips treated with these two cryoprotectants, with the exception in rapid cooling following application of 10% glycerol (Table 3.2). Plant vitrification solution 2 contains some components that are highly penetrative (DMSO and glycerol) and osmotically active (ethylene glycol and sucrose), which upon prolonged exposure can cause a cell to be plasmolysed and lose intercellular communication with the adjacent cells as the connections plasmodesmata would be broken (Helliot *et al.*, 2003). Findings of the current study (Figures 3.1 and

3.2) confirmed that glycerol on its own is highly penetrative like PVS2 (Fuller, 2004) which is a mixture of several chemicals. It can be said that cells lining the outer layer of the embryonic axes are highly permeable to glycerol (Gao *et al.*, 1995; Fuller, 2004; Benson, 2008), thus promoting more intake of this cryoprotectant. Eastmond (2004) showed using *Arapidopsis*, that the accumulation of glycerol can promote resistance to a variety of abiotic stresses linked with dehydration. Moreover, Garcia-Jimenez *et al.* (1998) showed that plant growth regulators (e.g. gibberellin) could also influence the effect of glycerol on growth. Irrespective of the ability of glycerol in helping plant tissues to tolerate desiccation and ultra-low temperatures (Sershen *et al.*, 2012c), the advantage in embryonic axes from many recalcitrant seeds is also in the fact that they can withstand considerable dehydration to a certain extent without damage. Only when dehydration is rapid explants have chances of survival, as rapid dehydration can curtail metabolism-linked damage that is detrimental especially in slow cooling (Pammenter *et al.*, 1998; Walters *et al.*, 2001; Berjak and Pammenter 2004; Varghese *et al.*, 2011). Berjak *et al.* (1989), and Diegel (1991) described that rapid drying can promote dehydration to levels lower than those which can be attained through slow dehydration of several species (e.g. *Landolphia kirkii*, *Castanospermum austral*, *Scadoxus membranaceus*, *Hevea brasiliensis*, and *Camellia sinensis*).

4.1.1 Shoot tips

Shoot tips consisting of shoot meristems and the apical dome with one or two pairs of leaves, are considered suitable explants for cryopreservation (Karthi *et al.*, 1980; Shibli *et al.*, 1999; Varghese *et al.*, 2009; Gebashe, 2015). Prior to cooling, retention of viability in shoot tips of *T. emetica* was > 75% across all treatments (Table 3.1). Following slow cooling and post cryo-recovery, only PVS2 treated shoot tips retained viability of 68% (Table 3.1, Figure 2.6) at WC = 1.4 g g⁻¹ (Table 3.2).

Findings of this study were consistent with the reports by Varghese *et al.* (2009) and Gebashe (2015), who also reported successful cryopreservation of shoot tips of the same species. However, those authors reported the requirement of a three-day pre-conditioning before the explants were exposed to cryoprotection and cooling. The pre-culture medium (MS with 0.3 M sucrose + 0.5 M glycerol) used was reported to yield high shoot recovery of *T. emetica* shoot meristems (Varghese *et al.*, 2009; Gebashe,

2015) following retrieval from LN. Similarly, pre-culture medium was found to be beneficial for embryonic axes as well; Thammasiri (1999) showed that pre-culture on this medium was indispensable for high survival of embryonic axes of *Artocarpus heterophyllus* following retrieval from LN. Successful cryopreservation of shoot tips using PVS2 has been reported in many other species by many researchers (e.g. Volk *et al.*, 2006; Volk and Walters, 2006; Chua and Normah, 2011; Teixeira *et al.*, 2013, 2014). According to Mroginski *et al.* (1991) shoot meristems consist of small number of relatively undifferentiated cells, which are believed to keep genetic stability during the regeneration process. However, according to Harding (2004), the idea of keeping genetic stability during regeneration process remains to be rigorously tested. Moreover, shoot tips consist of a relatively identical population of small cells that possesses the ability actively divide. Engelmann (2000) described that these cells consist of limited vacuoles and a high nucleo-cytoplasmic ratio that makes them better able to tolerate desiccation than differentiated cells. However, it must be noted that Gebashe (2015), using the same protocol established by Varghese *et al.* (2009) (78% survival), did not achieve similar cryo survival (40%) which highlights the challenge to cryopreserve explants from recalcitrant seeds in general and *T. emetica* in particular due to their high inter- and intra-seasonal variation (Berjak and Pammenter, 2008). Hence, studies like these enhances our understanding of cryopreservation, which could possibly increase survival and shoot production after retrieval from the cryogen.

4.1.2 Embryonic axes

In this study, viability assessments provide evidence that partial physical dehydration in all treatments had an additive effect as shoot production in embryonic axes with cotyledonary segments attached of *T. emetica* declined with an increasing degree of drying (Figures 3.1 - 3.4) irrespective of pre-condition treatment. Embryonic axes with cotyledonary segments attached were tolerant to initially moderately rapid removal of water, where > 55% viability was retained at WCs $\geq 0.45 \text{ g g}^{-1}$, across all treatments in the absence of 3d preconditioning (Figures 3.3 and 3.5). On the other hand, embryonic axes with cotyledonary segments attached were tolerant to initially moderately rapid removal of water, where > 55% viability was retained at WCs $\geq 1.00 \text{ g g}^{-1}$ (and $\geq 0.9 \text{ g g}^{-1}$ in PVS2), across all treatments that received 3 d preconditioning. Moreover, pre-conditioning lowered the viability of pre-cultured embryonic axes of *T.*

emetica with cotyledonary segments attached (Figure 3.4). Irrespective of pre-conditioning, prolonged dehydration below these WCs proved to be highly damaging. These findings confirmed the desiccation sensitivity of *T. emetica* that probably contributed to their cryo-recalcitrance, and were consistent with findings in *Amaryllid* spp. by von Fintel (2006) and Ngobese (2013). Contrary, to the current study, Sershen *et al.* (2007) showed that partial cryoprotectant-mediated dehydration before air drying was effective for embryos of some *Amaryllid* species. Moreover, the results contradict those of Choinski (1990), who showed that the rate of drying of *T. dregeana* seeds did not have any effect on the desiccation response. Wesley-Smith *et al.* (1992) showed that excessive drying of recalcitrant tissues led to some dehydration damage, which is exacerbated by freezing. Many studies have suggested that drying below an optimum WC may accelerate ageing rate (Buitink *et al.*, 1998a; Walters, 1998; Walters *et al.*, 2005).

The inability to retain viability or shoot production is suggested to be a consequence of stresses incurred at various procedural steps of cryopreservation, which can include a burst of ROS that is accompanied by a declining antioxidant potential (Naidoo *et al.*, 2011). Varghese *et al.* (2011) suggested that retention of viability at lower WCs in embryonic axes from recalcitrant seeds where water has been rapidly removed, can be partly attributed to the retention of functional antioxidant system, that are responsible for quenching ROS. Although Berjak and colleagues in 2011b, showed that cathodic water can afford a non-toxic means of amelioration of oxidative, stress related damage, there was no improvements with this species, as axes were treated with cryoprotectants made with cathodic water and also rehydrated in cathodic water. Varghese *et al.* (2011) suggested that the decline of viability in embryonic axes of *T. dregeana* that have been rapidly dried to very low WCs was also a non-oxidative process. In the current study, when these embryonic axes with cotyledonary segments attached were subjected to cryogenic condition irrespective of treatment and cooling rate, no shoots or any recovery was observed, which indicated that cryoprotectants were probably not well controlled to enable sufficient cellular dehydration without causing an injury from chemical toxicity (Mycock *et al.*, 1995; Volk and Walters, 2006).

4.2 The effect of cryoprotectants and cooling rates on properties of water in embryonic axes shoot segments of *T. emetica* in the absence of a 3d pre-conditioning

Previous studies (Wesley-Smith *et al.*, 2004, 2014 and 2015), have shown that for any cryopreservation protocol to be successful, there must be a suitable balance between tissue WC and cooling rate. Wesley-Smith *et al.* (2004; 2014) suggest that the cooling rate affects the location, magnitude and number of ice crystals formed in biological tissues exposed to ultra-low temperature. It is generally accepted that properties of water and ice relevant for cryopreservation may be revealed through the enthalpy of melting transitions, (which are known to give accurate information than that of freezing transitions). The standard enthalpy of melt for pure water is $333 \text{ J g}^{-1} \text{ H}_2\text{O}$, and a slight reduction of this value is suggested to be a consequence of a decrease in melting temperature (Vertucci, 1990). Lower enthalpies of melt ($<333 \text{ J g}^{-1} \text{ H}_2\text{O}$) can also signify the presence of increased solutes or shift to matrices that potentially limit ice formation or promote re-crystallisation events (Leprince *et al.*, 1999; Volk and Walters, 2006; Benson, 2008). The current study via use of DSC, provides partial evidence supporting why shoot tips of *T. emetica* established *in vitro* survives cryopreservation, whereas no embryonic axes with cotyledonary segments attached survives cryopreservation.

4.2.1 Treatment of embryonic axis shoot segments with glycerol increases the overall melt enthalpy and depresses the point at which unfreezable water appears, under a slow cooling rate

In the current study, slow cooling was used following cryoprotection with PVS2 and glycerol. This study displayed that there was a strong linear relationship between the enthalpy of melt and WC in all treatments (Figure 3.10). The current study reveals that under a slow cooling rate ($10 \text{ }^\circ\text{C min}^{-1}$) properties of water in fresh and glycerol treated embryonic axis shoot segments showed a slight non-significant degree of change ($220.8; 230.9 \text{ J g}^{-1} \text{ DW}$, respectively) (Figure 3.10), that were still relatively high when compared to melt enthalpy of pure water ($333 \text{ J g}^{-1} \text{ H}_2\text{O}$). These findings were consistent with previous studies (Vertucci *et al.*, 1991; Wesley-Smith *et al.*, 1992; Farrant and Walters, 1998) that have described, highly recalcitrant and hydrated axes have melting enthalpies that are near $333 \text{ J g}^{-1} \text{ H}_2\text{O}$ or sometimes signified by the

presence of a sharp peak (pure water) around 0 °C. Such high enthalpy values (Figure 3.10) correspond with low tolerance to various stresses by embryonic axes. The enthalpies of melting transitions did not reveal properties of water that are suitable for cryopreservation, as unfreezable water appeared at low WCs ($< 0.35 \text{ g g}^{-1}$, Figures 3.7-3.8 and 3.10) below the critical WC range. In 1990, Vertucci revealed that the states of water in seed tissues show that water that is unfreezable is tightly associated with hydrophilic surface.

Slightly higher overall melting enthalpy in embryonic axis shoot segments (Figures 3.10) suggests evidence that cryoprotection using glycerol (5% followed by 10%) probably decreased intra cellular viscosity of cryopreserved axes by increasing the WC and the overall enthalpy of melt, and therefore not altering freezing behaviour of water. Moreover, treatment of embryonic axis shoot segments with glycerol increased magnitude freezing transitions (Figure 3.8) and depressed the point where unfreezable water is first observed (Figure 3.10), which is not ideal for successful cryopreservation. This study did not agree with work by Sershen *et al.* (2012a, b) on zygotic embryos from *Haemanthus montanus* and *Amaryllis belladonna* who showed that glycerol enabled cryoprotection via maintaining integrity of cellular ultrastructure that will enable survival of cryogenic condition. Findings via DSC regarding embryonic axis shoot segments in all treatments (irrespective of cryoprotectants and cooling rate) were in agreement with those of Wesley-Smith *et al.* (1992) who showed that the possibility of ice damage in *Camellia sinensis* was reduced as moisture content decreased, where optimal survival was around water content of 0.45 g g^{-1} (Figure 3.3). At 0.40 g g^{-1} (Figure 3.8), a WC considered to be within the critical range for successful cryopreservation, there are still freezing events that occur, and probably causing freezing damage (Wesley-Smith *et al.*, 1992; Volk and Walters, 2006). The inability to alter the freezing behaviour of water around the critical WC, can be a possible explanation for no survival of embryonic axes with cotyledonary segments attached of *T. emetica* from cryopreservation.

Preliminary DSC studies revealed that glycerol solution (5% and 10%) used, on their own do not exhibit of any second order transition which simply indicated that solution did not vitrify. In contrast, previous work including that by Ablett *et al.* (1992) reported that complete amorphous glycerol (28%) forms a second order transition

(e.g. glass transition around $-78\text{ }^{\circ}\text{C}$) in biological systems. Lower glycerol concentrations (5% plus 10%) and the inability to reveal any second order transition (protective mechanism) can also be partial evidence for no recovery of embryonic axes with cotyledonary segments attached from exposure to cryogenic temperatures, irrespective of a slow cooling rate.

4.2.2 Treatment of embryonic axis shoot segments with PVS2 lowers the overall melt enthalpy and increases the point at which unfreezable water appears, under slow cooling rate

This study provides DSC evidence that under a slow cooling rate, PVS2 lowers the WC and alters the freezing behaviour of water in embryonic axis shoot segments ($191.2\text{ J g}^{-1}\text{ DW}$, Figures 3.9 and 3.10). The behaviour of PVS2 was contrary to that of glycerol, however, consistent with the study by Volk and Walters (2006). Those authors provided evidence that PVS2 changed the properties by lowering WC and the enthalpy of melt in shoot tips of *Mentha* and *Allium sativum*, and increasing the point where freezable water is observed, and this aid in cryoprotection of explants. At 0.45 g g^{-1} (Figure 3.9), a WC within the critical range for successful cryopreservation, there was a freezing event observed. It is established (Vertucci, 1990; Buitink *et al.*, 1996; Farrant and Walters, 1998) that germplasm with naturally acquired tolerance to low temperature and desiccation reveals enthalpy of melt transitions of $150 \pm 50\text{ J g}^{-1}\text{ H}_2\text{O}$ at the WC close to the unfrozen WC. This research provides insight that embryonic axes of *T. emetica* have no natural acquired tolerance to low temperature and desiccation (melt enthalpy $> 150 \pm 50\text{ J g}^{-1}\text{ DW}$, in all three treatments including PVS2). This can be a partial explanation for no survival of embryonic axes with cotyledons from cryopreservation.

Plant Vitrification Solution 2 on its own, is well known for its ability to undergo a second order transition e.g. glass (Volk *et al.*, 2004; Volk and Walters, 2006; Teixeira *et al.*, 2013, 2014; Nadarajan and Pritchard, 2014; also revealed in preliminary studies). However, this study provides DSC evidence that a PVS2 treatment does not promote a second order transitions (protective mechanism) in embryonic axis shoot segments (Figure 3.9), under a slow cooling rate. Volk and Walters (2006) suggested that prevention of water freezing without occurrence of glass is an indication that

PVS2 imparts its effects in pre-vitrified solutions. It is described that at 0 °C, these cryoprotective solutions have adequate mobility to infiltrate cells and therefore allowing water to be displaced (Volk and Walters, 2006). Components of PVS2, dimethyl sulphoxide (DMSO) and glycerol can penetrate the cell wall and plasma membrane to limit cellular WC and avoid ice nucleation (Benson, 2008).

As the temperatures decrease below 0 °C, molecular reorganisation required to nucleate water and allow ice crystals to grow are limited by these cryoprotective solutions (Volk and Walters, 2006; Teixeira *et al.*, 2014). Moreover, cytoplasmic viscosity can be enhanced by increased drying and increased solute concentration (Leprince *et al.*, 1999) that possibly limit ice nucleation during cooling. A decrease in the viscosity of any system allows an opportunity for quick molecular rearrangement (Leprince and Walters-Vertucci, 1995) that can cause intracellular crystallisation, and therefore, not suitable for successful cryopreservation. Previous studies (Roos and Karel, 1991; Slade and Levine, 1991) have reported that many degradative reactions are believed to rely on the viscosity of the system. Thus, viscosity is asserted to be a primary factor that controls seed ageing in storage (Vertucci and Roos, 1990; Vertucci, 1992; Williams *et al.*, 1993; Sun and Leopold, 1994). Regardless of a slow cooling rate or application of cryoprotectants, or a combination of both, no embryonic axes with cotyledonary segments attached survived cryopreservation which simply suggests that axes probably lack efficient freezing defence mechanisms.

4.2.3 Rapid cooling rate has a slightly higher degree of change on the behaviour of water within embryonic axis shoot segments that were treated with cryoprotectants

In the current study, rapid cooling was used following cryoprotection with glycerol and PVS2 of embryonic axis shoot segments. A direct plunge of embryonic axis shoot segments into LN greatly reduced the amount of water that can undergo ice crystals (Figures 3.11-3.13) and increased the enthalpies of melt in fresh and glycerol treatments (Figure 3.14), in comparison to those of slow cooling procedures (Figure 3.10). The enthalpies of melt in embryonic axis shoot segments for fresh and glycerol treated axes were not broadly different (265.98; 297.77 J g⁻¹ DW respectively), though lower than expected for pure water, however, PVS2 greatly lowered the enthalpy of melt (160.24 J g⁻¹ DW) (Figure 3.14). Here also, the enthalpies of melting transitions

did not reveal properties of water that are suitable for cryopreservation, as unfreezable water appeared at $\leq 0.4 \text{ g g}^{-1}$. The present study provides evidence that melt endotherms of cryoprotected and dried embryonic axes shoot segments crystallisation occurs at approximately 0.45 g g^{-1} (Figures 3.11-3.13) (respectively for fresh, glycerol and PVS2). Rapid cooling at this WC higher than point of unfreezable water, probably caused formation of lethal ice crystals (Dumet and Benson, 2000) that potentially affects the survival of the embryonic axes with cotyledonary segments attached following retrieval from LN. Despite the remarkable ability to lower the melt enthalpy (Figure 3.14), rapid cooling significantly decreased point where unfreezable water is observed (Figure 3.13), and this was contrary to the reported behaviour of PVS2, where this solution is reported to aid in cryopreservation of shoot tips (Volk *et al.*, 2004, 2006; Volk and Walters, 2006; Teixeira *et al.* 2013). However, the mode of action of each cryoprotectant or components of cryoprotectants mixture may differ with germplasm type, species, temperature, and other solution components (Fahly *et al.*, 1984; Finkle *et al.*, 1985).

There is considerable evidence that cooling rate affects survival in hydrated biological systems (Levitt, 1980; Fahy *et al.*, 1984; Fujikawa, 1987), and rapid cooling is believed to promote more retention of viability. Findings of this study (Figure 3.3) are contrary to that of Sisunandar *et al.* (2010) who showed that the zygotic embryos of coconut can be cryopreserved successfully via use of rapid dehydration and rapid cooling. Therefore, evidence of current study did not support the view (Bejark *et al.*, 1992; Pammenter *et al.*, 1998; Berjak *et al.*, 2001b; Wesley-Smith *et al.*, 2001), that suggest rapid dehydration prior to rapid cooling, is a step necessary for preparing tissues from recalcitrant explants for cryopreservation. A study by Wesley-Smith *et al.* (1992) using *Cammellia sinensis* showed that drying below a certain level was associated with loss of viability which is attributed to the dehydration damage and aggravated by rapid cooling. Sun (1999) suggested that the lower WC limit (above point of unfreezable water) is probably constrained by desiccation sensitivity of recalcitrant explants, whilst upper WC limit is linked with freezing injury that results from ice crystallisation at high WC. Moreover, cellular dehydration can still continue during cooling because of ice crystallisation. Sun (1999) showed using embryonic axes from recalcitrant seeds that the vitrified intracellular matrix was extremely unstable during heating at $10^{\circ}\text{C min}^{-1}$, where WC was at 0.50 g g^{-1} . The current study

provides evidence that the use of different cooling rates has an effect in the behaviour of water remaining in tissue. In PVS2 treated embryonic axis shoot segments, slow cooling procedures increased the point at which unfreezable water is first observed, whilst rapid cooling decreased the point of unfreezable water remaining with the tissues.

4.3 The effect of pre-conditioning followed by cryoprotection of embryonic axis shoot segments

In the current study, cooling was used following 3 d pre-conditioning, cryoprotection via use of glycerol and PVS2. A three-day pre-conditioning on medium that consisted of Murashige & Skoog medium, 0.3 M sucrose + 0.5 M glycerol had a marked effect on the properties of water within embryonic axis shoot segments. Under a slow cooling procedure, properties of water in embryonic axis shoot segments treated as fresh, glycerol and PVS2 treated changed to varying degrees (enthalpy 349.32, 284.34 and 263.98 J g⁻¹ DW, respectively) (Figure 3.18). Melt enthalpies lower than expected for pure water but still relatively high, with the exception of control (fresh) which was slightly higher than 333 J g⁻¹ H₂O. These were much higher than those observed in absence of a 3 d pre-conditioning, with the exception of glycerol (297.77 J g⁻¹ DW) treated (Figure 3.18). Such high melt enthalpies provide insight that embryonic axes of *T. emetica* has no acquired tolerance to low temperature and desiccation (melt enthalpy > 150 ± 50 J g⁻¹ DW) (Vertucci, 1990; Buitink *et al.*, 1996; Farrant and Walters, 1998). This is possibly explanation for decline viability with increased drying (Figure 3.4) and also no survival following retrieval from LN.

The enthalpies of melting transitions reveal properties of water that are suitable for cryopreservation, as unfreezable water increased and appeared at WCs (≥ 0.63 g g⁻¹, Figure 3.18). However, there were no crystallisation events below unfreezable water levels, where explants were cryopreserved at about 0.45 g g⁻¹ did not recover. This implies that damage incurred at this lower WC is not a consequence of ice formation as water is not freezable at these WCs (Wolfe *et al.*, 2002). These findings are in agreement with those of Vertucci (1989) who described that seeds of *Glycine max* and *Helianthus annulus* incurred damage at unfreezable WCs. Pammenter *et al.* (1991) described that recalcitrant seeds do not survive dehydration below point of

unfreezable water, where all intracellular water in recalcitrant axes is probably unfreezable (Pammenter *et al.*, 1993). Vertucci (1989) showed that pea seeds with a high water content were mostly damaged by slow cooling rate. Investigations by Varghese *et al.* (2009) and Gebashe (2015) on shoot tips of *T. emetica*, show that a 3 d pre-conditioning is a necessary step for successful cryopreservation. Despite pre-conditioning of explants, there is accumulative evidence that suggests slow cooling, which promote extracellular ice growth, can also result in mechanical forces that can cause deformation of cells or induce membrane structural changes (Fujikawa, 1987; Gordon-Kamm and Steponkus, 1984a, b). This may be a possible explanation why there was no survival of embryonic axes with cotyledonary segments attached from cryopreservation conditions, irrespective of pre-conditioning.

In contrast, under rapid cooling procedure, a three-day pre-conditioning had a marked effect, where properties of water in fresh and glycerol and PVS2 treated embryonic axis shoot segments did change (enthalpies; 209.44, 275.94, and 47.98 J g⁻¹ DW, respectively) (Figure 3.22), lower than expected for pure water. What was notably, was the response of embryonic axis shoot segments to PVS2 treatment where rapid cooling reduced the melt enthalpy (47.98 J g⁻¹ DW), lower than all previously mentioned PVS2 treatments. This melt enthalpy was much lower even for that observed in meristems of *Allium sativum* L. cryoprotected using PVS2 (Volk *et al.*, 2004). Contrary to finding on shoot tips that survives cryopreservation, these embryonic axes with cotyledonary segments attached treated with PVS2 did not survive cryopreservation.

Sarkar and Naik (1998) reported that pre-conditioning of excised shoot tips was indispensable for successful cryopreservation of potato shoot tips by vitrification. Also, Thammasiri (1999) has shown that pre-conditioning is important for high survival of embryonic axes of *Artocarpus heterophyllus* retrieved from LN. Pre-culture of explants on MS with 0.3 M sucrose and 0.5 M glycerol medium for 3 d had an added effect in that it increased WC across all treatments (Figure 3.4). It is generally accepted that sucrose and glycerol are imperative pre-growth additives for the attainment of desiccation tolerance during cryopreservation of explants by vitrification. It has been show that sucrose is non-penetrative to cells and lowers WC via osmotic dehydration (Engelmann *et al.*, 1994; Fuller, 2004; Quain *et al.*, 2009).

On the other hand, glycerol is highly penetrative and can have dehydrating effects as well (Gao *et al.*, 1995; Fuller, 2004; Benson, 2008), however this effect was not observed in the present study. Previous studies (Dumet *et al.*, 1993; Gonzalez-Arno *et al.*, 1996) have shown that a high level of sugars (e.g. sucrose, mannitol) are important for attaining a high survival rates of cryopreserved shoot tips. These sugars, sometimes in combination are most effective in reducing the water content and increasing the freeze resistance of suspension cells during cryopreservation by conventional methods (Withers and Street, 1977; Pritchard *et al.*, 1986a). Pre-conditioning in these high sugar levels has been suggested to include solute accumulation, decreasing isotonic water content, reduction in vacuolar volumes (Pritchard *et al.*, 1986) and alters membrane structure (Webb and Steponkus, 1990; Steponkus *et al.*, 1992). In this study, irrespective of pre-conditioning, cooling rate and cryoprotectants, embryonic axes with cotyledonary segments attached did not survive cryopreservation. A study by Ding *et al.* (2008) on meristem shoot tip that has been pre-culture on sucrose containing media, revealed several ultra-structure changes that included, breaking up of large vacuoles into smaller one, differentiation of proplastids into amyloplasts, and disruption of plasma membrane as a consequence of a partial plasmolysis. Fragmentation of nuclear envelope, creates vacuole-like lucent areas in some nucleoli, and this could result in the halt of nucleolar activity or enabling small ice crystals (Helliot *et al.*, 2003).

4.4 The effect of cooling rates and pre-conditioning treatment on in vitro grown shoot tips.

4.4.1 Impact of glycerol and cooling rates on the behaviour of water within shoot tips

Thermodynamic studies showed that the melt enthalpies of pre-conditioned *in vitro* grown shoot tips were 386 and 273.43 J g⁻¹ DW (rapid cooling and slow cooling, respectively) prior to cryoprotection (Figure 3.26). In all procedural steps of cryopreservation investigated, melt enthalpy was consistently higher in rapidly cooled shoot tips, and this was contrary to that which was observed for embryonic axis shoot segments, with the exception of two treatments observed in Figure 3.14. It is described that rapid cooling results in rapid removal of water within cells (Sakkar and

Naik, 1998; Volk and Walters, 2006). Generally, rapid cooling can also result in small but numerous ice nuclei within explants. Treatment with 5% followed by 10% glycerol slightly decreased the WC (Table 3.2), however, it did not alter the freezing behaviour of water (Figure 3.26), as the system was probably highly viscous. The enthalpy of melt for glycerol treatment is higher (401.3 and 389.32 J g⁻¹ DW) than the melt of enthalpy for pure water (Figure 3.26), which probably indicated decreased viscosity that partially explains why glycerol treated shoot tips do not survive cryopreservation. This was not consistent with work by Sershen *et al.* (2012a, 1212b) on zygotic embryos from *Haemanthus montanus* and *Amaryllis belladonna* who showed that glycerol maintains integrity of cellular ultrastructure, hence leading to successful cryopreservation. Furthermore, melt endotherms showed no second order transitions irrespective of cooling rate (Figures 3.23 and 3.24), as glycerol was not highly concentrated enough to induce a second order event or any signal typifying any protective mechanism. These shoot tips may be damaged as a results of freezing damage and other mechanical stresses (Wesley-Smith *et al.*, 1992).

4.4.2 Impact of PVS 2 and cooling rates on the behaviour of water within shoot tips

Plant vitrification solution 2 had a marked effect in that it lowers the WC (especially in slow cooling, Table 3.2) and melt enthalpies to 94.8 and 68.64 J g⁻¹ DW (for rapid cooling and slow cooling respectively, Figure 3.26), where no melt transitions from water were exhibited, only melt transition of the components of PVS2 were exhibited (Figures 3.23 and 3.24). These values were below the 150 J g⁻¹ H₂O a point of naturally acquired protection (Vertucci, 1990; Buitink *et al.*, 1996; Farrant and Walter, 1998), suggesting that these values might fall in the ranges of intermediate (limited) endogenous protection. When *in vitro* shoot tips were subjected to slow cooling (two-step) procedures, shoots were produced (Table 3.1), and were consistent with findings by Varghese *et al.* (2009) and Gebashe (2015) who both worked on *T. emetica*.

An effective balance between WC and cooling rate is essential (Normah and Makeen, 2008) which might possible explain survival via use of slow cooling (Table 3.1). According to Walters (1998) and Walters *et al.* (2005), the survival of desiccated tissue is said to be a consequence of the association of water with biomolecules and

the influence on biomolecule stability and mobility within the aqueous areas of tissue. Ballesteros and Walters (2007) asserted that above a certain level that could vary with species, presences of too much intracellular water will lead to lethal ice crystal formation and damage during freezing or thawing. On the other hand, there is a lower limit below which extreme dehydration will lead to dehydration damage. Therefore, it can be assumed that the treatment of shoot tips with PVS2 especially in slow cooling, retained tissue water within these critical ranges, changing the properties of water. Enhanced cytoplasmic viscosity as a consequence of cell dehydration and increase in solute concentration (Leprince *et al.*, 1999) limits the possibility of ice nucleation during cooling. A 68% survival (Table 3.1), indicated that mortality observed after exposure to cryoprotection and cryogenic temperatures was not a consequence of intracellular ice, as no freezing event was observed in shoot tips treated 50% followed by 100% PVS2 exposure. Moreover, better survival with slow cooling could probably be due to initial slow rate of cooling that possibly caused extracellular ice crystals that allows a flow of water from the cells as a consequence of osmotic imbalance, efficiently increasing cytoplasmic viscosity (Wesley-Smith *et al.*, 2004; Kaczmarczyk *et al.*, 2012), and this allows vitrification during cooling.

On the other hand, findings of the current studying on rapid procedures are consistent with findings by Volk and Walters (2006) who asserted damage to shoot tips is most likely to be a consequence of extreme desiccation allowed by PVS2, and possible aggravation by rapid cooling (Wesley-Smith *et al.*, 1992). Moreover, toxicity from the components of PVS2 (Volk and Walters, 2006), is more likely to cause damage of shoot tips. It can be said that no survival of shoot tips treated by PVS2 and rapidly cooled (Table 3.1), can be a results of these combined effects with a “greater magnitude” of damage exacerbated by direct exposure to liquid nitrogen. A study by Vertucci (1989) reported that rapid cooling improves the germination of hydrated seeds, however, lower the germination of some dry seeds such as soybean and sunflower. This sensitivity of dry seeds to rapid cooling is a consequence of a lipid component that can vitrify and also impart damage to the seed. However, in the current study there were no obvious signals of lipids detected via use of DSC. Moreover, Vertucci (1989) showed that above an unfreezable point, soybean seeds were damaged by either extreme cooling ($700\text{ }^{\circ}\text{C min}^{-1}$) or very low cooling ($1\text{ }^{\circ}\text{C min}^{-1}$).

Differential scanning calorimetry experiments revealed that cryoprotected *in vitro* established shoot tips of *T. emetica* display vitrified transitions (Figures 3.23 and 3.24), typifying an existence of a protective mechanism against desiccation and freezing damage. Those findings were similar to those of (Volk *et al.*, 2004; 2006; Volk and Walters, 2006; Teixeira *et al.*, 2013, 2014) who also worked with shoot tips from various species. In current study, irrespective of cooling rate, treatment with PVS2 resulted in a glass transition in shoot tips of *T. emetica* (Figures 3.23 and 3.24). In 2006, Volk and Walters were led to propose three mechanisms by which PVS2 aid in the cryoprotection of shoot tips. One, it replaces cellular water as ethylene glycol and dimethyl sulfoxide components permeate into shoot tips where it replaces water and also increasing the amount of unfrozen water. Secondly, it alters freezing behaviour of the residual water in cells, where enthalpies of melt transitions were substantial lower than that of pure water. Thirdly, it hinders the loss of water during air drying, where evaporative drying was slower in PVS2 treated shoot tips than in shoot tips receiving no cryoprotection treatments. Another possible explanation is that rapid cooling induces additional physical damage. It has been suggested that successful cryopreservation depends on the induction of tolerance in the direction of dehydration, instead of tolerance to freezing alone (Wesley-Smith *et al.*, 1992).

4.4.3 Occurrence of glass in pre-conditioned shoot tips in relation to survival from cryopreservation

In this study, the residual water in shoot tips of *T. emetica* treated with PVS2 does not crystallise when subjected to freezing temperatures as the water molecules are probably restrained sufficiently to limit the molecular reorganisation necessary for a crystallisation event (Wolfe *et al.*, 2002). Water molecule restriction is associated with or enabled by the formation of glass (Figures 3.23 and 3.24). Irrespective of the cooling rate formation of glass transitions was a consequence of PVS2 treatment *in vitro* grown shoot tips, and behaviour of this solution was partially associated with survival from cryopreservation, with the exception of rapidly cooling procedures. In this study, only T_g as a parameter of glass could be determined via DSC, however, more experiments are required in order determine if restricted ice crystallisation was a

consequence of reduction in mobility, signified as the primary function of glasses, or the unsettling of crystalline structure.

4.4.3.1 Parameters of intracellular glasses that probably affect survival during cryopreservation

An insight of the molecular kinetics and stability of any glassy matrix is essential for successful cryopreservation. It has been described that glasses can exhibit different molecular (or structural) relations (e.g. Buitink *et al.*, 1998a; Ballesteros and Walters, 2011). Angel (1991) asserted that glass fragility describes the change in the slope of viscosity in relation to temperature below T_g . According to that author fragility glasses are known to have larger slopes. In 2004, it was established by Walters that dry seeds formed 'strong' glasses, and later DMA work by Ballesteros and Walters (2011) suggested that increasing hydration level resulted in seed glasses becoming more fragile. This may hint why rapidly cooled shoot tips did not survive as they were at higher WC (Table 3.2). The current held view (Buitink and Leprince, 2004; Walters, 2004; Ballesteros and Walters, 2011) is that, occurrence of glasses in biological system is vital for storage, but more importantly understanding the stability of formed glasses has more potential for extended storage.

Angel (1991) stated that the temperature ranges where structural relations happen may correspond to the concept of glass fragility. Therefore, the stability of material over a narrow temperature range may be impacted, particularly in cryopreservation of hydrated seeds (Wesley-Smith *et al.*, 2001). Molecular mobility is an important factor that influence longevity in cryogenic storage, as intracellular viscosity is increased, molecular mobility decreases and this probably increase long-term survival (Walters, 2004; Buitink and Leprince, 2004). In this study, a slight difference in T_g existed, where slowly cooled shoot tips showed the onset of the glass transition around -118.8 °C ($T_g = 106.25$ °C), and whilst in rapidly cooled shoot tips the onset of the glass transition appeared at -112.5 °C ($T_g = 100$ °C) (Figure 3.25). According to previous studies (Chang and Baust, 1991; Buitink and Leprince, 2004; Walters, 2004) storage of preserved material must be well below the glass transition temperature as molecules are mobile at T_g if viability is to be maintained for extended periods. Therefore, it can be said that at T_g following rapid cooling, there were probably

greater molecular relaxations, that affected survival from LN (Ballesteros and Walters, 2011).

An understanding the molecular mobility/relaxations in seed is vital as mobility controls physiological activities such as seed aging (Buitink *et al.*, 1998a; Walters *et al.*, 2001, 2010; Ballesteros and Walters, 2011). Measuring molecular mobility has only been performed using artificially introduced and indirect methods or probes which have been suggested to unsettle the natural structure of seed glasses, where these methods or probe emitted signals not specific to seed glassy matrix (e.g. Buitink *et al.*, 1998a; 2000). The rate as which glasses decompose varies widely (William and Leopold.,1989), and it can be influenced by both the composition and temperature of glass. Moreover, decomposition of the glassy state in a seed may be gradually, and can associated with the loss of viability as the seed ages in relation its known to temperature.

In this study, no survival of shoot tips from direct exposure to liquid nitrogen. This indicated that evidence which suggested that PVS2 precluded ice formation by forming a glass is weak. Consistent with the studies on shoot tips (Volk *et al.*, 2004; Volk and Walters, 2006; Teixeira *et al.*, 2013, 2014) T_g occurred at -112 °C to 118 °C in PVS2 treated shoot tips (Figure 3.25), however, this temperature range was the same as that with PVS2 solution on its own. Therefore, protective mechanism in PVS2 treated and slowly cooled shoot tips can be based on a partial restriction of molecular and/or disorganisation of ice crystal structure, just as also observed in Volk and Walters (2006).

Conclusions and recommendations

In this study, embryonic axes of *T. emetica* with cotyledonary segments attached, treated with cryoprotectants did retain viability in the critical WC ranges. Cryoprotection of embryonic axes with PVS2 was consistent in that it not only reduced WC and enthalpy of melt prior to rapid dehydration, but also exhibited a glass transition in pre-cultured material at both slow and rapid cooling rates. Pre-conditioning revealed properties that might be suitable for cryopreservation, as unfreezable water in PVS2 treated embryonic axis shoot segments was increased above the critical WC. However, irrespective of cooling rates, embryonic axis did not survive cryopreservation, and this was an indication that survival from cryopreservation does not depend only on the avoidance of freeze damage, but dehydration damage in desiccation sensitive embryos/embryonic axes needs to be taken in to consideration. In contrast, treating embryonic axis shoot segments with glycerol increased the WC as well as the enthalpy of melt prior to dehydration. Though the unfreezable water in pre-conditioned and glycerol treated embryonic axis shoot segments was above critical WC, none of these embryonic axes survived exposure to LN. The melt enthalpy of water in axes treated with glycerol was relatively higher compared with control and PVS2 treated explants, and in many instances was not significantly different from the enthalpy of pure water. Glycerol did not promote survival of *in vitro* grown shoot tips after cryopreservation, as this cryoprotection increased the WC, and shoot tips were probably freeze damaged by ice crystallisation, irrespective of cooling rates used. In contrast, PVS2 altered the behaviour of water in shoot tips, via lowering the WC as well as the enthalpy of melt, and promoting a glass event during slow cooling, known to be a protective mechanism against stresses such as low temperatures. However, rapidly cooled shoot tips did not exhibit cryosurvival regardless of formation of glass in pre-cultured and PVS2 treated tissues. It is likely that rapid cooling worsened osmotic dehydration/damage and hence shoot tips were unable to withstand exposure to LN.

In this study, only one parameter (T_g) of glass was measured using DSC scans, therefore future research can use DMA, ERP techniques etc. to determine other

parameters of glassy matrix formed. Additionally, Teixeira *et al.* (2013) showed that the information on ice formation and vitrification data attained using Cryo Scanning Electron Microscopy and DSC was in perfect agreement. In addition to determining the water content suitable for cryopreservation, determining the stability of glass formed in explants from recalcitrant material can be very useful. Current views state that it not a matter of forming glass in biological material, but determining a how stable the glass formed is essential. Such studies done on explants from recalcitrant seeds will enhance our understanding on success of cryopreservation. It can be recommended that the glycerol concentration be increased, as solution used was not completely amorphous, as it did not display any second order transition, thus, not offering protection at cryogenic conditions. As this study has shown that physical drying results in dehydration damage, osmotic dehydration should be the preferred method of drying in *T. emetica*. In future work on *T. emetica*, use of low temperature SEM can be useful in monitoring ice crystallisation in cells and tissues, under conditions suitably for developing cryopreservation protocols. Lastly, use of Cryo-SEM associated with DSC can produce complementary information (e.g. about glass formation) that has more potential for successful preservation.

References

- Ablett, S., Izzard, M.J, Lillford, P.J.,** 1992. Differential Scanning Calorimetric study of frozen sucrose and glycerol solutions. *Journal of the Chemical Society, Faraday Transactions*, 88(6), 789-794.
- Acker J.P., McGann L.E.,** 2001. Membrane damage occurs during the formation of intracellular ice. *CryoLetters*, 22(4), 241-54.
- Adinew, B.,** 2014. Proximate nutritional composition, characterization of some selected physicochemical properties and comparative compositional analysis of *Trichilia emetica* oilseeds with some selected commercial oil seeds. *African Journal of Agricultural Research*, 9(28), 2177-2184.
- Allaby, M.,** 1998. A Dictionary of Plant Sciences. Oxford University Press, Oxford.
- Ambika S.R.,** 2006. Physiology of recalcitrant seeds. ICAR Short course on *In Vitro* Conservation and Cryopreservation-New options to Conserve Horticultural Genetic resources, Bangalore, India, 21-30.
- Angell, C.A.,** 1988. Perspective on the glass transition. *Journal of Physics and Chemistry of Solids*, 49, 863-871.
- Angell, C.A.,** 1991. Relaxation in liquids, polymers and plastic crystals-strong/fragile patterns and problems. *Journal of Non-Crystalline Solids*, 131, 13-31.
- Angell, C.A., Bressel, R.D., Green, J.L., Kanno, H., Oguni, M., Sare, E.L.,** 1994. Liquid fragility and the glass transition in water and aqueous solutions. *J Food Engineering*, 22, 115-142.
- Atkins, P.W.,** 1982. Physical chemical. Freeman, W.H, San Francisco, CA.
- Ballesteros, D., Walters, C.,** 2007. Calorimetric properties of water and triacylglycerol in fern spores relating to storage at cryogenic temperature. *Cryobiology*, 55, 1-9.
- Ballesteros, D., Walters, C.,** 2011. Detailed characterization of mechanical properties and molecular mobility within dry seed glasses: relevance to the physiology of dry biological systems. *The Plant Journal*, 68, 607-619.
- Ballesteros, D., Sershen, Varghese, B., Berjak, P., Pammenter, N.W.,** 2014. Uneven drying of zygotic embryonic axes of recalcitrant seeds: challenges and considerations for cryopreservation. *Cryobiology*, 69,100-109.
- Benson, E.E.,** 1990. Free Radical Damage in Stored Plant Germplasm. International Board for Plant Genetic Resources, Rome.

- Benson, E.E.**, 2004. Cryo-conserving algal and plant diversity: historical perspectives and future challenge. In Fuller, B., Lane, N., Benson, E.E (Eds) *Life in the frozen state*, London, CRC Press, 299-328.
- Benson, E.E.**, 2008. Cryopreservation of Phytodiversity: A critical appraisal of theory & practice. *Critical Reviews in Plant Science*, 27, 141-219.
- Benson, E.E., Bremner, D.**, 2004. Oxidative stress in the frozen plant: A free radical point of view. In: Fuller, B.J., Lane, N., Benson, E.E (Eds) *Life in the frozen state*. Boca Raton, CRC Press, 205-241.
- Benson, E.E., Harding, K. and Johnston J.W.** 2007. Cryopreservation of shoot tips and meristems. In: Stacey, G.N., Day, J.G (Eds) *Methods in Molecular Biology, Cryopreservation and Freeze-Drying Protocols*. Humana Press Inc., Totowa, NJ. 368, 163-183.
- Berjak, P.**, 2006. Unifying perspectives of some mechanisms basic to desiccation tolerance across life forms. *Seed Science Research*, 16, 1-15.
- Berjak, P., Dumet, D.**, 1996. Cryopreservation of seeds and isolated embryonic axes of neem (*Azadirachta indica*). *CryoLetters*, 17, 99-104.
- Berjak, P., Pammenter, N.W.**, 2000. What ultrastructure has told us about recalcitrant seeds. *Revista Brasileira de Fisiologia Vegetal*, 12, 22-55.
- Berjak, P., Pammenter, N.W.**, 2001a. An analysis of non-orthodox behaviour, with particular reference to truly recalcitrant seeds. In: Vozo, J. (Ed.) *Tropical Tree Seeds*. U.S Dept of Agriculture, Washington, DC. 1-25.
- Berjak, P., Pammenter, N.W.**, 2001b. Seed recalcitrant- current perspectives. *South African Journal of Botany*, 67, 79-89.
- Berjak, P., Mycock, D.**, 2004. Calcium, with magnesium, is essential for normal seedling development from partially dehydrated recalcitrant axes: a study on *Trichilia dregeana* Sond. *Seed Science Research*, 14, 217-231.
- Berjak, P., Pammenter, N.W.**, 2004. Recalcitrant seeds. In: Benech-Arnold, R.L., Sánchez, R.A (Eds.), *Handbook of Seed Physiology: applications to agriculture*. Imprints of the Haworth Press., London, Oxford, 305-345.
- Berjak, P., Pammenter, N.W.**, 2008. From *Avicennia* to *Zizania*: seed recalcitrance in perspective. *Annals of Botany*, 101, 213-228.
- Berjak, P., Pammenter, N.W.**, 2013. Cryostorage of germplasm of tropical recalcitrant-seeded species: approaches and problems. *International Journal of Plant Sciences*, 175 (1), 29-39.

- Berjak, P., Farrant, J.M., Pammenter, N.W.**, 1989. The basis of recalcitrant seed behaviour. In: Tylorson, R.B (Ed.) Recent advances in the development and germination of seeds. New York, Plenum Press, 89-108.
- Berjak, P., Pammenter, N.W., Vertucci, C.W.**, 1992. Homoiohydrous (recalcitrant) seeds: developmental status, desiccation sensitivity and the state of water in axes of *Landolphia Kirkii* Dyer. *Planta*, 186, 24-261.
- Berjak, P., Vertucci, C.W., Pammenter, N.W.**, 1993. Effects of developmental status and dehydration rate on characteristics of water and desiccation-sensitive in recalcitrant seeds of *Camellia sinensis*. *Seed Science Research*, 3, 155-166.
- Berjak, P., Farrant, J.M., Mycock, D.J., Pammenter, N.W.**, 1990. Recalcitrant (homoiohydrous) seeds: the enigma of their desiccation sensitivity. *Seed Science and Technology*, 18, 297-310.
- Berjak, P., Bradford, K.J., Kovach, D.A., Pammenter, N.W.**, 1994. Differential effects of temperature on ultrastructural responses to dehydration in seeds of *Zizania palustris*. *Seed Science Research*, 4, 111-121.
- Berjak, P., Walker, M., Watt, M.P., Mycock, D.J.**, 1999. Experimental parameters underlying failure or success in the plant germplasm cryopreservation: a case study on zygotic axes of *Quercus robur* L. *CryoLetters*, 20, 251-262.
- Berjak, P., Sershen, Varghese, B., Pammenter, N.W.**, 2011b. Cathodic amelioration of the adverse effects of oxidative stress accompanying procedures necessary for cryopreservation of embryonic axes of recalcitrant-seeded species. *Seed Science Research*, 21, 187-203.
- Berjak, P., Mycock, D.J., Wesley-Smith, J., Dumet, D., Watt, M.P.**, 1996. Strategies for *in vitro* conservation of hydrated germplasm. In: Normah, M.N., Narimah, M.K., Clyde MM (Eds) *In vitro* Conservation of Plant Genetic Resources, Percetakan Watan SDN. BHD, Kuala Lumpur, 199-52.
- Berjak, P., Mycock, D.J., Walker, M., Kioko, J., Pammenter, N.W., Wesley-Smith, J.**, 2000. Conservation of genetic resources naturally occurring as recalcitrant seeds. In: Black, M., Bradford, K.J., Vásquez-Ramos, J (Eds.), *Seed Biology: Advances and Applications*, CAB International, Wallingford, UK (2000), 223-228 ISBN 0 85199 404 0.
- Berjak, P., Bartels, P., Benson, E., Harding, K., Mycock, D.J., Pammenter, N.W., Sershen, and Wesley-Smith, J.**, 2011a. Cryo-conservation of South African plant genetic diversity. *In vitro Cellular & Developmental Biology - Plant*, 47, 65-81.

- Block W.**, 2003. Water status and thermal analysis of alginate beads used in cryopreservation of plant germplasm. *Cryobiology*, 47, 59-72.
- Bryant, G., Koster, K.L., Wolfe, J.**, 2001. Membrane behaviour in seeds and other systems at low water content: the various effects of solutes. *Seed Science Research*, 11, 17-25.
- Buitink, J., Leprince, O.**, 2004. Glass formation in plant anhydrobiotes ; survival in the dry state. *Cryobiology*, 48, 215-228.
- Buitink, J., Leprince, O.**, 2008. Intracellular glasses and seed survival in the dry state. *C R Biologies*, 331, 788-795.
- Buitink, J., Walters-Vertucci, C., Hoekstra, F.A., Leprince, O.**, 1996. Calorimetric properties of dehydrating pollen : analysis of a desiccation-tolerant and an intolerant species. *Plant Physiology* 111, 235-242.
- Buitink, J., Claessens, M.A.E., Hemmings, M.A., Hoekstra, F.A.**, 1998a. Influence of water content and temperature on molecular mobility and intracellular glasses in seeds and pollen. *Plant Physiology*, 118, 531-541.
- Buitink, J., C. Walters, F. A. Hoekstra, and J. Crane.** 1998b. Storage behavior of *Typha latifolia* pollen at low water contents: interpretation on the basis of water activity and glass concepts. *Physiologia Plantarum*, 103, 145–153.
- Buitink, J., Leprince, O., Hemminga, M.A., Hoekstra, F.A.**, 2000. Molecular mobility in the cytoplasm: an approach to describe and predict lifespan of dry germplasm. *Proceedings of the National Academy of Science USA*, 97, 2385-2390.
- Burke, M.J.**, 1986. The glassy state and survival of anhydrous biological systems. In: Leopold, A.C (Eds) Membranes, metabolism and dry organisms. Ithaca, New York, Comstock Publishing Associates, 358-363.
- Calistru, C., McLean, M., Pammenter, N.W., Berjak, P.**, 2000. The effects of mycofloral infection on the viability and ultrastructure of wet-stored recalcitrant seeds of *Avicennia marina* (Forssk.) Vierh. *Seed Science Research*, 10, 341-353.
- Carrington, A.K., Goff, H.D., Stanley, D.W.**, 1996. Structure and stability of the glassy state in rapidly and slowly cooled carbohydrate solution. *Food Research International*, 29, 207-213.
- CBD.**, 1992. Convention on Biological Diversity. Interim Secretariat for the Convention on Biological Diversity, Geneva, Switzerland.
- Clegg, J.S., Szwarnowski, S., McClean, V.E.R., Sheppard, R.J., Grant, E.H.**, 1982. *Biochimica et Biophysica Acta*, 721, 458-468.

- Chaitanya, K.S.K., Naithani, S.C.**, 1994. Role of superoxide, lipid peroxidation and superoxide dismutase in membrane perturbation during loss of viability in seeds of *Shorea robusta* Gaertn. F. *New Phytologist*, 126, 623–627.
- Chang, Z., Baust, J.G.**, 1991. Further inquiry into the cryobehavior of aqueous solutions of glycerol. *Cryobiology*, 28, 268-278.
- Chin, H.F.**, 1995. Storage of recalcitrant seeds. In: Basra, A.S (Ed.), *Seed Quality: Basic Mechanisms and Agriculture Implications*. Haworth Press Inc., New York, 209-222.
- Chin, H.F., Roberts, E.H.**, 1980. Recalcitrant crop seeds. Kuala Lumpur, Malaysia, Tropical Press Sdn, Bhd, 152.
- Chin, H.F., Krishanapillay, B., Alang, Z.C.**, 1988. Media for embryo culture of some tropical recalcitrant species. *Pertanika*, 11, 357-363.
- Cho, H.J., Hor, Y.L., Kim, H.H., Ramanatha Rao, V., Engelmann, F.**, 2002. *CryoLetters*, 23, 325-332.
- Choinski, J.S.**, 1990. Aspects of viability and post-germinative growth in seeds of tropical tree, *Trichilia dregeana* Sonder. *Annals of Botany*, 437-442.
- Chua, S.P., Normah, M.N.**, 2011. Effect of preculture, PVS2 and vitamin C on survival of recalcitrant *Nephelium ramboutan-ake* shoot tips after cryopreservation by vitrification. *CryoLetters*, 32, 506-515.
- Coates-Palgrave, K.**, 2000. *Trees of Southern Africa*. Stuik Publishers, Cape Town.
- Crowe, J.H., Carpenter, J.F., Crowe, L.M.**, 1998. The role of vitrification in anhydrobiosis. *Annual Review of Physiology*, 60, 73-103.
- Crowe, J.H., Crowe, L.M., Carpenter, J.F., Aurell, Wistrom, C.**, 1987. Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochemical J*, 242, 1-10
- Day, J.G., Harding, K., Nadarajan, J., Benson, E.E.**, 2008. Cryopreservation: conservation of bioresources at ultra-low temperatures, in: Walker, J.M., Rapley, R. (Eds.), *Molecular Biomethods Handbook*. Humana Press Inc., Clifton, New Jersey, 915-945.
- De Leeuw, F.E., De Leeuw, A.M., Den Daas, J.H., Colenbrander, B., Verkleij, A.J.**, 1993. Effects of various cryoprotective agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing. *Cryobiology*, 30(1)-32-44.

- Diegel, A.**, 1991. M.Sc Thesis. Biology Department, University of Natal, Durban, South Africa.
- Ding, F., Jin., Hong, J.N., Zhong, Y.**, 2008. Vitrification-cryopreservation, an efficient method for eliminating *Candidatus Liberobacter asiaticus*, the citrus Huanglongbing pathogen, from in vitro adult shoot tips. *Plant Cell Reports*, 27, 241-250.
- Dumet, D., and Benson, E. E.**, 2000. The use of physical and biochemical studies to elucidate and reduced cryopreservation-induced damage in hydrated/desiccated plant germplasm. In: Engelmann, F., Takagi, H (eds) Cryopreservation of Tropical Plant Germplasm, IPGRI, Italy.
- Drew, P.J., Pammenter, N.W., Berjak, P.**, 2000. 'Sub-imbibed' storage is not an option for extending longevity of recalcitrant seeds of the tropical species, *Trichilia dregeana* Sond. *Seed Science Research*, 10, 355-363.
- Dumet, D., Engelmann, F., Chabrillange, N., Duval, Y., Dereuddre, J.**, 1993. Importance of sucrose for the acquisition of tolerance to desiccation and cryopreservation of oil palm somatic embryos. *CryoLetters*, 14, 243-250.
- Dumont, F., Marechal, P.A., Gervais, P.**, 2004. Cell size and water permeability as determining factors for cell viability after freezing at different cooling rates. *Applied Environmental Microbiology*, 70(1), 268-72.
- Eastmond, P.J.**, 2004. Glycerol-insensitive *Arabidopsis* mutants *gli1* seedlings lack glycerol kinase, accumulate glycerol and are more resistant to abiotic stress. *The Plant Journal*, 37, 617-625.
- Eggers, S.K.**, 2007. Sub-imbibed storage of recalcitrant seeds of four species. M.Sc. Dissertation, University of KwaZulu-Natal, Durban, South Africa.
- Ellis, R.H., Hong, T.D., Roberts E.H.**, 1989. A comparison of the low-moisture-content limit to the logarithmic relation between seed moisture and longevity in twelve species. *Annals of Botany*, 63, 601-611.
- Engelmann, F.**, 1991. In vitro conservation of tropical plant germplasm: a review. *Euphytica*, 57, 227-243.
- Engelmann, F.**, 1997. Importance of desiccation for cryopreservation of recalcitrant seed and vegetatively propagated apices. *Plant Genetic Resource News* 112, 9-18.
- Engelmann, F.**, 2000. Importance of cryopreservation for the conservation of plant genetic resources. In: Engelmann, F., Takagi, H (eds). Cryopreservation of Tropical germplasm: Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, 8-20.

- Engelmann, F.**, 2004. Plant Cryopreservation: Progress and Prospects. In *In Vitro Cellular & Developmental Biology – Plant*, 40, 427-433.
- Engelmann, F.**, 2011a. Use of biotechnologies for the conservation of plant biodiversity. In *in-vitro-Cellular and Development Biodiversity*, 47, 5-16.
- Engelmann, F.**, 2011b. Cryopreservation of zygotic embryos: an overview. In: Thorpe, T. and Yeung, E. (eds.), *Plant Embryo Culture: Methods and Protocols*, Methods in Molecular Biology Series. Humana Press, Totowa, NJ, 155-184.
- Engelmann, F., Engels, J.M.M.**, 2002. Technology and strategies for ex situ conservation. In: Ramanatha Rao V., Brown, A.H.D., Jackson, M. T (eds) *Managing plant genetic diversity*, Wallingford, Rome, CAB International, IPGRI, 89-104.
- Engels, J.M.M., Visser, L.**, 2003. A guide to effective management of germplasm collections. IPGRI Handbooks for Genebanks, IPGRI, Rome, Italy.
- Engelmann, F., Dambier, D., Ollitrait, P.**, 1994. Cryopreservation of cell suspension and embryogenic callus of citrus using a simple freezing process. *CryoLetters*, 15, 53-58.
- Fahy, G.M.**, 1986. The relevance of cryoprotectant “toxicity” to cryobiology. *Cryobiology*, 23, 1-13.
- Fahy, G.M., MacFarlane, D.R., Angel, C.A., Meryman, C.A.**, 1984. Vitrification as an approach to cryopreservation. *Cryobiology*, 21, 407–426.
- Fahy, G.M., Lilley, T.H., Linsdell, H., Douglas, M.S., Meryman, H.T.**, 1990. Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms. *Cryobiology*, 27, 247–268.
- Farrant, J.M., Walters, C.**, 1998. Ultrastructural and biophysical changes in developing embryos of *Aesculus hippocastanum* L. in relation to the acquisition of tolerance to drying. *Physiologia Plantarum*, 104, 513-524.
- Farrant, J.M., Pammenter, N.W., Berjak, P.**, 1988. Recalcitrance - a current assessment. *Seed Science and Technology*, 67, 155-166.
- Farrant, J.M., Pammenter, N.W., Berjak, P.**, 1992. Development of the recalcitrant (homoiohydrous) seeds of *Avicennia marina*: Anatomical, ultrastructural and biochemical events associated with development from histodifferentiation to maturation. *Annals of Botany*, 70, 75-86.
- Farrant, J.M., Pammenter, N.W., Berjak, P.** 1989. Germination associated events and the desiccation sensitivity of recalcitrant seeds: a study on three unrelated species. *Planta*, 178, 189-198.

- Farrant, J.M., Pammenter, N.W., Berjak, P., Walters, C.,** 1986. The increasing desiccation sensitivity of recalcitrant *Avicenna marina* seeds with storage time. *Physiologia Plantarum*, 67, 291-298.
- Farrant, J.M., Pammenter, N.W., Berjak, P., Walters, C.,** 1993. Seeds development in relation to desiccation tolerance: A comparison between desiccation-sensitive (recalcitrant) seeds *Avicennia marina* and desiccation-tolerant types. *Seed Science Research*, 3, 1-13.
- Farrant, J.M., Pammenter, N.W., Berjak, P., Walters, C.,** 1997. Subcellular organization and metabolic activity during the development of seeds that attain different levels of desiccation tolerance. *Seed Science Research*, 7, 135-144.
- Farrant, J., Walters, C.A., Lee, H., Morris, G.J., Clarke, K.J.,** 1977. Structural and functional aspects of biological freezing techniques. *Journal of Microscopy*, 111, 17-34.
- Food and Agriculture Organization of the United Nations, FAO.,** 1996. Global Plan of Action for the Conservation and Sustainable Utilization of Plant Genetic Resources for Food and Agriculture. FAO, Rome, Italy.
- Food and Agriculture Organization of the United Nations, FAO.,** 2010. The state of the world's plant genetic resources for food and agriculture. FAO, Rome, Italy.
- Finkle, B.J., Zavala, M.E., Ulirich, J.M.,** 1985. Cryoprotective compound in the viable freezing of plant tissue, in: Karkha, K.K (Ed), Cryopreservation of plant tissue cell and organs, CRC Press, Boca Raton, 75-113.
- Finch-Savage, W.E.,** 1996. The role of developmental studies in research on recalcitrant and intermediate seeds. In: Ouédraogo, A.S, Poulsen, K., Stubsgaard, F (Eds.), Intermediate/Recalcitrant Tropical Forest Tree Seeds. IPGRI, Rome, 83-97.
- Finch-Savage, W.E., Blake, P.S.,** 1994. Indeterminate development in desiccation-sensitive Seeds of *Quercus robur* L. *Seed Science Research*, 4, 127-133.
- Finch-Savage, W.E., Hendry, G.A.F., Atherton, N.M.,** 1994. Free radical activity and loss of viability during drying of desiccation-sensitive tree seeds. Proceedings of the Royal Society of Edinburgh, 102, 257-260.
- Franks, F.,** 1985. Biophysics and Biochemistry at Low Temperatures. Cambridge University Press, New York, 39-52.
- Fujikawa, S.,** 1987. Mechanical force by growth of extracellular ice crystals is widespread cause for slow freezing injury in tertiary hyphae of mushrooms. *CryoLetters*, 8, 156-161.

Fuller, B.J., 2004. Cryoprotectants: The essential antifreezes to protect life in the frozen state. *CryoLetters*, 25 (6), 375-388.

Gao, D. Y., Liu, J., Lui, C., McGann, L.E., Watson, P. F., Kleinhans, F.W., Mazur, P., Critser, E.S., Critser, J.K., 1995. Prevention of somatic injury to human spermatozoa during addition and removal of glycerol. *Human Reproduction*, 10, 1109-1122.

Garcia-Jimenez, P., Rodrigo, M., Robaina, R.R., 1998. Influence of plant growth regulators, polyamines and glycerol interaction on growth and morphogenesis of carposporelings of *Grateloupia* cultured *in vitro*. *Journal of Applied Phycology*, 10, 95-100.

Gebashe, F.C., 2015. Studies on the cryopreservation of shoot apices from recalcitrant-seeded *Trichilia emetica* Vahl. and *Trichilia dregeana* Sond. M.Sc. Thesis, University of KwaZulu-Natal, Durban, South Africa.

Gonzalez-Arno, M.T, Moreira, T, Urra, C., 1996. Importance of pre-growth with sucrose and vitrification for the cryopreservation of sugarcane apices using encapsulation-dehydration. *CryoLetters*, 17, 141-148.

Gonzalez-Benito, M.E., Clavero-Ramirez, I., Lopez-Aranda, J.M., 2004. The use of cryopreservation for germplasm conservation of vegetatively propagated crops. *Spanish Journal of Agricultural Research*, 2, 341-351.

Gordon-Kamm, W.J., Steponkus, P.L., 1984a. Lamellar-to-hexagonal II phase transitions in the plasma membrane of isolated protoplasts after freeze-induced dehydration. *Proceedings of National Academy of Sciences USA*, 81, 6373-6377.

Gordon-Kamm, W.J., Steponkus P.L., 1984b. The behavior of the plasma membrane following osmotic contraction of isolated protoplasts: implications in freezing injury. *Protoplasma*, 123, 83-94.

Goveia, M., 2007. The effect of developmental status and excision injury on the success of cryopreservation of germplasm from non-orthodox seeds. M.Sc. thesis, University of KwaZulu-Natal, Durban, South Africa.

Goveia, M., Kioko, J.L., Berjak, P., 2004. Developmental status is a critical factor in the selection of excised recalcitrant axes as explants for cryopreservation: a study on *Trichilia dregeana* Sond. *Seed Science Research*, 14, 241-248.

Grant, R., Thomas, V., 1998. Sappi Tree Spotting, KwaZulu-Natal, Coast and Midlands. *Jacana Education (Pty) Ltd.*, Johannesburg, South Africa.

- Grout, B.W., Shelton, K., Pritchard, H.W.,** 1983. Orthodox behaviour of oil palm seed and cryopreservation of the excised embryo for genetic conservation. *Annals of Botany*, 52, 351-384.
- Gumede, Z., Mehar, V., Berjak, P.,** 2003. Effect of desiccation on the microfilament component of the cytoskeleton in the zygotic embryonic axes of *Trichilia dregeana*, Sond. In: 4th International Workshop on desiccation tolerance and sensitivity of seeds and vegetative plant tissues, Blouwaterbaai, South Africa, 22.
- Hanaoka, A.,** 2001. Antioxidant effects of reduced water produced by electrolysis of sodium chloride solutions. *Journal of Applied Electrochemistry*, 31, 1307-1313.
- Harding, K.,** 2004. Genetic integrity of cryopreserved plant cells: a review. *CryoLetters*, 25, 3-22.
- Hawkes, J.G., Maxted, N., Ford-Lloyd, B.V.,** 2000. The Ex Situ Conservation of Plant Genetic Resources. Springer Science + Business Media, B.V. The University of Birmingham, U.K.
- Helliot, B., Swennen, R., Poumay, Y., Frison, E., Lepoivre, P., Panis, B.,** 2003. Ultrastructural changes associated with cryopreservation of banana (*Musa* spp.) highly proliferating meristems. *Plant Cell Reports*, 21(7), 690-698.
- Hendry, G.A.F.,** 1993. Oxygen, free radical processes and seed longevity. *Seed Science Research*, 3, 141-153.
- Hendry, G.A.F., Finch-Savage, W.E., Thorpe, P.C., Atherton, N.M., Buckland, S. M., Nilsson, K.A., Seel, W.E.,** 1992. Free radical processes and loss of seed viability during desiccation in the recalcitrant species *Quercus robur* L. *New Phytologist*, 122, 273-279.
- Heywood, V.H., Dulloo, M.E.,** 2005. *In situ* conservation of wild plant species: a critical global review of best practices. IPGRI Technical Bulletin 11. IPGRI, Rome, Italy.
- Hirsh, A.G.,** 1987. Vitrification in plants as a natural form of cryoprotection. *Cryobiology*, 24, 214-228.
- Hirsh, A.G., Williams, R.J., Meryman, H.T.,** 1985. A novel method of natural cryoprotection, *Plant Physiology*, 66, 40-45.
- Hobbs, P.V.,** 1974. Ice Physics. Clarendon Press, Oxford, UK.
- Hoekstra, F.A., Golovina, E.A., Buitink, J.,** 2001. Mechanisms of plant desiccation tolerance. *Trends in Plant Science*, 6, 431-438.

- Hong, T.D., Ellis, R.H.**, 1996. A Protocol to Determine Seed Storage Behaviour. IPGRI Technical Bulletin No. 1, International Plant Genetic Resource Institute, Rome.
- Hong, T.D., Linington, S., Ellis, R.H.**, 1996. Seed storage behaviour: a compendium. Handbooks for genebanks, 4, IPGRI, Rome, 101.
- International Union for Conservation of Nature, IUCN.**, 2010. Guidelines for Using the IUCN Red List Categories and Criteria Version 8.1.
- Kaczmarczyk, A., Zanke, C., Senula, A., Grube, M., Keller, E.R.J.**, 2011. Thermal analyses by differential scanning calorimetry for cryopreservation of potato shoot tips. *Acta Horticulturae*, 908, 39-46.
- Kaczmarczyk, A., Funnekotter, B., Menon, A., Ye Phang, P., Al-Hanbali, A., Bunn, E., Mancera, R.L.**, 2012. Current issues in plant cryopreservation. In: Katkov, I. (Ed.). *Current Frontiers in Cryobiology*. Available Online from: <http://www.intechopen.com/books/current-frontiers-in-cryobiology/current-issues-in-plant-cryopreservation>
- Karow, A.M.**, 1969. Cryoprotectants - a new class of drugs. *Journal of Pharmacy and Pharmacology*, 21, 209-223.
- Kartha, K.K.**, 1981. Meristem culture and cryopreservation methods and applications. In: Thorpe, T.A. (Ed.), *Plant Tissue Culture, Methods and Applications in Agriculture*. Academic Press, New York, 181-212.
- Kartha, K.K.**, 1985. Meristem culture and germplasm preservation. In: Kartha, K.K. (Ed.), *Cryopreservation of Plant Cells and Organs*. CRC Press, Boca Raton, 116-134.
- Kartha, K.K. and Engelmann, F.**, 1994. Cryopreservation and germplasm storage. In: Vasil, I.K. and Thorpe, T.A. (Eds.), *Plant Cell and Tissue Culture*. Kluwer Academic Publishers, The Netherlands, 195-230.
- Kartha, K.K., Leung, N.L., Pahl, K.**, 1980. Cryopreservation of strawberry meristems and mass propagation of plantlets. *Journal of American Society of Horticulture Science*, 105(4), 481-484.
- Kartha K.K, Leung, N.L., Mroginski, L.A., 1982.** In vitro growth responses and plant regeneration from cryopreserved meristems of cassava (*Manihot esculenta* Crantz). *Zeitschrift für Pflanzenphysiologie*, 107, 133-140.
- Kaviani, B.**, 2011. Conservation of plant genetic resources by cryopreservation. *Australian of Journal of Crop Science*, 5(6), 778-800.
- Kermode, A.R.**, 1990. Regulatory mechanisms involved in the transition from seed development to germination. *Critical Reviews in Plant Science*, 9, 155-195.

- Kermode, A.R., Finch-Savage, B.E.**, 2002. Desiccation sensitivity in orthodox and recalcitrant seeds in relation to seed development. In: Bewley, M. and Pritchard, H.W (Eds), *Desiccation and Survival in Plants: Drying Without Dying*. CABI Publishing, Wallingford, Oxon, 149-184.
- King, M.W., Roberts, E.H.**, 1979. The Storage of Recalcitrant Seeds: Achievements and Possible Approaches. Rome: International Board for Plant Genetic Resources, 96.
- King, M.W., Roberts, E.H.**, 1980. Maintenance of recalcitrant seeds in storage. In: Chin, H.F., Roberts, E.H (Eds) *Recalcitrant Crop Seeds*. Tropical Press, Kuala Lumpur, Malaysia, SDN. BHD, 53-89.
- Kioko, J.L.**, 2003. Aspects of post-harvest seed physiology and cryopreservation of the germplasm of three medicinal plant species indigenous to Kenya and South Africa, PHD thesis, University of Natal, Durban, South Africa.
- Kioko, J.I., Berjak, P., Pammenter, N.W.**, 2006. Viability and ultrastructural responses of seeds and embryonic axes of *Trichilia emetica* to different dehydration and storage conditions. *South African Journal of Botany*, 72, 167-176.
- Kioko, J.I., Berjak, P., Pammenter, N.W., Watt, P., Wesley-Smith, J.**, 1998. Desiccation and cryopreservation of embryonic axes of *Trichilia dregeana* Sond. *CryoLetters*, 19, 5-11.
- Kistnasamy, P., Berjak, P., Pammenter, N.W.**, 2011. Desiccation and exposure to cryogenic temperatures of embryonic axes of *Landolphia kirkii*. *CryoLetters*, 32, 28-39.
- Komane, B.M., Oliever, E.L., Vijoer, A.M.**, 2011. *Trichilia emetica* (Meliaceae) - A review of traditional uses, biological activities and phytochemistry. *Phytochemistry Letters*, 4, 4-9.
- Koster, K.L.**, 1991. Glass formation and desiccation tolerance in seeds. *Plant Physiology*, 96, 302-304.
- Koster, K.L., Leopold, A.C.**, 1988. Sugars and desiccation tolerance in seeds. *Plant Physiology*, 88, 829-832.
- Lane, N.**, 2004. The future of cryobiology. In: Fuller, B.J., Benson, E.E. and Lane, N. (Eds.), *Life in the Frozen State*. CRC Press, UK, 645-657.
- Lee, M.Y., Kim, Y.K., Ryoo, K.K., Lee, Y.B., Park, E.J.**, 2006. Electrolyzed-reduced water protects against oxidative damage to DNA, RNA and protein. *Applied Biochemistry and Biotechnology*, 135, 273-289.

- Leopold, A.C., Vertucci, C.W.**, 1986. Physical attributes of desiccated seeds. In: Membranes, metabolism and dry organism. Leopold, AC (Ed), Comstock Publishing Associates, Ithaca, London, 22-34.
- Leopold, A.C., Sun, W.Q., Bernal-Lugo, I.**, 1994. The glassy state in seeds: analysis and function. *Seed Science Research*, 4, 267-274.
- Leprince, O., Walters-Vertucci, C.**, 1995. A calorimetric study of glass transition behaviour in axes of bean seeds with relevance to storage stability. *Plant Physiology*, 109, 1471-1481.
- Leprince, O., Hendry, G.A.F., McKersie, B.D.**, 1993. The mechanisms of desiccation tolerance in developing seeds. *Seed Science Research*, 3, 231-246.
- Leprince, O., Buitink, J., Hoekstra, F.A.**, 1999. Axes and cotyledon of recalcitrant seeds of *Castanea sativa* Mill. exhibit contrasting responses of respiration to drying in relation to desiccation sensitivity. *Journal of Experimental Biology*, 50, 1515-1524.
- Leprince, O., Harren, F.J.M., Buitink, J., Alberda, M., Hoekstra, F.A.**, 2000. Metabolic dysfunction and unabated respiration precede the loss of membrane integrity during dehydration of germinating radicles. *Plant Physiology*, 122, 597-608.
- Levine, H., Slade, L.**, 1988. Water as a plasticizer: physico-chemical aspects of low-moisture polymer systems. In Franks, F (Ed) Water Science Reviews, 3, Cambridge University Press, Cambridge, UK, 79-185.
- Levitt, J.**, 1980. Responses of plants to environmental stress, second ed. Academic Press, New York.
- Liang, Y. H., Sun, W. A.**, 2000. Desiccation tolerance of recalcitrant *Theobroma cacao* embryonic axes: the optimal drying rate and its physiological basis. *Journal of Experimental Botany*, 51(352), 1911-1919.
- Lin, T.P., Chen, M.H.**, 1995. Biochemical characteristics associated with the development of the desiccation-sensitive seeds of *Machilus thunbergii* Sieb. & Zucc. *Annals of Botany*, 76, 381-387.
- Lin, L., Yuan, B., Wang, D.D., Lin, W.Q.**, 2014. Cryopreservation of adventitious shoot tips of *Paraisometrum mileense* by droplet vitrification. *CryoLetters*, 35(1), 22-28.
- Luyet, B., Tanner, J. and Rapatz, G.**, 1962. X-ray diffraction study of the structure of rapidly frozen gelatine solutions. *Biodynamica*, 9, 21-46.
- Mandal, B.B., Dixit-Sharma, S.**, 2007. Cryopreservation of in vitro shoot tips of *Dioscorea deltoidea* Wall., an endangered medicinal plant: effect of cryogenic procedure and storage duration. *CryoLetters*, 28(6),460-70.

- Marikkar, J.M.N., Ghazali, H.M., Che Man, Y.B., Lai, O.M.,** 2002. The use of cooling and heating thermograms for monitoring of tallow, lard and chicken fat adulterations in canola oil. *Food Research International*, 35, 1007-1014.
- Matsumoto, T., Sakai, A., Nako, Y.,** 1998. A novel pre-culturing for enhancing the survival of *in vitro*- grown meristems of wasabi (*Wasabia Japonica*) cooled to -196 °C by vitrification. *CryoLetters*, 19, 27-36.
- Martinez D., Revilla M.A., Espina A., Garcia J.A.,** 2000. Differential scanning calorimetry applied to the storage at ultra-low temperatures of olive and hop *in vitro* grown shoot-tips. *Thermochimica Acta*, 349, 147-151.
- Martinez D., Revilla M.A., Espina A., Jaimez E., Garcia J.A.,** 1998. Survival cryopreservation of hop shoot tips monitored by differential scanning calorimetry. *Thermochimica Acta*, 317, 91-94.
- Mayeza, N.,** 2005. Effects of various potentially anti-fungal pre-treatments on hydrated storage life span of recalcitrant seeds. M.Sc Thesis. University of KwaZulu-Natal, South Africa
- Mazur, P.,** 1963. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *Journal of Genetic Physiology*, 47, 347-369.
- Mazur, P.,** 1970. Cryobiology: the freezing of biological systems. *Science*, 168, 939-949.
- Mazur, P.,** 1984. Freezing of living cells: Mechanisms and implications. *American Physiological Society*, 16, 125-142.
- Mazur, P.,** 1990. Equilibrium, quasi-equilibrium, and non-equilibrium freezing of mammalian embryos. *Cell Biophysics*, 17, 53-92.
- Mazur, P.,** 2004. Principles of cryobiology. In: Fuller, B.J., Lane, N. and Benson, E.E. (Eds.), *Life in the Frozen State*. CRC Press, UK, 3-65.
- Mazur, P., Rall, W.F., Rigopoulos, N.,** 1981. The relative contribution of the fraction of unfrozen water and of salt concentration to the survival of slowly frozen human erythrocytes. *Biophysical Journal*, 35, 653-675.
- McGann, L.E.,** 1978. Differing actions of penetrating and non-penetrating cryoprotective agents. *Cryobiology*, 15, 382-390.
- McGee, H.A. and Martin, W.J.,** 1962. Cryochemistry. *Cryogenics*, 2, 1-11.
- Moor, H.,** 1973. Cryotechnology for the structural analysis of biological material. In: Benedetti, E.L. and Favard, P (Eds.), *Freeze-Etching*. Société Française de Microscopie Electronique, Paris, 11-19.

- Motete, N., Pammenter, N.W., Berjak, P., Frederic, J.C.,** 1997. Response of recalcitrant seeds of *Avicennia marina* to hydrated storage: events occurring at the root primordia. *Seed Science Research*, 7, 1-10.
- Mroginski, L.A., Roca, W.M., Kartha, K.K.,** 1991. Crioconservacion de germoplasma. In: Roca, W.M., Kartha, K.K. and Mroginski, L.A (Eds). *Cultivo de Tejidos en la Agricultura: Fundamentos Aplicacions*, CIAT, Cali, Colombia, 715-730.
- Muldrew, K., Acker, J.A.C., Elliot, J.A.W., Mcgann, L.E.,** 2004. The water to ice transition: Implications for living cells. In Fuller, B.J., Lane, L., Benson, E.E. (eds) *Life in a frozen state*, CRC Press LLC, Boca Raton, London, NY, 67-103.
- Mullen, S.F., Critser, J.K.,** 2007. The Science of Cryobiology (Chapter 7). Woodruff, T.K., Snyder, K. A (Eds.) *Oncofertility* © Springer.
- Murashige, T., Skoog, F.,** 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15, 473-497.
- Mycock, D.J.,** 1999. Addition of calcium and magnesium to a glycerol and sucrose cryoprotectant solution improves the quality of plant embryo recovery from cryostorage. *CryoLetters*, 20, 77-82.
- Mycock, D.J, Blakeway, F.C., Watt., M.P, 2004.** General applicability of in vitro storage technology to the conservation and maintenance of plant germplasm. *South African Journal of Botany*, 70, 31-36.
- Mycock, D.J., Wesley-Smith, J, Berjak, B.,** 1995. Cryopreservation of somatic embryos of four species with and without cryoprotectant pre-treatment. *Annals of Botany*, 75, 331-336.
- Mycock, D.J., Berjak, P., Finch-Savage, W.E.,** 2000. Effects of desiccation on the subcellular matrix of the embryonic axes of *Quercus robur*. In: Black, M., Bradford, K.J. and Vázquez-Ramos, J (Eds.), *Seed Biology: Advances and Applications*. CABI Publishing, Wallingford, Oxon, 197-203.
- Nadarajan, J., Pritchard, H.W.,** 2014. Biophysical characteristics of successful oilseed embryo cryoprotection and cryopreservation using vacuum infiltration vitrification: an innovation in plant cell preservation. Seed Conservation Department, Royal Botanic Gardens Kew, Wellcome Trust Millenium Building, Ardingly, West Sussex, United Kingdom, 9.
- Nadarajan, J., Mansor, M., Kirshna Pillay, B., Staines, H.J., Benson, E.E., Harding, K.,** 2008. Applications of differential scanning calorimetry in developing

cryopreservation strategies for *Parkia speciosa*; A tropical tree producing recalcitrant seeds. *Cryoletters*, 29, 95-110.

Naidoo, C., 2012. Oxidative status and stress associated with cryopreservation of germplasm of recalcitrant-seeded species. MSc Dissertation. University of KwaZulu-Natal, South Africa.

Naidoo, C., Benson, E., Berjak, P., Goveia, M., Pammenter, N.W., 2011. Exploring the use of DMSO and ascorbic acid to promote shoot development by excised embryonic axes of recalcitrant seeds. *CryoLetters*, 32, 166-174.

Naidoo, C., Berjak, P., Pammenter, N.W., Varghese, B., 2016. The role of reactive oxygen species and antioxidants during precooling stages of axis cryopreservation in recalcitrant *Trichilia dregeana*. *Botany*, 94(5), 391-403.

Ngobese, N.Z., 2013. Effects of some of the procedural steps of cryopreservation on cryo-recalcitrant zygotic embryos of three amaryllis species producing desiccation-sensitive seeds. M.SC thesis. University of KwaZulu-Natal, South Africa

Nishizawa, S., Sakai, A., Amano, Y., Matuzawa, T., 1993. Cryopreservation of asparagus (*Asparagus officinalis* L.) embryonic suspension cells and subsequent plant regeneration by vitrification. *Plant Science*, 91, 67-73.

Noel, T.R., Ring, S.G., Whittam, M.A., 1990. Glass transition in low moisture foods. *Trends in Food Science and Technology*, 1, 62-67.

Ntuli, T.M., Pammenter, N.W., 2009. Dehydration kinetics of embryonic axes from desiccation-sensitive seeds: an assessment of descriptive models. *Journal of Integrative Plant Biology*, 51, 1002-1007.

Ntuli, T.M., Berjak, P., Pammenter, N.W., Smith, M.T., 1997. Effects of temperature on the desiccation responses of seeds of *Zizania palustris*. *Seed Science Research*, 7, 145-160.

Nuc, K., Marszałek M., Pukacki P.M., 2016. Cryopreservation changes the DNA methylation of embryonic axes of *Quercus robur* and *Fagus sylvatica* seeds during in vitro culture. *Trees* 30, 1831-1841.

Oliver, M.J., Bewley, J.D., 1997. Desiccation-tolerance of plant tissues: a mechanistic overview. *Horticultural Reviews*, 18, 171-213.

Oliver, M.J., Tuba, Z., Mishler, B.D., 2000. The evolution of vegetative desiccation tolerance in land plants. *Plant Ecology*, 151, 85-100.

Ozkavukcu, S., Erdemli, E., 2002. Cryopreservation: basic knowledge and biophysical effects. *Journal of Ankara Medical School*, 24, 187-196.

- Pammenter, N.W., Berjak, P.,** 1999. A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms. *Seed Science Research*, 9, 13-37.
- Pammenter, N.W., Adamson, J.H., Berjak, P.,** 1974. Viability of stored seed: extension by cathodic protection. *Science*, 186, 1123-1124.
- Pammenter, N.W., Farrant, J.M., Berjak, P.,** 1984. Recalcitrant Seeds: Short-term Storage Effects in *Avicennia marina* (Forsk.) Vierh. may be Germination-associated. *Annals of Botany*, 54 (6), 843-846.
- Pammenter, N.W., Vertucci, C.W., Berjak, B.,** 1991. Homoiohydrous (recalcitrant) seeds: dehydration, the state of water and viability characteristics in *Landolphia Kirkii*. *Plant Physiology*, 96, 1093-1098.
- Pammenter, N.W., Vertucci, C.W., Berjak, B.,** 1993. Responses to dehydration in relation to non-freezable water in desiccation-sensitive and tolerant seeds. In: Côme, D., Corineau, F (Eds) Proc. Of the 4th International Workshop on Seeds: Basic and Applied Aspects of Seed Biology. AFSIS, Paris, 667-672.
- Pammenter, N.W., Berjak, P., Walters, C.,** 1999. The effect of drying rate, and processes leading to viability loss in recalcitrant seeds. In: Marzalina, M., Khoo, K.C., Jayanti, N., Tsan, F.Y. and Krishnapillay, B. (Eds.), Recalcitrant Seeds. Forest Research Institute, Kuala Lumpur, pp. 14-24.
- Pammenter, N.W., Berjak, P., Farrant, J.M., Smith, M. T., Ross, G.,** 1994. Why do stored, hydrated recalcitrant seeds die? *Seed Science Research*, 4, 187-191.
- Pammenter, N.W., Berjak, P., Wesley-Smith, J., van der Willigen, C.,** 2002. Experimental aspects of drying and recovery. In: Bewley, M. and Pritchard, H.W (Eds.), Desiccation and Survival in Plants: Drying Without Dying. CABI Publishing, Wallingford, Oxon, UK, 93-110.
- Pammenter, N.W., Greggains, V., Kioko, J.I., Wesley-Smith, J., Berjak, P., Finch-Savage, W.E.,** 1998. Effects of differential drying rates on viability retention of recalcitrant seeds of *Ekerbegia capensis*. *Seed Science Research*, 8, 363-461.
- Panis, B., Lambardi, M.,** 2005. Status of cryopreservation technologies in plants (crops and forest trees). Villa Gualino, Turin, Italy, 5-7.
- Paunescu, A.,** 2009. Biotechnology for endangered plant conservation: a critical overview. *Romanian Biotechnological Letters*, 14, 4095-4103.
- Pegg, D.E.,** 1995. Principles of cryopreservation. In: Day, J.G., Stacey, G.N (Eds.), Cryopreservation and Freeze-Drying Protocols. Humana Press., Totowa, New Jersey, 39-57.

- Pegg, D.E., Dieper, M.P.** 1988. On the mechanisms of injury to slowly frozen erythrocytes. *Biophysical Journal*, 54, 471-488.
- Perez, J.**, 1994. Theories of liquid-glass transition. *Journal of Food Engineering*, 22, 89-114.
- Polge, C., Smith, A.U., Parkes, A.S.**, 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature*, 164, 166.
- Pooley, E.**, 1993. The complete field guide to trees of Natal, Zululand and Transkei. Natal Flora Publication Trust, Durban, 204-419.
- Potts M.**, 1994. Desiccation tolerance of prokaryotes. *Microbiology Reviews*, 58, 755-805.
- Prescott-Allen, R.Y., Prescott-Allen, C.**, 1981. In situ conservation of crop genetic resources, IBPGR and IUCN.
- Prickett, R.C., Marquez-Curtis, L.A., Elliot, J.A.W., McGann, L.E.**, 2015. Effect of supercooling and cell volume on intracellular ice formation. *Cryobiology*, 70, 156-163.
- Pritchard, H.W.**, 1995. Cryopreservation of seeds. In: Day, J.G., McLellan, M.R (Eds) *Methods in Molecular Biology: Cryopreservation and Freeze-Drying Protocols*. Humana Press Inc., New Jersey, 133-144.
- Pritchard, H.W., Grout, B.W.W., Short, K.C.**, 1986a. Osmotic stress as a pre-growth procedure for cryopreservation. 3. Cryobiology of sycamore and soybean cell suspensions. *Annals of Botany*, 57, 379-387.
- Pritchard, H.W., Grout B.W.W., Short, K.C.**, 1986b. Osmotic stress as a pre-growth procedure for cryopreservation 1. Growth and ultrastructure of sycamore and soybean cell suspensions. *Annals of Botany*, 57, 41-48.
- Pritchard, H.W., Tompsett, B.B., Manger, K.**, 1996. Development of a thermal time model for the quantification of dormancy loss in *Aesculus hippocastanum* seeds. *Seed Science Research*, 6, 127 -135.
- Pritchard, H.W., Tompsett, P.B., Manger, K., Smidt, W.J.**, 1995. The effects of moisture content on the low temperature responses of *Araucaria hunsteinii* seed and embryos. *Annals of Botany*, 76, 79-88.
- Quain, M.D., Berjak, P., Acheampong, E., Kioko, J.I.**, 2009. Sucrose treatment and explant water content: critical factors to consider in development of successful cryopreservation protocols for shoot tip explants of the tropical species *Dioscorea rotundata* (Yam). *CryoLetters*, 30, 212-223.

- Raimondo, D.C., von Staden, L., Donaldson, J.S.,** 2013. Lessons from the conservation assessments of South African megaflora. *Annals of Missouri Botanical Garden*, 99, 221-230.
- Rajasegar, A., mansor, A., Poobathy, R., Sivalingam, E., Sinniah, U.R., Subramaniam, S.,** 2015. An improved PVS2 cryopreservation technique for Ascocenda Wangsa Gold orchid using protocorm-like bodies. *Turkish Journal of Biology*, 39, 202-209.
- Rajasekharan, P.E.,** 2006. *In Vitro* conservation of horticultural crops. Paper presented at the ICAR Short Course on *In Vitro* Conservation and Cryopreservation- New Options to Conserve Horticultural Genetic Resources, Bangalore, India, 21-30.
- Ramanatha, R.V., Riley, R.,** 1994. The use of biotechnology for conservation and utilization of plant genetic resources. *Plant Genetic Resources Newsletter*, 97, 3-20.
- Rao, N.K.,** 2004. Plant genetic resources: Advancing conservation and use through biotechnology. *African Journal of Biotechnology*, 3(2), 136-145.
- Rathwell, R., Popova, E., Shukla, M.R., Saxena, P.K.,** 2016. Development of cryopreservation methods for cherry birch (*Betula lenta* L.), an endangered tree species in Canada. *Canadian Journal of Forest Research*, 46(11), 1284-1292.
- Rawat, D.S.C., Nautiyal, A.R.,** 1997. Seed viability in *Syzygium cuminii* in response to drying. In Naithani, S.C., Varghese, B., Sahu, K.K (Eds) Proceedings of the IUFRO Symposium on Innovations in Forest Trees Seed Science and Nursery technology. Raipur, India, Jai Printers, Raipur, India, 59-61.
- Reed, B.M.,** 2008. Plant cryopreservation: A Practical Guide. Springer, New York.
- Reed, B.M., Sarasen, V., Kane, M., Bunn, E., Pence, V.C.,** 2011. Biodiversity conservation and conservation biotechnology tools. *In Vitro Cellular and Developmental Biology-Plant*, 47, 1-4.
- Reinhold, P.J., Van Iren, F., Kijne, W.,** 2000. Cryopreseravtion of undifferentiated plant cells. In: Englman, F., Takagi, H. (eds) Cryopreservation of Tropical Plant germplasm. Current Research Progresss and Application. Japan International Research Center for Agricultural Sciences, Tsukuba, Japan / International Plant Genetic Resources Institute Rome, Italy, 89-102.
- Roberts, E.,** 1973. Predicting the storage life of seeds. *Seed Science and Technology*, 1, 499-514.
- Rogerson, N.E., Matthews, S.,** 1977. Respiratory and carbohydrate changes in developing pea (*Pisum sativum*) seeds in relation to their ability to withstand desiccation. *Journal of Experimental Botany*, 28, 304-313.

- Roos, Y., Karel, M.,** 1991. Amorphous state and delayed ice formation in sucrose solutions. *International Journal of Food Science and Technology*, 26, 553-566.
- Sakai, A.,** 1965. Survival of plant tissue at super low temperatures. III. Relation between effective prefreezing temperatures and degree of frost hardiness. *Plant Physiology*, 40, 882-87.
- Sakai, A.,** 1993. Cryogenic strategies for survival of plant cultured cells and meristems cooled to -196 °C. In: Cryopreservation of plant genetic resources: technical assistance activities for genetic resources projects, JICA, 5-30.
- Sakai A.,** 1997. Potentially valuable cryogenic procedures for cryopreservation of cultured plant meristems. In: Razdan, M.K., Cocking, E.C (Eds.) Conservation of Plant Genetic Resources *In Vitro*, Science Publishers, USA, 53-66.
- Sakai, A.,** 2004. Plant cryopreservation. In: Fuller, B., Lane, N., Benson, E.E (Eds) Life in the frozen state, London, CRC Press, 329-346.
- Sakai, A., Yoshida, S.,** 1967. Survival of Plant Tissue at Super-Low Temperature VI: Effects of Cooling and Rewarming Rates on Survival. *Plant Physiology*, 42(12), 1695-1701.
- Sakai, A., Kobayashi, S., Oiyama, I.,** 1990. Survival by vitrification of nucellar cells of navel orange (*Citrus sinensis* var. *Brasiliensis* Tanaka) cooled to -196 °C. *Plant Physiology*, 137, 465-470.
- Sakai, A., Kobayashi, S. and Oiyama, I.** 1991. Survival by vitrification of nucellar cells of navel orange (*Citrus sinensis* var. *brasiliensis* Tanaka) cooled to -196 °C. *Journal of Plant Physiology*, 137, 465-470.
- SANBI, 2010.** Red List of South African Plant. Available online from: www.redlist.sanbi.org
- Sarkar, D., Naik, P.S.,** 1998. Cryopreservation of Shoot Tips of Tetraploid Potato (*Solanum tuberosum* L.). *Annals of Botany*, 82, 455-461.
- Schmidt, E., Lotter, M., McClelland, W.,** 2002. Trees and shrubs of Mpumalanga and Kruger National Park. *Jacana Education (Pty) Ltd.*, Johannesburg, South Africa.
- Senaratna, T., McKersie, B.D.,** 1986. Loss of desiccation tolerance during seed germination: a free radical mechanism of injury. In Leopold, A.C. (Ed.), Membranes, Metabolism and Dry Organisms. Cornell University Press., New York, 85-101.
- Sershen, Berjak, P., Pammenter, N.W.,** 2008. Desiccation sensitivity of excised embryonic axes of selected *amaryllid* species. *Seed Science Research*, 18, 1-11.

- Sershen, Berjak, P., Pammenter, N.W.**, 2011. Effects of partial dehydration of recalcitrant *Amaryllis belladonna* zygotic embryos on vigour of recovered seedlings: a case of ‘hangover’? *Physiologia Plantarum*, 139, 193-202.
- Sershen, Berjak, P., Pammenter, N.W., Wesley-Smith, J.**, 2012a. The effects of various parameters during processing for cryopreservation on the ultrastructure and viability of recalcitrant zygotic embryos of *Amaryllis belladonna*. *Protoplasma*, 249, 155-169.
- Sershen, Berjak, P., Pammenter, N.W., Wesley-Smith, J.**, 2012b. Rate of dehydration, state of subcellular organisation and nature of cryoprotection are critical factors contributing to the variable success of cryopreservation: studies on recalcitrant zygotic embryos of *Haemanthus montanu*. *Protoplasma*, 249, 171-186.
- Sershen, Varghese, B., Pammenter, N.W., Berjak, P.**, 2012c. Cryo-tolerance of zygotic embryos from recalcitrant seeds in relation to oxidative stress – a case study on two *amaryllid* species. *Journal of Plant Physiology*, 169, 999-1011.
- Sershen, Pammenter, N.W., Berjak, P., Wesley-Smith, J.**, 2007. Cryopreservation of embryonic axes of selected *amaryllis* species. *CryoLetters*, 28, 387-399.
- Sharp, K.A.**, 2001. Water: Structure and Properties. Johnson, E.R Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania, USA.
- Shibli, R. A., Smith, M.A.L., Swatnawi, M.A.**, 1999. Pigment recovery from encapsulated-dehydrated *Vaccinium pahalae* (ohelo) cryopreserved cells. *Plant Cell Tissue Culture and Organ Culture*, 55, 119-123.
- Simione, F.P Jr.**, 1992. Key issues relating to the genetic stability and preservation of cells and cell banks. *Journal of Parenteral Science and Technology*, 46(6), 226-32.
- Sisunandar, A., Rival, P., Turquay, Y., Samosir, S.W., Adkins.**, 2010. Cryopreservation of coconut (*Cocos nucifera* L.) zygotic embryos does not induce morphological, cytological or molecular changes in recovered seedlings. *Planta*, 232, 435-447.
- Slade, L., Levine, H.**, 1991. A food polymer science approach to structure-property relationships in aqueous food systems: non equilibrium behaviour of carbohydrate-water systems. In Levine, H., Slade, L (Eds). *Water Relationship in Food*. Plenum Press, New York, 29-101.
- Slade, L., Levine, H.**, 1994. Water and glass transition: dependence of the glass transition on composition and chemical structure: special implications for flour functionality and cookie baking. *Journal of Food Engineering*, 22, 143-188.

- Srimathi, P., Malarkodi, K., Sasthri, G.,** 1999. Water flotation technique to upgrade the quality of jamun (*Syzygium cuminii*) seeds. *Progressive Horticulture*, 31, 20-22.
- Srinivasan, R., Gopalan, P., Zarriello, P.R., Myles-Tochko, C., Meyer, J.H.,** 1996. Designs of cathodic protection of rebars in concrete structures: an electrochemical engineering approach. *Johns Hopkins Applied Technical Digest*, 17, 362-370.
- Stanwood, P.C.,** 1985. Cryopreservation of seed germplasm for genetic conservation. In: Kartha, K.K (Ed) Cryopreservation of plant cells and organs, CRC Press, Boca Raton, Florida, 199-226.
- Steponkus, P.L., Langis, R., Fungiwawas.,** 1992. Cryopreservation of plant tissues by vitrification in: Steponkus, P.L (Ed). *Advances in Low-temperature biology*, JAL Press, Greenwich, Connecticut, 1-61.
- Steponkus, P.L.,** 1984. Role of the Plasma Membrane in Freezing Injury and Cold Acclimation. *Annual Review of Plant Physiology*, 35, 543-584.
- Sun, W.Q.,** 1999. State and phase transition behaviors of *Quercus rubra* seed axes and cotyledonary tissues: relevance to the desiccation sensitivity and cryopreservation of recalcitrant seeds. *Cryobiology*, 38, 372-385.
- Sun, W.Q.,** 2000. Dielectric relaxation of water and water-plasticized biomolecules in relation to cellular water organization, cytoplasmic viscosity, and desiccation tolerance in recalcitrant seed tissues. *Plant Physiology*, 124, 1203-1215.
- Sun, W.Q., Leopold, A.C.,** 1993. The glassy state and accelerated aging of soybean seeds. *Plant Physiology*, 89, 767-774.
- Sun, W.Q., Leopold, A.C.,** 1994. Glassy state and seed storage stability: a viability equation analysis. *Annals of Botany*, 74, 601-604.
- Suranthran, P., Gantait, S., Sinniah, R.U., Subramaniam, S., Alwee, S.S.RS., Roowi, S.H.,** 2012. Effect of loading and vitrification solutions on survival of cryopreserved oil palm polyembryoids. *Journal of Plant Growth Regulation*, 66(2), 101-109.
- Takagi, H.,** 2000. Recent developments in cryopreservation of shoot apices of tropical species, in: Engelmann, F., Takagi, H. (Eds.), *Cryopreservation of tropical plant germplasm*. JIRCAS International Agricultural Series., Tsukuba, Japan, 178-193.
- Takagi, H., Thinh, N.T., Islam, O.M., Senboku, T., Sakai, A.,** 1997. Cryopreservation of *in vitro*-grown shoot tips of taro (*Colocasia esculenta* [L] Schott) by vitrification. *Plant Cell Reports*, 16, 594-599.

- Teixeira, A., Gonzalez-Benito, M.E., Molina-Garcia, A.D.,** 2013. Glassy State and Cryopreservation of Mint Shoot Tips. *Biotechnology Progress* 29(3), 707-717.
- Teixeira, A., González-Benito, M.E., Molina-García, A.D.,** 2014. Determination of glassy state by cryo-SEM and DSC in cryopreservation of mint shoot tips by encapsulation–dehydration. *Plant Cell Tissue and Organ Culture*, 119(2), 269-280.
- Thammasiri, K.,** 1999. Cryopreservation of embryonic axes of jackfruit. *CryoLetters*, 20, 21-28.
- Thinh, N.T., Takagi, H., Yashima, S.,** 1999. Cryopreservation of *in vitro*-grown shoot tips of banana (*Musa* spp) by vitrification. *CryoLetters*, 20, 163-174.
- Towill, L.E., Waters, C.,** 2000. Cryopreservation of pollen. In: Engelmann, F., Takagi, H (eds), Cryopreservation of Tropical Plant Germplasm. International Plant Genetic Resources Institute, Rome, 115-129.
- Tweddle, J.C., Dickie, J.B., Baskin., C.C, Baskin, J.M.,** 2003. Ecological aspects of seed desiccation sensitivity. *Journal of Ecology*, 91, 294-304.
- Uragami, A., Sakai, A., Nagai, M., Takahashi, T.,** 1989. Survival of cultured-cells and somatic embryos of *Asparagus officinalis* cryopreserved by vitrification. *Plant Cell Reproduction*, 9, 418-421.
- Uyoh, E.A., Nkang, A.E., Eneobong, E.E.,** 2003. *Biotechnology, genetic*, 30,704-709.
- van Wyk, B., van Wyk, P., van Wyk, B.E.,** 2000. Photographic Guide to Trees of South Africa. Struik Publishers, Pretoria.
- Varghese, B., Naithani, S.C.,** 2000. Desiccation induced loss of vigour and viability during storage in Neem (*Azadirachta indica* A. Juss) seeds. *Seed Science and Technology*, 28, 485–96.
- Varghese, B., Naithani, S.C.,** 2002. Desiccation-induced changes in lipid peroxidation, superoxide level and antioxidant enzymes activity in neem (*Azadirachta indica* A.Juss) seeds. *Physiologia Plantarum*, 24, 79-87.
- Varghese, B., Wuehlisch, G. V., Naithani, S. C.,** 2004. Effect of differential rates of drying on viability and storability in recalcitrant Sal (*Shorea robusta* C.F. Gaertn) seeds. *Seed Technology*, 6, 51-64.
- Varghese, D., Berjak, P., Pammenter, N.W.,** 2009. Cryopreservation of shoot tips of *Trichilia emetica*, a tropical recalcitrant- seeded species. *CryoLetters*, 30 (3), 280-290.

- Varghese, B., Sershen, Berjak, P., Varghese, D., Pammenter, N.W.,** 2011. Differential drying rates of recalcitrant *Trichilia dregeana* embryonic axes: study of survival and oxidative stress metabolism. *Physiologia Plantarum*, 142, 326-338.
- Vertucci, C.W.,** 1989. Relationship between thermal transitions and freezing injury in pea and soybeans seeds. *Plant Physiology*, 90, 1121-1128.
- Vertucci, C.W.,** 1990. Calorimetric studies of the state of water in seed tissues. *Biophysical Journal Biophysical Society*, 58, 1463-1471.
- Vertucci, C.W.,** 1992. A calorimetric study of the changes in lipids during seed storage under dry conditions. *Plant Physiology*, 99, 310-316.
- Vertucci, C.W., Farrant, J.M.,** 1995. Acquisition and loss of desiccation tolerance. In: Kigel J., Galili, G (Eds) seed development and germination. Marcel Dekker, New York, 237-271.
- Vertucci, C.W., Leopold, A.C.,** 1986. Physiological activities associated with hydration level in seeds. In: Leopold, A.C (Ed.), Membranes, Metabolism and Dry Organisms. Cornell University Press., New York, 35-49.
- Vertucci, C.W., Roos, E.E.,** 1990. Theoretical basis of protocols for seed storage. *Plant Physiology*, 64, 1019-1023.
- Vertucci, C.W., Berjak, B., Pammenter, N.W., Crane, J.,** 1991. Cryopreservation of embryonic axes of an *Hemiohydrous* (recalcitrant) seed in relation to calorimetric properties of tissue water. *CryoLetters*, 12, 339-350.
- Volk, G.M., Walters, C.,** 2006. Plant vitrification solution 2 lowers water content and alters freezing behaviour in shoot tips during cryoprotection. *Cryobiology*, 52, 48-61.
- Volk, G.M., Maness, N., Rotondo, K.,** 2004. Cryopreservation of garlic (*Allium sativum* L.) using plant vitrification solution 2. *CryoLetters*, 25, 219-226.
- Volk, G.M., Harris, J.L., Rotida. K.E.,** 2006. Survival of mint shoot tips after exposure to cryoprotectant solution components. *Cryobiology*, 52, 305-308.
- von Breitenbach, F.,** 1965. The Indigenous Trees of Southern Africa. Pretoria, Department of Forestry.
- von Fintel, G.T.,** 2006. Towards the long-term germplasm conservation of selected *Amaryllidaceae*. M.Sc Thesis. University of KwaZulu-Natal, Durban, South Africa.
- Walters, C.,** 1998. Understanding the mechanisms and kinetics of seed aging. *Seed Science Research*, 7, 223-244.

- Walters, C.**, 2004. Temperature-dependency of molecular mobility in preserved seeds. *Biophysical Journal*, 86, 1253-1258.
- Walters, C., Hill, L.M., Wheeler, L.M.**, 2005. Dying while dry: kinetics and mechanisms of deterioration in desiccated organisms. *Integrative and Comparative Biology*, 45, 751-758.
- Walters, C., Ballesteros, D., Vertucci, V.A.**, 2010. Structural mechanics of seed deterioration: standing the test of time. *Plant Science*, 179, 565-573.
- Walters, C., Pammenter, N. W., Berjak, P., Crane, J.**, 2001. Desiccation damage, accelerated ageing and respiration in desiccation tolerant and sensitive seeds. *Seed Science Research*, 11, 135-148.
- Walters, C., Volk, G.M., Stanwood, Ph.C., Towill, L.E.**, 2009. Long-term survival of cryopreserved germplasm: contributing factors and assessments from thirty-year-old experiments. Proceedings of the First International Symposium on Cryopreservation in Horticultural Species. *Acta Horticulture*, 908, 113-121.
- Walters, C., Farrant, J.M., Pammenter, N.W., Berjak, P.**, 2002a. Desiccation stress and damage. In: Black, M. and Pritchard, H.W. (Eds.), *Desiccation and Survival in Plants: Drying Without Dying*. CABI Publishing, Wallingford, Oxon, 263-291.
- Walters, C., Touchel, D.H., Power, P., Wesley-Smith, J, Antolin, M.F.**, 2002b. A cryopreservation protocol for embryos of the endangered species *Zizania texana*. *CryoLetters*, 23, 291-298.
- Wang, Y.L., Fan, M.J., Liaw, S.L.**, 2005. Cryopreservation of *in vitro*-grown shoot-tips of papaya (*Carica papaya* L.) by vitrification. *Botanical Bulletin of Academia Sinica*, 46, 29-34.
- Watterson, J.G.**, 1987. A role for water in cell structure. *Biochem. J.*, 248, 615-617.
- Webb, M.S., Steponkus, P.L.**, 1990. Dehydration-induced hexagonal phase formation in phospholipid bilayers. *Cryobiology*, 27, 666-667.
- Wesley-Smith, J.**, 2003. Investigations into the responses of axes of recalcitrant seeds to dehydration and cryopreservation. Ph.D. thesis, University of Natal, Durban.
- Wesley-Smith, J., Vertucci, C.W., Berjak, P., Pammenter, N.W.**, 1991. Freezing rate, ultrastructural preservation and survival of embryonic axes of *Camellia sinensis*. *Electron Microscopy Society of Southern Africa*, 21, 175-176.
- Wesley-Smith, J., Berjak, P., Pammenter, N.W. and Vertucci, C.W.**, 1995. Ultrastructural evidence for the effect of freezing in embryonic axes of *Pisum sativum* L. at various water contents. *Annals of Botany*, 75, 59-64.

- Wesley-Smith, J., Walters, C., Pammenter, N.W., Berjak, P.,** 1999. Rapid freezing of embryonic axes of recalcitrant species. In: Marzalina, M., Khoo, K.C., Jayanthi, N., Tsan, F.Y. and Krishnapillay, B (Eds.), *Recalcitrant Seeds*. FRIM, Kuala Lumpur, 132-139.
- Wesley-Smith, J., Pammenter, N.W., Berjak, P., Walters, C.,** 2001. The effects of two drying rates on the desiccation tolerance of embryonic axes of recalcitrant jackfruit (*Artocarpus heterophyllus* Lamk.) seeds. *Annals of Botany*, 88, 653-664.
- Wesley-Smith, J., Walters C., Berjak P., Pammenter N.W.,** 2004. The influence of water content, cooling and warming rate upon survival of embryonic axes of *Poncirus trifoliata* (L.). *Cryoletters*, 25(2), 129-138.
- Wesley-Smith, J., Berjak, P., Pammenter, N.W., Walters, C.,** 2014. Intracellular ice and cell survival in cryo-exposed embryonic axes of *Acer saccharinum*: an ultrastructural study of factors affecting cell and ice structures. *Annals of Botany*, 113, 695-709.
- Wesley-Smith, J., Pammenter, N.W., Walters, C., Berjak, P.,** 2015. Why is intracellular ice lethal? A microscopical study showing evidence of programmed cell death in cryo-exposed embryonic axes of recalcitrant seeds of *Acer saccharinum*. *Annals of Botany*, 115, 991-1000.
- Wesley-Smith, J., Vertucci, C.W., Berjak, B., Pammenter, N.W., Crane, J.,** 1992. Cryopreservation of desiccation- sensitive axes of *Camellia sinensis* in relation to dehydration, freezing rate and the thermal properties of tissue water. *Journal of Plant Physiology*, 140, 596-604.
- Wiersum, K.F., Dold, A.P., Husselmann, M., Cocks, M.,** 2006. Cultivation of medicinal plants as a tool for biodiversity conservation and poverty alleviation in the amatola region, South Africa. In: Bogers, R.J., Craker, L.E. and Lange, D. (Eds.), *Medicinal and Aromatic Plants*. Springer, The Netherlands, 43-57.
- Williams, R.J., Leopold, A.C.,** 1989. The Glassy State in Corn embryos. *Plant Physiology*, 89, 977-981.
- Williams, R.J., Hirsh, A.G., Takahashi, T.A, Meryman, H.T.,** 1993. What is vitrification and how can it extend life? *Japanese Journal of Freeze Drying*, 39, 3-12.
- Withers, L.A.,** 1991. *In Vitro* Conservation. *Biological Journal of the Linnean Society*, 43, 31-42.
- Withers, L.A.,** 2001. Biotechnology and plant genetic resource conservation. In: Paroda, R.S., Arora, R.K (Eds.), *Plant Genetic Resources Conservation and Management*. IBPGR Regional Office, New Dehli, 273-297.

- Withers, L.A., Street, H.E.,** 1977. Freeze preservation of cultured plant cells. III. The pregrowth phase. *Physiologia Plantarum*, 39, 171-178.
- Withers, L.A., King, P.J.,** 1979. Proline: a novel cryoprotectant for the freeze preservation of culture cells of *Zea mays* L. *Plant Physiology*, 64, 675-678.
- Wolfe, J., Bryant, G.,** 1999. Freezing, drying and/or vitrification of membrane-solutewater systems. *Cryobiology*, 39, 103-129.
- Wolfe, J., Bryant, G., Koster, K.L.,** 2002. What is 'unfreezable water', how unfreezable is it and how much is there? *CryoLetters*, 23, 157-166.
- Wolkers, W.W.F.,** 1998. The role of macromolecular stability in desiccation tolerance. Wageningen Agricultural University.
- Yamamoto, S., Fukui, K., Rafique, T., Khan., Martinez, C.R.C., Sekizawa, K.,** 2012. Cryopreservation of in vitro-grown shoot tips of strawberry by the vitrification method using aluminium cryo-plates. *Plant Genetic Resources: Characterization and Utilization*, 10(1), 14-19.
- Yonezawa, F.,** 1991. Glass transition and relaxation of disordered structures. In Ehrenreich, H., Turnbull, D (eds) *Solid State Physics: Advances in Research and Application*, 45, Academic Press, San Diego, CA, 179-254.
- Zamecnik, J., Faltus, M.,** 2009. Evaluation of Thermograms from Differential Scanning Calorimeter. *Cryoletters*, 30(5), 388-388.
- Zamecnik, J., Faltus, M., Kotkova R.,** 2009. Glass transition determination in *Allium* shoot tips after dehydration. Paper presented at the 1st International Symposium on Cryopreservation in Horticultural Species, Leuven, Belgium, 5-9 April 2009.
- Zamecnik, J., Faltus, M., Bilavcik, A., Kotkova, R.F.,** 2012. Comparison of cryopreservation methods of vegetatively propagated crops based on thermal analysis. In: *Agricultural and biological sciences: Current frontiers in cryopreservation*. In Tech. Available from: <http://www.intechopen.com/books/current-frontiers-in-cryopreservation/comparison-of-cryopreservation-methods-of-vegetatively-propagated-crops-based-on-thermal-analysis>.
- Zhao, M.A., Dhital, S.P., Fang, Y.L., Khu, D.M., Song, Y.S., Park, E.J., Kang, C.W, Lim, H.T.,** 2005. Application of slow-freezing cryopreservation method for the conservation of diverse potato (*Solanum tuberosum* L.) genotypes. *Journal of Plant Biotechnology*, 3, 183-186.