UNIVERSITY OF KWAZULU-NATAL

THE POTENTIAL USE OF EMBRYONIC SHOOT APICES AS EXPLANTS FOR CRYOPRESERVATION OF SELECTED RECALCITRANT-SEEDED SPECIES

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The potential use of embryonic shoot apices as explants for cryopreservation of selected recalcitrant-seeded species

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

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PREFACE

The experimental work contained in this Master's dissertation was completed by the author while based at the School of Life Sciences, of the University of KwaZulu-Natal, Durban – Westville Campus, South Africa, at the Plant Germplasm Conservation Research Unit. This work was initially conducted under the supervision of the late Professor Patricia Berjak (from February 2014 to December 2014) as the main supervisor; Dr Dalia Varghese and Prof Norman W. Pammenter as co-supervisors and was later carried on by Dr Sershen Naidoo as the main supervisor from January 2015 to November 2017.

This study represents original work by the author and where previous work has been used, appropriate acknowledgements have been made in the text. The study herein has not been submitted to any other university for any degree or diploma, the results and outcomes reported are due to investigations by candidate.

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Anathi Nkayi

November 2017

DECLARATION

I, **Anathi Asanda Nkayi**, declare that:

(i)   The research reported in this dissertation, except where otherwise indicated, is my original research.

(ii) This dissertation has not been submitted for any degree or examination at any other university.

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

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Signed:

_____

November 2017

DEDICATION

This dissertation is dedicated to my pillow of strength, my late mother and my late supervisor, the most kind hearted, strong women. I will forever love and cherish you.

In loving memory of:

Tenjiwe Gloria Nkayi (mother)

and

Patricia Berjak (supervisor)

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ABSTRACT

Seeds can belong to two major categories, *viz.*, orthodox or recalcitrant. Recalcitrant seeds are desiccation, and often, chilling sensitive and thus cannot be stored for any meaningful time using conventional methods. The current decline in plant diversity necessitates urgent *in* and *ex situ* conservation measures but the challenges associated with the storage of recalcitrant seeds impedes *ex situ* germplasm conservation in a number of species. Cryopreservation seems to be the most promising way of conserving the germplasm of these species in the long-term. However, cryopreservation of zygotic embryos, embryonic axes and vegetative shoot meristems of many of these species has been largely unsuccessful: lack of shoot production, lack of root production or no survival following the various procedural steps involved in cryopreservation. This abnormal or lack of growth following cryopreservation has been attributed to variable combinations of excision damage, uneven drying, lethal ice crystal formation and metabolic stress. Reducing the size of the explants used has been suggested as a way of alleviating some of the potentially harmful consequence of cryopreservation in some recalcitrant-seeded species. This motivated the present study which assessed the potential use of embryonic shoot apices of three recalcitrant-seeded species, *Ammocharis coranica*, *Trichilia emetica* and *Castanospermum australe*, as explants for cryopreservation. These species were also selected for cryopreservation studies due to their medicinal value. *A. corani*ca contains alkaloid and triterpenoids, *T. emetica* seeds have oil used for treating rheumatism and broken bones, and seeds of *C. australe* contain alkaloids that exhibit anti-HIV properties.

The main objectives of this study were to assess the potential of excised embryonic shoot apices to develop into plantlets (root and shoot production) prior to exposing the explants to the various procedural steps of cryopreservation. Secondly, to assess the desiccation sensitivity of excised shoot apices, with the intention of identifying water contents (WCs) that are suitably low for cryopreservation and thirdly, to optimise cooling rates and regeneration conditions for successful cryopreservation of the excised shoot apices. The explants used were excised 2 mm away from the root pole of the embryonic axes and cultured on suitable medium to assess its potential to develop into full plantlets. These explants were then cryoprotected with three cryoprotectant solutions, *viz.*: 5 and 10% glycerol; 5 and 10%

glycerol + sucrose and plant vitrification solution 2 (PVS2), followed by dehydration by flash drying. The treated explants were subjected to three cooling treatments, *viz.*: slow cooling (using Mr. Frosty®); faster cooling (direct immersion into liquid nitrogen) and rapid cooling (using nitrogen slush). A cathodic water solution supplemented with ascorbic acid was used as a rehydration solution following each cryo-procedure. The selection of this solution was based on its anticipated antioxidant properties and its ability to ameliorate reactive oxygen species (ROS) produced as a result of excision injury and other pre-treatment steps.

Embryonic shoot apices of *A. coranica*, *T. emetica* and *C. australe* showed high potential to develop into full plantlets. The shoot apices of *A. coranica* and *T. emetica* produced complete plantlets *in vitro* on full strength MS medium with 0.8% Agar, 3% sucrose, supplemented with 1 mg L^{-1} BAP (6-benzylaminopurine) for shoot production and 0.1 mg L^{-1} IBA for root production. For *C. australe* shoot apices, full strength MS medium with 0.8% Agar, 3% sucrose, supplemented with 1 mg L^{-1} BAP and 0.1 mg L^{-1} GA$_3$ was found to be the best to induce shoot production and the same medium but with 0.1 mg L^{-1} IBA resulted in root production. The ability to produce complete plantlets validated the use of these explants for further cryopreservation studies.

For the assessment of desiccation sensitivity, two of the three species were used, *viz.*, *T. emetica* and *C. australe*. The results obtained from these studies showed that shoot apices of *T emetica* could be dried to WCs between *ca.* 0.51 and *ca.* 0.37 g g^{-1} with 15% viability loss and shoot apices of *C. australe* to WCs between *ca.* 0.40 and *ca.* 0.37 g g^{-1} with 10% viability loss. Water content and viability were also assessed after cryoprotection and flash drying. Shoot apices of *T. emetica* were treated with PVS2 only, but viability was lost completely; no further cryoprotection assessments were performed for this species, due to contamination during hydrated storage that resulted in loss of seeds. However, shoot apices of *C. australe* managed to retain viability after treatment with cryoprotectants and subsequent flash drying. The results obtained showed that 15% viability was lost after cryoprotecting explants with 5 and 10% of glycerol + flash drying to WCs between *ca.* 0.41 and *ca.* 0.37 g g^{-1} while there was a 20% loss of viability when explants were cryoprotected with 5 and 10% of glycerol and sucrose + flash drying to WCs between *ca.* 0.45 and *ca.* 0.36 g g^{-1}.

Cryopreservation was attempted for *C. australe* and *T. emetica* explants only, with survival (greening of explants and opening of leaf primordia) after cooling being limited to 16% in *C. australe* only. Faster cooling was the only cooling rate that resulted in survival of explants after cryopreservation. The findings of this study have made significant contribution towards the cryopreservation of genetic resources of recalcitrant-seeded species through exploring use of alternate explants (embryonic shoot apices) that have not been commonly used in cryopreservation studies. It can be concluded that shoot apices excised from embryos of recalcitrant-seeded species can result into full plantlets, thus serving as potential explants for cryopreservation of recalcitrant germplasm. The explants of two of the species investigated also withstood, to an extent, the impact of various stresses imposed during the procedural steps of cryopreservation. This was justified by the viability retention of these explants throughout the various steps of cryopreservation. That said, there is a need to further standardise the various steps of cryopreservation in order to obtain higher survival after cooling. Ultrastructural and biochemical assessments also could be carried out in future to understand the reasons for the poor survival of these explants following cryopreservation.

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explants and opening of leaf primordia) and explants that did not survive (cream-white and brown explants) cryopreservation taken 9 weeks after retrieval from liquid nitrogen.

ABBREVIATIONS, SYMBOLS AND UNITS OF MEASUREMENTS

APX	ascorbate peroxidase
ANOVA	analysis of variance
AsA	ascorbic acid
BAP	6-benzylaminopurine
CAT	catalase
CaMg	calcium magnesium solution
CaCl$_2$	calcium chloride
CaCl$_2$.2H$_2$O	calcium chloride dihydrate
CO$_2$	carbon dioxide
CP	cryoprotectant
CP1, 2 and 3	cryoprotectant treatment 1, 2 and 3
CW	cathodic water
^{o}C	degrees celcius
DEAT	Department of Environmental Affairs and Tourism
dmb	dry mass basis
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
FAO	Food and Agriculture Organization
FD	flash drying
g	gram
g g^{-1}	gram per gram
g L^{-1}	gram per litre
GA$_3$	gibberellic acid
GSH	glutathione
h	hours
H$_2$O	water
H$_2$O$_2$	hydrogen peroxide
IBA	indole-3-butyric acid
IPGR	International Plant Genetic Resources Institute
IUCN	International Union for Conservation of Nature
KCl	potassium chloride

L	litre
LN	liquid nitrogen
mg	milligram
mg L^{-1}	milligram per litre
mm	millimetre
ml	millilitre
ml L^{-1}	millilitre per litre
min	minutes
min^{-1}	per minute
M	molar
mM	millimolar
MgCl$_2$.6H$_2$O	magnesium chloride hexahydrate
MS	Murashige and Skoog
NaOCl	sodium hypochlorite
NaDCC	sodium dichloro-isocynaruate
no.	number
$^\bullet$O$_2^-$	superoxide
$^\bullet$OH	hydroxyl radical
^{1}O$_2$	singlet oxygen
pH	hydrogen ion concentration
ppm	parts per million
pt.	points
PVS2	plant vitrification solution 2
RH	relative humidity
ROS	relative oxygen species
RO$^-$	alkoxyl
ROO$^-$	peroxyl
rpm	revolutions per minute
SANBI	South African National Biodiversity Institute
SCR	seed coat/covering ration
SOD	superoxide dismutase
μM	micromolar
V	volt

v/v	volume per volume
w/v	weight per volume
WC	water content
wmb	wet mass basis
WPM	Woody Plant Medium
%	per cent

CHAPTER 1: INTRODUCTION

Plants play a crucial role in the basic functioning of ecosystems by providing shelter, serving as a food source and fixing carbon dioxide. Habitat destruction and unsustainable harvesting of plants for medicinal use has had a negative impact on plant biodiversity in many parts of the world (Chapin *et al*., 2000). According to Tandon *et al*. (2009), loss in biodiversity is mainly attributed to human population growth together with unsustainable patterns of consumption, increasing production of waste and pollutants, deforestation, urban development, developmental activities such as hydroelectric plants and roads and finally modern agriculture which involves clearing of land and the introduction of new and uniform varieties. Due to human's alteration of the global environment, the atmospheric carbon dioxide (CO_2) and methane concentrations have since increased (Chapin *et al*., 2000), and this contributes towards global climate change which is predicted to also impact on plant biodiversity (Bellard *et al*., 2012). In this regard, human activities and various disturbances have already resulted in the extinction of 5-20% of various groups of organisms, which includes plants (Chapin *et al*., 2000). Of the world's 270, 000 estimated plant species, 34, 000 are endangered (Tandon *et al*., 2009) and many are presently threatened or endangered (Berjak *et al*., 2011a; IUCN, 2011).

Given the unprecedented rate of climate change, some species may not be able to adapt to environmental conditions in a given region and this failure to adapt could lead to their local or global extinction (Bellard *et al*., 2012). Thus, in order to persist, individual species must produce adaptive responses (Bellard *et al*., 2012). In the wake of these treats, it is of paramount importance to find reliable ways to preserve the genetic resources. South Africa is known for its rich plant diversity, hosting 10% of the world's plant species (DEAT, 2005; Berjak, *et al*., 2011a). However, land use patterns, agricultural practices and unsustainable harvesting for medicinal use could erode South Africa's plant diversity (Rao, 2004). The major concern with loss of plant diversity is the impact that it could have on food security as plant genetic resources are the main source of food (Rao, 2004). It is therefore of utmost importance that the plant genetic resources be conserved. Conservation entails supporting sustainable development through protection and use of biological resources in ways that will not destroy the ecosystem or impair the world's genetic and species diversity (Tandon *et al*., 2009).

Plant diversity can be sustained and preserved through conservation of plant genetic resources. Several programs have been initiated for the conservation of genetic resources and many of these involve the use of gene banks to conserve genetic diversity (Rao, 2004). There are two ways in which plant genetic resources can be conserved: (1) *in situ* conservation, and (2) *ex situ* conservation (Engelmann and Engels, 2002; González-Benito *et al*., 2004). *In situ* conservation involves the maintenance of genetic resources in their natural habitats and *ex situ* conservation involves conservation of genetic resources outside their natural habitat (Altieri and Merrick, 1987; Engelmann and Engels, 2002; Rao, 2004; Tandon *et al*., 2009). These two conservation strategies have their own specific

techniques, where *ex situ* involves mainly seed storage, *in vitro* storage, storage of DNA, pollen, field gene banks and botanic garden storage, while *in situ* strategies include storage in protected areas, on-farm and home garden conservation (Engels and Wood, 1999; Engelmann and Engels, 2002). Seed storage is the most efficient and convenient method of long term storage of plant genetic resources, as this method allows for storage of seeds under low relative humidity (RH) and low temperatures for extended periods of time (years to centuries [González-Benito *et al*., 2004; Rao, 2004]). However, not all plant species produce seeds that can be stored using conventional seed storage methods (González-Benito *et al*., 2004).

In this regard, seeds produced by orthodox-seeded species can generally be stored in the dry state under low RH conditions and sub-zero temperatures (Chin *et al*., 1989). Orthodox seeds are shed at low WCs, undergo maturation drying prior to shedding and can be dried down to very low moisture contents (around 5% or 0.05 g water per gram on fresh mass basis) without losing viability (Chin *et al*., 1989; Han *et al*., 1997; Berjak and Pammenter, 2002). However, some tropical, subtropical and temperate species produce recalcitrant or intermediate seeds that cannot be stored in this manner for any significant period of time (Rao, 2004). These types of seeds are known as non-orthodox seeds (Chin *et al*., 1989). Recalcitrant seeds are shed at high WCs ranging from *ca.* 0.43 to 4.0 grams water per gram dry mass (g g^{-1}) depending on the species (Berjak and Pammenter, 2002; Berjak and Pammenter, 2008). Recalcitrant seeded-species are known for their variability in embryonic axis WC, within and across species as well as inter- and intra- seasonally (Berjak and Pammenter, 2013). Studies on *Camellia sinensis* seeds for example, showed that seeds harvested from the same tree population exhibited WCs that ranged from 2.0 to 4.4 g g^{-1} (Berjak and Pammenter, 2008; Berjak and Pammenter, 2013). According to Berjak and Pammenter (2002), recalcitrant seeds undergo little to no maturation drying causing them to remain desiccation-sensitive during their development and after they are shed. Upon shedding, these seeds are quick to lose viability, are prone to fungal contamination and cannot survive desiccation (Rao, 2004; Berjak and Pammenter, 2013).

Although all recalcitrant seeds are considered to be desiccation sensitive, their degree of tolerance to water loss differs across species (Farrant *et al*., 1989; Han *et al*., 1997). In addition to being desiccation sensitive, the seeds of some recalcitrant-seeded species are also chilling sensitive (Farrant *et al*., 1988; Han *et al*., 1997). Studies have suggested that chilling sensitivity may also be provenance related, however, further investigations on the nature of the chilling injury are needed (Berjak and Pammenter, 2008). Given the above, recalcitrant seeds cannot be stored for long periods of time under conditions used for storage of orthodox seeds. Thus, storage of recalcitrant seeds can only be for short to medium term, by maintaining seeds at the lowest temperatures that they can withstand and at high relative humidity that maintains them at WCs close to that at shedding (Berjak and Pammenter, 2008; 2013). Intermediate seeds represent another group of non-orthodox seeds that are unamenable to long term storage but partially dried seeds of some intermediate-seeded species can be stored for short periods of time (González-Benito *et al*., 2004).

As a consequence of the difficulties associated with seed storage in recalcitrant- and intermediate-seeded species, other methods for long term germplasm storage of such species have to be explored. Currently, cryopreservation is the only feasible option for long term storage of the germplasm of species producing recalcitrant and intermediate seeds (Engelmann, 2011a). Cryopreservation entails the storage of plant germplasm at ultra-low temperatures commonly in liquid nitrogen (LN) at -196°C, in the vapour phase of LN at -140°C to -160°C (Wesley *et al*., 1992; Sershen *et al*., 2007; Berjak, *et al*., 2011b; Varghese, *et al*., 2011). In this way long term storage of recalcitrant germplasm can be achieved, since cell division and metabolic activities are suspended at these temperatures and there is presumably no alteration or modification of the material for theoretically long periods of time (Mycock *et al*., 1995; Engelmann, 2004; Rao, 2004).

However, for successful cryostorage, the tissue needs to be sufficiently dehydrated and cooled at an appropriate rate to prevent lethal ice crystal formation (Walters *et al*., 2013). Ice crystal formation is one of the main reasons for the limited success in cryostorage of recalcitrant seed germplasm (Berjak and Pammenter, 2013). Lethal ice crystal formation can be avoided by using explants that are sufficiently small in size, facilitating more rapid diffusion of water and heat transfer, and partially drying these to WCs that avoid lethal ice crystal formation (Walters *et al*., 2013). The problem with recalcitrant seeds is that, unlike orthodox and intermediate types, successful cryopreservation of whole seeds is impossible due to their large size and their highly hydrated nature encourages lethal ice crystal damage (Berjak *et al*., 2011b; Walters *et al*., 2013). Hence, there is a need for the use of explants that are smaller in size such as zygotic embryos, embryonic axes, shoot apices or meristems and in some cases dormant buds (González-Benito *et al*., 2004). The embryonic axes are usually the explant of choice for cryopreservation of recalcitrant-seeded species (Berjak and Pammenter, 2013) as they are small, contain the genetic information of the maternal plant and have the ability to produce a whole plant (Chandel *et al*., 1995). Studies have also shown that zygotic embryos and embryonic axes of a number of recalcitrant-seeded species can be dried to WCs sufficiently low for successful cryopreservation using a flash dryer which involves placing samples over activated silica gel and allowing dry air to pass over them (Berjak *et al*., 1990; Pammenter *et al*., 2002). In some cases, the combination of flash drying and cryoprotection is used, which involves exposing samples to chemical substances that reduce tissue WC osmotically and/or concentrate the cell contents, allowing the intracellular contents to vitrify without causing ice crystal formation during freezing (Berjak and Pammenter, 2013).

The procedural steps involved in the cryopreservation of seed derived explants generally include the following: (1) excision of the explant; (2) decontamination; (3) cryoprotection; (4) flash drying; (5) cooling; (6) cryostorage; (7) thawing and rehydration; (8) decontamination; (9) recovery on specific germination media depending on the species and lastly; (10) hardening off (Berjak *et al*., 2011b; Varghese *et al*., 2011). Each of these steps, particularly excision (Goveia *et al*., 2004) and partial

dehydration (Berjak *et al*., 2011b) can impose physical, biochemical and in most cases both these types of stresses/damage on the explants, resulting in abnormal, incomplete, poor or even no survival in many species (Goveia *et al.*, 2004; Berjak *et al*., 2011b). This stress/damage is most often related to the over-production of reactive oxygen species (ROS) (Berjak *et al*., 2011b; Varghese *et al*., 2011). Reactive oxygen species are known to be highly toxic and damaging in plant tissues if unquenched (Varghese and Naithani, 2008; Whitaker *et al*., 2010).

Many authors have claimed that oxidative stress associated with the various procedural steps involved in cryopreservation can hinder post-cryo shoot and/or root production (Varghese and Naithani, 2008; Berjak *et al.*, 2011b). The most commonly produced ROS are superoxide ($^{\bullet}O_2^-$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2), where $^{\bullet}O_2^-$ has been observed to be associated with shoot meristem necrosis, which leads to the failure of shoot production (Berjak *et al.*, 2011b). Furthermore, as embryos have been selected as the explant of choice for most cryopreservation studies, they face some challenges. Most embryos experience the problem of inability to produce shoots due to excision of the cotyledonary body (Pammenter *et al*., 2011; Ballesteros *et al*. 2014). This is known as excision damage, which is a consequence of a burst in ROS following excision, in close proximity to the shoot apex, resulting in a failure to produce shoots (Pammenter *et al*., 2011; Ballesteros *et al*., 2014).

These threats of oxidative stress, physical damage to the embryo and uneven drying across the embryo tissues may explain why post-cryo embryonic axis/zygotic embryo survival in a number of recalcitrant-seeded species is limited to root production only, as in *T. dregeana* (Pammenter *et al*., 2011), *E. capensis* (Perán *et al*., 2006) and *L. kirkii* (Kistnasamy *et al*., 2011), and/or poor vigour, as in shoot meristems of *T. emetica* (Varghese *et al*., 2009). Post-cryo success in recalcitrant-seeded species is therefore largely dependent on explant of choice, e.g. zygotic embryo/embryonic axis (Berjak *et al*., 1995; Goveia *et al*., 2004; Sershen *et al*., 2007) and shoot meristems (Varghese *et al*., 2009; Gebashe, 2015). There are, however, challenges associated with choosing embryonic axes/embryos: they are of complex tissue composition with differential drying rates across the different tissues (Ballesteros *et al*., 2014) and they are constantly changing in terms of their developmental stage and consequent degree of resistance to desiccation and cooling (Engelmann, 2004). So, even though zygotic embryos/embryonic axes have been the most commonly used explant for cryopreservation of recalcitrant seed germplasm, it is not always easy to achieve successful cryopreservation using these explants, for reasons discussed above.

Thus, alternative explants need to be explored for cryopreservation of a number of recalcitrant-seeded species. Use of alternative explants such as shoot apices excised from embryos, adventitious buds or somatic embryos have been suggested as potential alternative explants to overcome the problems posed by embryos and embryonic axes (Pence, 1995; Engelmann, 2004). Shoot meristems of *Trichilia emetica* from *in vitro* germinated plants have been successfully used as explants for cryopreservation but the protocol is extremely labour intensive and prone to failure at certain stages

(Varghese *et al*., 2009; Gebashe, 2015). In light of this, the current study therefore explores the use of embryonic shoot apices as alternative explants for cryopreservation of selected recalcitrant-seeded species. However, it is of paramount to mention that similar explants have been used before in a study by Chmielarz *et al.* (2011). Those authors used shoot apical meristems from embryonic axes (plumules) to successfully cryopreserve the germplasm of *Quercus robur* (Chmielarz *et al.*, 2011).

In order to achieve success in cryopreservation, optimisation of various variables such as size of the explant, correct type and concentration of cryoprotectants, WC of the explant and rate of cooling and thawing (Mycock *et al*., 1995), is required. These variables were therefore optimised in the current study. The main aim of the current study was to investigate the potential use of embryonic shoot apices as explants for cryopreservation of three recalcitrant-seeded species, *viz*. *Ammocharis coranica*, *Trichilia emetica* and *Castanospermum australe*, which have proven impossible to successfully cryopreserve to date. The reasons behind the selection of the embryonic shoot apices as candidate explants were as follows: they allow for a reduction in explant size relative to the embryonic axes traditionally used as smaller explants, which may favour more rapid dehydration; the selection of shoot tissue only (and hence, homogenous tissue) may facilitate more even drying (Ballesteros *et al*., 2014) across the explant. The specific objectives of the study included:

- Ensuring that shoot apices from the three selected recalcitrant-seeded species have the potential to develop into full plantlets (roots and shoots) prior to implementation of the cryo-stages.
- Assessing the impact of various procedures preceding and following cryopreservation of excised shoot apices of *Trichilia emetica* and *Castanospermum australe* on their ability to produce plantlets.

It was therefore hypothesised that embryonic shoot apices can be used as explants for the conservation of germplasm of recalcitrant-seeded species.

CHAPTER 2: LITERATURE REVIEW

2.1 Biodiversity in South Africa

According to Tandon *et al*. (2009), biodiversity refers to the variability among living organisms from all sources, which include terrestrial, marine and aquatic ecosystems. Biodiversity as a whole constitutes the biological basis for the world's food security because it provides genetic resources for food and agriculture (Tandon *et al*., 2009). South Africa takes up only 2% of the total land globally but is known to possess high biodiversity due to its geographic positioning and varying climatic conditions (Thuiller *et al*., 2006; Berjak *et al*., 2011a). According to the Department of Environment and Tourism (DEAT), 2005, both plants and animals contribute towards South Africa's rich diversity, as it hosts various biomes and almost 10% of the global plant species (Berjak *et al*., 2011a).

The biodiversity as we see it in South Africa together with the rest of the world is currently under threat and is declining at an unprecedented rate (Sarasan *et al*., 2006; Tandon *et al*., 2009). This is mainly due to anthropogenic pressures, invasive alien species, natural disturbances and unpredictable climatic changes (DEAT, 2005; Berjak *et al*., 2011a). According to Heywood and Iriondo (2003), human interactions with the environment has led to habitat loss, degradation and fragmentation of land as well as subsequent loss of species and genetic resources. This is mainly due to land use change, pollution and unsustainable harvesting of natural resources (Tandon *et al*., 2009; Berjak *et al*., 2011a; Reed *et al*., 2011). Because of these reasons, many species are facing the threat of extinction and according to the Red Data List (2009), quite a number of unique taxa are already extinct, with some being categorized as being critically endangered/possibly extinct and some as being endangered (Berjak *et al*., 2011a). Provided below is a table (Table 2.1), taken from South Africa's Red Data List (SANBI, 2006), which shows how the number of threatened plant species in South Africa has risen from 1980 to 2005. This gives a clear understanding and validation of the effects disturbances have on the ecosystem and raises concerns for remediation.

Table 2.1: Threatened South African plant taxa as listed in the Red Data List from 1980 to 2005 (Adapted from SANBI, 2006).

Category	1980	1996	2002 (only 25% of flora assessed)	2005 (inter-mediate list)
Extinct	39	56	15	48
Extinct in the wild	*n/a*	*n/a*	*n/a*	7
Critically endangered	n/a	*n/a*	19	175
Endangered	104	241	58	216
Vulnerable	165	422	322	814
Rare	521	1 322	*n/a*	*n/a*
Data deficient	*n/a*	*n/a*	108	610
Total assessed	1 893	3 268	948	18 057
Total threatened	790	1 985	399	1 205

The decline in biodiversity which is attributed by ecosystem degradation will in the long term have a major negative impact on the economy at large (Adams *et al*., 2004) and will also affect the use of medicinal plants and herbs in traditional medicine (Berjak *et al*., 2011a). This will affect at least 70% of the South African population that use plants for medicinal purposes (von Ahlefeldlt *et al*., 2003). In order to reduce the rate of plant species extinction more effective conservation strategies need to be employed. These strategies must support sustainable development through protection and use of biological resources in ways that do not diminish genetic variation or destroy important habitats and ecosystems (Tandon *et al*., 2009).

2.2 Conservation strategies

According to the World Conservation Strategy (1980), the term conservation was broadly explained as 'the management of human use of the biosphere so that it may yield the greatest sustainable benefit to the present generations, while maintaining it's potential to meet the needs of and aspirations of the future generations' (Heywood and Iriondo, 2003). Jordan (1995), further defined conservation in terms of 'preservation as a philosophy of managing the environment in such a way that does not despoil, exhaust, or destroy it or the resources and values it contains'. Conservation is, however, more than a concept but a method that can be implemented to preserve genetic resources for the future. The global concern about the decline of valuable genetic resources has promoted the exploration of many new conservation strategies (Paunescu, 2009). The primary aim of conservation is to maintain diversity of living organisms and this can be achieved through the use of two conservation strategies: *in situ* and *ex situ* conservation (defined below [Heywood and Iriondo, 2003; Tandon *et al*., 2009]). These

conservation strategies were put forth by the Society of Conservation Biology (Heywood and Iriondo, 2003).

In order to preserve plant genetic resources, it is worth considering that since plants and their propagules respond differently to environmental change, not all plants/propagules can be preserved using the same method or technique. The most ideal way of conserving plant genetic resources is by seed storage (discussed below [Reed *et al*., 2001]). However, plants produce seeds of different types, which behave differently, and identifying the seed type is one of the key requirements for plant germplasm conservation (Xin *et al*., 2010). Thus, the choice of the plant germplasm conservation strategy depends primarily on the post-harvest behaviour of the seeds, as well as the availability of resources and the primary objective of the particular conservation strategy (Tandon *et al*., 2009).

2.2.1 *In situ* conservation

This is a conservation strategy that entails the maintenance of genetic resources in their natural habitats (Engelmann and Engels, 2002; Tandon *et al*., 2009). As much as *in situ* conservation maintain plants in their natural environment, the threat of natural disasters such as forest fires, high temperatures, and other extreme weather conditions such as wind and precipitation do exist in this type of conservation (Tandon *et al*., 2009). Thus, in view of climate change and anthropogenic degradation of ecosystems, this type of conservation should be complemented by other conservation techniques.

2.2.2 *Ex situ* conservation

Ex situ conservation strategy refers to the storage of plant germplasm outside of their natural environments, where samples are collected, transferred and stored in desired areas (Engelmann and Engels, 2002). Amongst the various *ex situ* conservation approaches, such as seed storage, *in vitro* storage, DNA storage, pollen storage, field genebanks and botanic gardens (Engelmann and Engels, 2002; Paunescu, 2009), the three most commonly used *ex situ* techniques are cultivation in botanic gardens, seed storage and *in vitro* storage (Paunescu, 2009; Kaviani, 2011). Even though botanic garden conservation is an effective conservation strategy for threatened and endangered species, time and space are often limiting factors.

Seed storage is one of the most promising *ex situ* conservation strategies because it allows for long term storage (years to centuries) of the germplasm when carried out at under condition of low RH and temperatures (Paunescu, 2009). However, these storage conditions are only suitable for orthodox, i.e. desiccation tolerant, seeds (Engelmann and Engels, 2002). Seed storage is, however, not feasible for some species, as they produce non-orthodox, i.e. desiccation sensitive, seeds which quickly lose viability upon desiccation to relatively high WCs (Paunescu, 2009). Recalcitrant and intermediate seeds fall within this un-storable category but advancements in biotechnology have been made through the use of *in vitro* culture techniques (Paunescu, 2009) for alternate methods to conserve germplasm of such plant species.

2.3. Seed storage and behavior

Storage of seeds is the most common method of *ex situ* germplasm conservation because seeds are representative of genetic diversity and seed collection is a cost-effective way of storing genetic resources (Pritchard, 2004; Reed *et al.,* 2011; Berjak and Pammenter, 2014). However, seeds of different species can differ in terms of their post-harvest physiology, necessitating different methods of seed storage. As mentioned earlier, orthodox seeds can be stored for extended periods using conventional seed storage methods of low WCs and low RH (Ellis *et al.*, 1990; Pammenter and Berjak, 1999; Walters *et al.*, 2001; Berjak and Pammenter, 2008; 2013). Desiccation sensitive seeds of many species can tolerate neither dehydration, nor storage at low temperatures (Berjak *et al.*, 1984; 1990; Chaitanya and Naithani, 1994; Varghese and Naithani, 2002; Sershen *et al.*, 2008; Sershen *et al.*, 2016). Thus, the remarkable ability of desiccation tolerance in seeds is the primary basis of seed longevity and storability during *ex situ* storage (Berjak *et al.*, 2007; Walters, 2015). According to these storage characteristics, seeds can be classified as either orthodox or non-orthodox (recalcitrant and intermediate) seeds.

2.3.1 Orthodox seeds

Orthodox-seeded species produce seeds which can tolerate desiccation as a consequence of undergoing a period of maturation drying and entering a state of metabolic quiescence on the mother plant during their development (Berjak, 2002). They can be stored in this dry state at low temperatures for extended periods of time (Engelmann and Engels, 2002). This seed category consists of a large number of wild and agriculturally important plant species, which comprise approximately 90% of plant species studied thus far (Berjak *et al.*, 2011b). The ability of orthodox seeds to tolerant desiccation is achievable during the seed development (Bewley and Black 1994; Kermode and Finch-Savage, 2002; Kalemba *et al.*, 2009; Sahu *et al.*, 2017). There are three different phases during seed development of orthodox seeds (Figure 1) *viz.*, histo-differentiation, accumulation of reserves and maturation (Berjak and Pammenter, 2000; Kermode and Finch-Savage 2002; Berjak and Pammenter, 2013). Histo-differentiation is a phase that begins just after fertilization and intensive metabolic activity, where tissues differentiate causing seed morphology to become apparent (Berjak and Pammenter, 2000; Berjak and Pammenter, 2013).

It is then followed by the accumulation of reserves such as starch, protein and lipids that are needed to sustain seedling growth (Berjak and Pammenter, 2000). Studies show that it is during this phase where the ability to tolerate desiccation is acquired and this prepares the seed tissue to withstand the stresses associated with dehydration during the maturation drying phase (Berjak and Pammenter, 2013). Lastly, maturation drying is a phase where the seeds' dry mass stabilizes and the water is lost, consequently causing a decline in fresh mass (Berjak and Pammenter, 2000). The maturation drying phase of seed development is associated with metabolic changes that are required for transition from development to the germination phase and for providing protection to dry seeds from environmental stress (Kermode and Finch-Savage, 2002). In fact, germinability and desiccation tolerance are two key

attributes that appear in developing seeds during maturation and are essential for the successful perpetuation of the next generation (Pammenter and Berjak, 1999; Kermode and Finch-Savage, 2002; Berjak and Pammenter, 2008). Orthodox seeds are regarded as desiccation tolerant because they have the ability to express all the three steps of development which confer desiccation tolerance.
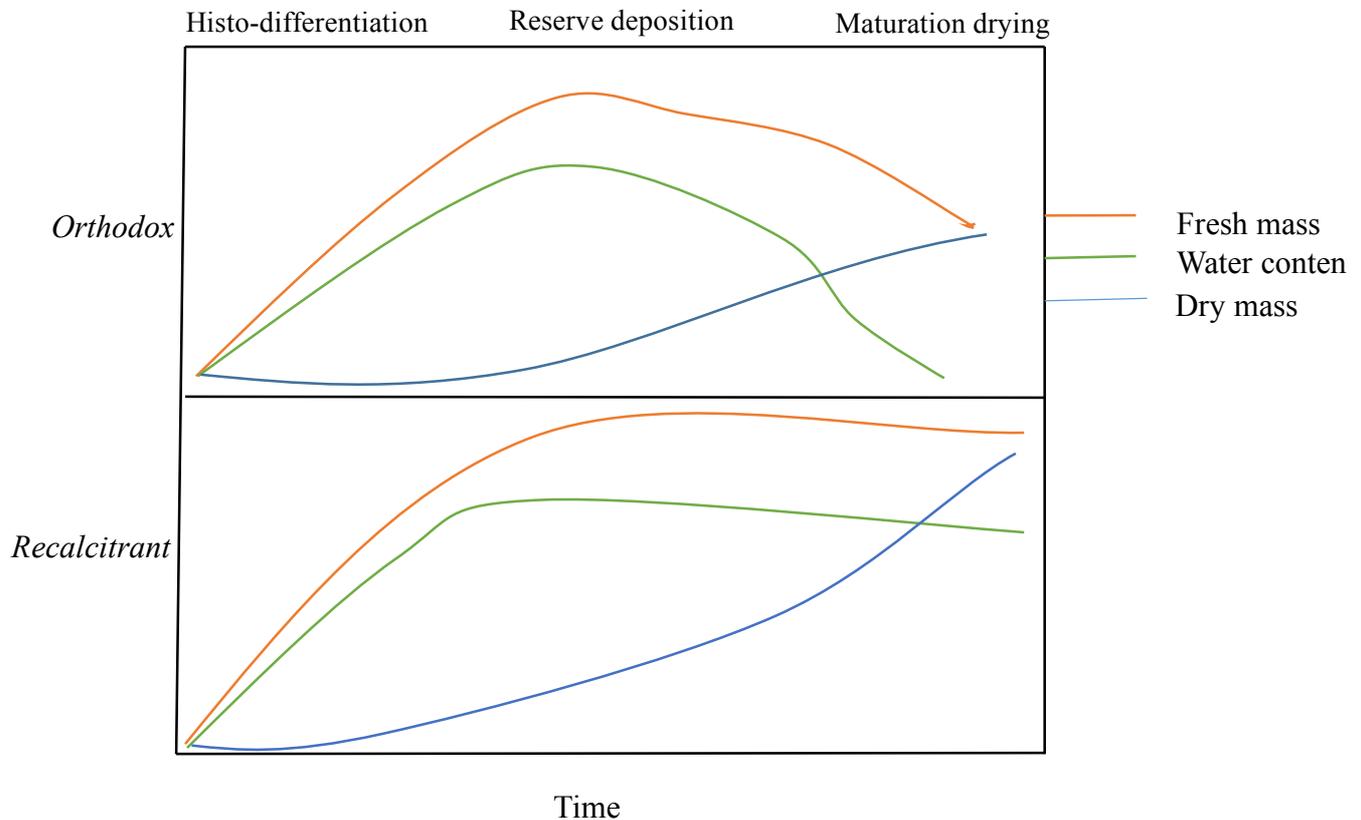
Figure 2.1: A schematic representation of changes in orthodox and recalcitrant seeds (fresh mass, dry mass and water content) during seed development (adapted from Berjak and Pammenter, 2000).

As highlighted earlier, conservation of plant species is done through seed storage and storage of orthodox seeds is usually achieved through drying seeds to WC of about 1-5% dry mass basis (dmb) (Han *et al*., 1997; Berjak and Pammenter, 2002) and storage at ultra-low temperatures of approximately -18°C or cooler [Food and Agriculture Organization and International Plant Genetic Resources Institute - (FAO and IPGR), 1994; Engelmann and Engels, 2002]. Because of the seed qualities mentioned above, orthodox seeds can maintain vigour and viability in storage until the next season and for many decades at -18°C (Berjak and Pammenter, 2000; Berjak, 2002) or in the vapour of liquid nitrogen at -120°C to -150°C (Walters *et al*., 2005). Recent developments with regards to storage of orthodox seeds entails desiccation of seeds to much lower WCs than usual (ultra-dry), thus allowing for the storage of seeds for even longer periods of time (Engelmann and Engels, 2002). Ultra-drying is the act of desiccating seeds to moisture contents less than 5 – 7%, affording safe storage at ambient temperatures

(Li *et al*., 2007; Bonner, 2008). The advantages of this technique are that it is cost efficient as it can reduce the cost of constructing and maintaining seed banks, it can be employed in cases where there is inadequate refrigeration for seed storage and has a promising application in germplasm conservation (Engelmann and Engels, 2002; Li *et al*., 2007).

Engelmann and Engels (2002) highlighted that long term storage of orthodox seeds is also determined by the initial quality of the seed, processing procedure as well as the storage conditions. Previous studies show that collecting orthodox seeds in their immature state causes them to rapidly lose vigour and viability while in storage, thus in order to achieve long term storage, seeds should be at their mature state (Bonner, 2008). This is because immature seeds have not completely developed, lack food reserves and there is incomplete morphological development and cell organization (Bonner, 2008). Reduced viability of orthodox seeds in storage may also be attributed to seeds damaged during extraction and conditioning and/or fungal contamination that progresses during storage (McLean and Berjak 1987; Bonner, 2008). Thus, retention of vigour and viability during long term storage of orthodox seeds depends on seed collection at the right developmental stage and prevention of damage to seeds as well as maintenance of proper storage conditions.

Depending on the seed physiology, orthodox seeds can be classified into two groups, as true orthodox or sub-orthodox seeds. Orthodox seeds with WCs reduced to about 5 – 10% wet mass basis (wmb), and storable at sub-zero temperatures for longer periods of time without losing vigour or viability are known as true orthodox seeds (Bonner, 2008). True orthodox seeds include mostly tree species from the Northern Temperate Zone (*Pinus* L., *Prunus* L., *Fraxinus* L., etc.), as well as species of the tropical and subtropical origin (*Acacia* L., and *Eucalyptus* L' Her.) (Bonner, 2008). Orthodox seeds that are also storable at sub-zero temperatures but for shorter periods are known as sub-orthodox seeds (*Fagus* L., *Populus* L., and some pine). Differentiation between the two classes of orthodox seeds is not easy but can assist in selecting the appropriate storage procedures as many orthodox species lose viability within a few years even under ideal conditions of storage.

2.3.2 Recalcitrant seeds

Recalcitrant-seeded plant species produce seeds that are shed hydrated and cannot be stored for any meaningful period of time (King and Roberts 1980; Berjak *et al*., 1984; Farrant *et al*., 1989; Pammenter and Berjak; 1999). This is because of their ongoing metabolic activity throughout development and even after they are shed (Berjak and Pammenter, 2008; 2013). These seeds are usually large, desiccation and/or chilling sensitive, and lose viability concomitantly with water loss (Dickie and Pritchard 2002; Berjak *et al*. 2011b), often within a few days (e.g. *Shorea robusta* [Chaitanya and Naithani, 1994] and *Avicennia marina* [Farrant *et al*., 1989]) to a few weeks (e.g. *Madhuca indica* [Varghese *et al*., 2002]). Recalcitrant seeds are unamenable to conventional seed storage techniques (i.e. reduced temperature, seed moisture content and RH), making it difficult to conserve. Seeds of some species can, however, be stored in the short to medium term (days to months) via hydrated storage which involves storage of

seeds in a 16°C freezer, in sterilized buckets moistened with 1% sodium hypochlorite (NaOCl) (Naidoo, 2012). Plant species which produce recalcitrant seeds are estimated to make up 70% of the rainforest plants, and are also mostly found in the tropics and sub-tropics (Berjak and Pammenter 2000; Pammenter and Berjak, 2014). With regards to South African plant species that have been screened, it was revealed that seeds of at least 17 herbaceous geophytic amaryllids and a further 13 unrelated species exhibited recalcitrant seed storage physiology (Erdey *et al.*, 2007; Sershen *et al.*, 2008; Berjak and Pammenter, 2008). More recent work which is still undergoing has revealed that the estimated number of recalcitrant species in South Africa is 81 (personal communication with Ashley Subbiah[1]).

One of the most important things to note about recalcitrant seeds is that they are highly variable in various aspects, including desiccation sensitivity (Farrant *et al.*, 1989; Sershen *et al.*, 2008; Ballesteros *et al.*, 2014) and seed storage longevity (Berjak and Pammenter, 2008; Moothoo-Padayachee *et al.*, 2016), both within or across species as well as inter- and intra-seasonally (Pammenter and Berjak, 1999; Sershen *et al.*, 2008; Berjak and Pammenter, 2013). Seeds of temperate species also appear to be less sensitive to desiccation (Berjak and Pammenter, 2004) and chilling than those of tropical species (Berjak and Pammenter, 2008). In order to understand desiccation sensitivity in recalcitrant seeds, one needs to understand the degree and the continuum of recalcitrance as this varies amongst species (Berjak and Pammenter, 2013).

Due to this degree of variation of recalcitrance, seeds are categorized into temperate-recalcitrant and tropical-recalcitrant seeds (Bonner, 2008). Seeds that are classified as temperate-recalcitrant seeds, e.g. *Aesculus hippocastanum, Q. robur, Q. rubra* (Pritchard, 1991), are unable to be dehydrated to low WCs like orthodox seeds, but can be stored at temperatures slightly below freezing for approximately 3 to 5 years (Bonner, 2008). Tropical recalcitrant seeds on the other hand have limited longevity and are storable at temperatures below 10 to 15°C for much shorter periods, often for a few days or weeks (Bonner, 2008). Based on a study by Daws *et al.* (2006), a model was developed to predict desiccation sensitivity of recalcitrant seeds based on seed mass and seed coat or covering ratio – SCR. According to the model, it was shown that there was a significant relationship between desiccation sensitivity and relatively low SCR, typified by large seed size with thin coverings (Daws *et al.*, 2006). Studies have tried to correlate desiccation sensitivity/tolerance with developmental stages (Goveia *et al.*, 2004; Sahu *et al.*, 2017); decline in pre-shedding WC (e.g., *Q. robur*, *E. capensis* and *A. hippocastanum* [Berjak and Pammenter, 2008]); heat sum of the environment during development (Daws *et al.* 2004), rate at which water is lost from the seed tissues (Farrant *et al.*, 1989; Pammenter *et al.*, 1998; Varghese *et al.*, 2011; Ballesteros *et al.*, 2014); and geographical location or provenance (Sershen *et al.*, 2008; Bharuth

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et al., 2007). However, the inter- and intra-species variability is so enormous that it is difficult to single-out the determinant of degree of recalcitrance.

Unlike orthodox seeds, recalcitrant seeds undergo little or no maturation drying during development on the mother plant, and as a consequence are shed at high WCs ranging from *ca.* 1.42 (*Protorhus longifolia*) to *ca.* 6.0 (*Crinum bulbispermum*) g g^{-1} on a dmb (Berjak, 2002; Berjak and Pammenter, 2008; Ballesteros *et al*, 2014). Because of this, recalcitrant seeds lack the ability to express desiccation tolerance because they do not express the genetic information that permits for desiccation tolerance due to them not undergoing the final stage of seed development (maturation drying; refer to Figure 2.1, [Berjak and Pammenter, 2002; Pammenter and Berjak, 2014]). Many factors have been reported to influence their post-harvest responses to dehydration which may vary amongst species, and includes shedding WC (Finch-Savage, 1996; Berjak 2002), rate at which water is lost during dehydration (Pammenter *et al*., 1991; Pammenter *et al*., 1998; Varghese *et al*., 2011), developmental status (Goveia *et al*., 2004) and chilling sensitivity (King and Roberts, 1980; Chaitanya and Naithani, 1998; Sershen *et al*., 2007). Thus, mechanisms or processes that confer desiccation sensitivity may vary across species, with recalcitrant seeds of different species showing different responses to the same drying regime (Farrant *et al*., 1989; Berjak and Pammenter, 2002; Pammenter and Berjak, 2014).Various recalcitrant seeds also show differences in the rates at which they lose water when subjected to the same dehydration conditions (Farrant *et al*., 1989; Berjak, 2002; Pammenter and Berjak, 2014; Subbiah *et al*., 2017). It was thus suggested that the rate at which water is lost may be inversely proportional to the water concentration at which viability was lost (Berjak, 2002; Pammenter and Berjak, 2014).

Rapid or ultra-rapid drying have allowed embryos/embryonic axes to reduce the WC to very low levels without significant loss in viability (Pammenter *et al*., 1998; Liang and Sun, 2002; Varghese *et al*., 2011). Drying of recalcitrant seeds to low WCs especially at slow drying rates has been widely reported to have deleterious effects on the ultrastructure of cells however, rapid dehydration may cause low WC to be reached before any adverse ultrastructural damage occurs (Liang and Sun, 2002; Berjak and Pammenter, 2008). During rapid dehydration explants get a rapid passage through the intermediate WC ranges which are the zone for aqueous-based metabolism linked damages (Berjak and Pammenter 2008). As highlighted earlier, recalcitrant seeds are highly metabolically active, thus they initiate germination around shedding and this increases desiccation sensitivity during dehydration as germination may still persist as the tissues are being dehydrated (Berjak and Pammenter, 2008). In a study by Liang and Sun (2002), it was shown that during desiccation, cumulative damage occurs to cell tissues due to physiological effect or metabolism alerations which result in lose in viablity. It was also suggested that recalcitrant seeds may lose vigour and viability after dehydration due to mechanical damage, metabolism-induced damage as well as macromolecular denaturation (Pammenter and Berjak, 1999; Varghese *et al*., 2011).

Recalcitrant seeds mostly lose viability at the desiccation stage because they are unable to withstand dehydration to levels necessary to avoid ice-crystallisation, thus the germplasm dies due to desiccation damage (Wesley-Smith *et al*., 1992). Since it is widely understood that desiccation of recalcitrant seeds, whether slow or rapid, results in the inevitable loss of viability, a reliable way to store these seeds (prior to attempting long term conservation) in the short-to medium-term is by maintaining seeds as close to the shedding WC as possible (Berjak and Pammenter, 2008; Moothoo-Padayachee *et al*., 2016) using hydrated storage. Hydrated storage entails storing seeds at high RH and lower temperatures but not low enough to cause chilling injury.

However, even with hydrated state many recalcitrant seeds do not store well, often resulting in storage germination (Sershen *et al*., 2008; Moothoo-Padayachee *et al*., 2016). According to Berjak and Pammenter (2008), viability loss of germplasm while in hydrated storage is brought about by slow water loss during on-going germination and proliferation of microorganisms (fungi). The proliferation of fungi in seeds placed under hydrated storage is also detrimental to recalcitrant seeds particularly in species of tropical/subtropical provenance (Berjak and Pammenter, 2013). Seeds undergoing germinative development in hydrated storage therefore lose viability due to a mild desiccation stress since additional water is not supplied to complete germination (Pammenter *et al.*, 1984; Farrant *et al*., 1986; Berjak *et al.*, 2000). Long term storage of recalcitrant germplasm has been observed to be only obtainable through cryopreservation (see section 2.4 below). The current study therefore focuses on plant species that haven't had much success with cryopreservation.

Another category of seeds known as 'intermediate' falls between orthodox and recalcitrant seeds based on their degree of desiccation tolerance. Like recalcitrant seeds, intermediate seeds (e.g. *Azadirachta indica* [Varghese and Naithani, 2000; 2002] and *Coffea Arabica* [Dussert *et al*. 1997]) are shed at relatively high WCs, but they are relatively desiccation tolerant; however not to the extent of orthodox seeds though (Berjak and Pammenter, 2008). More specifically, intermediate seeds have the ability to retain viability after dehydration to WCs as low as 12 to 15% dmb (Bonner, 2008), without any immediate detrimental effects (Goveia *et al*., 2004). These seeds can maintain vigour and viability in storage only for a few months (Varghese and Naithani, 2000) to sometimes a few years (Bonner, 2008). However, the misconception that recalcitrant and intermediate seeds cannot be stored for longer periods of time has since been dismissed due to advances in in the long term germplasm conservation of recalcitrant seeds via cryopreservation (Normah *et al*., 1986; Wesley-Smith *et al.,* 2001; Engelmann and Engels, 2002; Berjak and Pammnter, 2008; Sershen *et al*., 2012a).

2.4. Cryopreservation

Cryopreservation is the conservation of samples at ultra-low temperatures (Berjak *et* al., 1995; González-Benito *et al*., 2004; Rao, 2004; Benson, 2008; Varghese and Naithani, 2008; Varghese *et al*., 2009; Berjak and Pammenter, 2013) usually in liquid nitrogen (LN; -196°C) and/or its vapour phase (-140°C) (González-Benito *et al*., 2004; Benson, 2008). Cryopreservation is presently the most

promising solution for the long term storage of the germplasm of recalcitrant-seeded species (Normah *et al.* 1986; Sershen *et al.*, 2007).

The types of explants that can be cryopreserved include viable plant cells, seeds, tissues, somatic and zygotic embryos, shoot apices/meristems, nodal segments and pollen (Rao, 2004; González-Benito *et al.*, 2004; Benson 2008). The principle behind storing living material at ultra-low temperatures is that LN has the ability to 'freeze the biological time' by causing cell division and most chemical reactions and metabolic activities to be in a suspended state, thus affording storage without any changes to the material for long periods of time (Mycock *et al.*, 1995; Rao, 2004; Benson, 2008). Additionally, the cryogen will cause an arrest of ageing at the cellular, physiological and molecular level, thus preserving life indefinitely and this is an advantage over conventional conservation methods (Mycock *et al.*, 1995; Benson, 2008).

In order to ensure that the plant material survives cryo-storage, the material used needs to be small, dehydrated to sufficiently low WCs and cooled at the appropriate rate to avoid the formation of lethal ice crystals (Engelmann, 2011b). Partial dehydration of tissues is important as it reduces the amount of freezable water in cells, minimising ice crystal formation. It has been shown that partially dried embryos of recalcitrant seeds can be successfully cryopreserved (Normah *et al.*,1986). Since the first success report of cryopreservation of plant material (*Morus* spp.) by Sakai in 1956, various plant cryopreservation techniques have been developed and these include: freeze-induce dehydration, droplet-vitrification, vitrification, encapsulation-dehydration and encapsulation-vitrification (Shibli *et al.*, 2006; Benson, 2008; Engelmann, 2011b; Wang *et al.*, 2012). These techniques are discussed further below.

2.4.1 Cryopreservation techniques

Plant material used for cryopreservation (except for dry seeds) usually has high amounts of cellular water, making it sensitive to freeze-induced injury, thus it needs to be partially dehydrated to protect the cells from ice crystallisation (Mazur, 1984; Engelmann, 2011b). As mentioned earlier with recalcitrant seeds being shed highly hydrated and desiccation sensitive, they are likely to incur ice-crystal damage if stored at sub-zero temperatures (Pammenter and Berjak, 2014). However, this is not always the case, some recalcitrant-seeded species can survive desiccation to levels suitable for freezing, with partial dehydration (Sershen *et al.*, 2007). This was shown in various amaryllid species that were documented to have survived removal of freezable water in ranges of *ca.* 0.24±0.06 to *ca.* 0.14±0.08 g g^{-1} dmb without desiccation damage (Sershen *et al.*, 2007). There are thus, two cryopreservation techniques that can be employed; one being more classical and the other new technique being vitrification based. Over the years a transition has been made in cryopreservation from freeze-induced dehydration which is a classical technique to vitrification based techniques.

2.4.1.1 Classical techniques

This technique involves the initial slow cooling of plant material followed by rapid cooling. The difference between these two drying rates is that slow cooling entails cooling in defined pre-freezing temperatures and rapid cooling involves the direct immersion to LN (Engelmann, 2011b). Starting with slow cooling allows for the reduction of temperatures causing the cells and the external medium to be super-cooled and consequently followed by ice formation of the medium (Mazur, 1984). The cell membrane therefore acts as a barrier preventing ice from seeding into the cell interior, thus permitting cells to remain unfrozen but super-cooled (Engelmann, 2011b). A further decrease in temperature results in a conversion of the extracellular solution into ice crystals, and a removal of water from the intracellular solution, therefore leading to cellular dehydration and concentration of intracellular solutes (González-Benito *et al*., 2004; Engelmann, 2011b). Ice crystals which are larger and more damaging can reform during rewarming of the plant material; thus in order to avoid this thawing should be done rapidly (Mazur, 1984; Engelmann, 2011b). The disadvantages with classical techniques are that they use sophisticated and costly programmable freezers (Engelmann, 2011b). These techniques have been successfully applied to plant material such as cell suspensions and callus (Kartha and Engelmann, 1994) but are usually not effective for large plant material such as shoot apices (González-Benito *et al*., 2004). In the current study, embryonic shoot apices were the explants selected for cryopreservation, thus classical techniques were not employed.

2.4.1.2 Vitrification based techniques

Vitrification is defined as the transition of water from the liquid phase to an amorphous phase which is glassy solid or glass (Fahy *et al*., 1984; Charoensub *et al*., 1999; González-Benito *et al*., 2004; Engelmann, 2011b; Kulus and Zalewska, 2014). Vitrification is achieved through the use of cryoprotectants (CPs – detailed in 2.4.2.3). Cryoprotectants are substance that can lower the nucleation temperature and raise the recrystallization temperature (Wesley-Smith *et al*., 1992). Those authors also suggested that by using CPs and partially dehydrating plant material, the condition for optimal freezing can be maintained. Cryoprotectants can either be penetrating or non-penetrating; molecules of the penetrating CPs penetrate the cells by crossing through the plasmalemma while molecules of the latter do not cross the plasmalemma (Berjak and Pammenter 2014). Cryoprotectants (penetrating and non-penetrating cryoprotectants) are used in the initial step and subsequently followed by rapid cooling in LN, to reduce the intra- and extra- cellular freezable water, thus achieving the vitrified state (González-Benito *et al*., 2004; Panis and Lambardi, 2006). However, some explants are sensitive to vitrification solutions, and in such cases a step where a loading solution which is a glycerol-sucrose solution; is used to prepare the tissue prior to the exposure of the vitrification solution (González-Benito *et al*., 2004). Plant vitrification 2 (PVS2) is the most commonly used cryoprotectant mixture and is made up of glycerol, ethylene glycol, and dimethyl sulfoxide (DMSO) in a medium containing 0.4 M sucrose (Sakai *et al*., 1990; Kaczmarczyk *et al*., 2012).

The advantages of using the vitrification techniques for cryopreservation are that, unlike the classical techniques, they can be applied for complex tissues such as embryos and shoot tips (Engelmann, 2011b). In order to achieve survival with vitrification techniques, plant tissues should be successfully dehydrated to WCs that will allow them to survive freezing (Engelmann, 2011b). Furthermore, higher cooling rates can be achievable through the use of the 'droplet freezing protocol', where the plant material is placed onto aluminum foil strips and directly immersed into LN (Panis and Lambardi, 2006). High concentrations of the cellular solutions (cytosol), can also be obtained through the following techniques: air drying of explants, freeze drying, use of cryoprotectants, either penetrating or non-penetrating (Panis and Lambardi, 2006). In the current study explants were initially treated with a loading solution (2.0 M glycerol + 0.4 M sucrose; [Sakai, 2000]) and subsequently treated with a chilled PVS2 solution and cooled using three different cooling rates; slow, fast and rapid cooling (described below 2.4.2.2). The different vitrification techniques that can be used in cryopreservation are explained below:

Encapsulation-dehydration is a technique that was developed in 1990 by Fabre and Dereuddre, where explants such as shoot tips, somatic embryos or callus are used (Kaczmarczyk *et al.,* 2012). It entails the encapsulation of explants in alginate beads, incubating explants in sucrose enriched medium (0.7-1.5 M); partial dehydration in the air current of the laminar airflow or above silica gel and lastly rapidly cool the explants (González-Benito *et al*., 2004; Engelmann, 2011b). Explants are pre-cultured on media with high sucrose concentrations (0.3-0.7 M) so as to raise intracellular solute concentrations and promote vitrification, thus increasing chances of survival after desiccation and cooling (González-Benito *et al*., 2004; Kaczmarczyk *et al*., 2012). Use of alginate beads provides protection of the explant from damage that may be caused during handling, thus reducing chances of mechanical stress (Kaczmarczyk *et al*., 2009).

Encapsulation-vitrification is similar to encapsulation-dehydration, where explants are encapsulated in alginate beads and subsequently subjected to vitrification (González-Benito *et al*., 2004). The difference is that the physical dehydration step, where explants are dried in the laminar airflow or silica gel is omitted; rather, encapsulated explants are incubated to promote sufficient dehydration and vitrification (Kaczmarczyk *et al*., 2012). The *dehydration technique* involves desiccation of explants in the air current of the laminar airflow or by using flow of sterile compressed air/silica gel and then followed by direct immersion in LN (rapid cooling) [Engelmann, 2011b]. This technique was shown to be effective for cryopreservation of *P. lactifora* shoot tips (Seo *et al*., 2007) and somatic embryos (Kim *et al*., 2006).

The *pre-growth dehydration technique* involves pre-growing explants in the presence of CPs, followed by dehydration in the laminar airflow or over silica gel and rapid cooling by direct immersion in LN (Engelmann, 2011b; Kulus and Zalewska, 2014). The *droplet-vitrification technique* is a recently developed technique which is a modification of the vitrification technique, where explants are

pretreated with a vitrification solution and placed on aluminum foil in small droplets of vitrification solution and rapidly frozen in LN (Engelmann, 2011b; Kaczmarczyk *et al*., 2012).

2.4.2 Procedural steps involved in cryopreservation

Cryopreservation of plant germplasm involves procedural steps which may change from species to species and type of plant tissue used for cryopreservation. The protocol begins with the selection of a suitable explant which is at the optimal developmental stage and is a suitable size for rapid cooling (Engelmann, 1992). Once the suitable explant has been selected, it is generally taken through variable combinations of the following procedural steps for cryopreservation: excision of explants with minimum physical or oxidative damage, exposure to cryoprotectants to facilitate vitrification when dehydrated to WCs amenable for cooling, partial dehydration of the explants to low WCs suitable for cryo-storage, cooling followed by cryostorage, retrieval and rewarming, rehydration, decontamination and *in vitro* recovery (on a pre-established medium with or without plant growth regulators to promote growth). Provided below is a schematic diagram of the procedural steps involved in plant cryopreservation (Figure 2.2). All the steps involved in a cryopreservation protocol can influence on the success of cryopreservation (Shibli *et al*., 2006). More recently, Berjak and co-workers (2011b), have attributed this to the accumulation of stress associated with individual steps, amelioration of which determines the success of cryopreservation. The various procedural steps in cryopreservation are discussed in detail below.
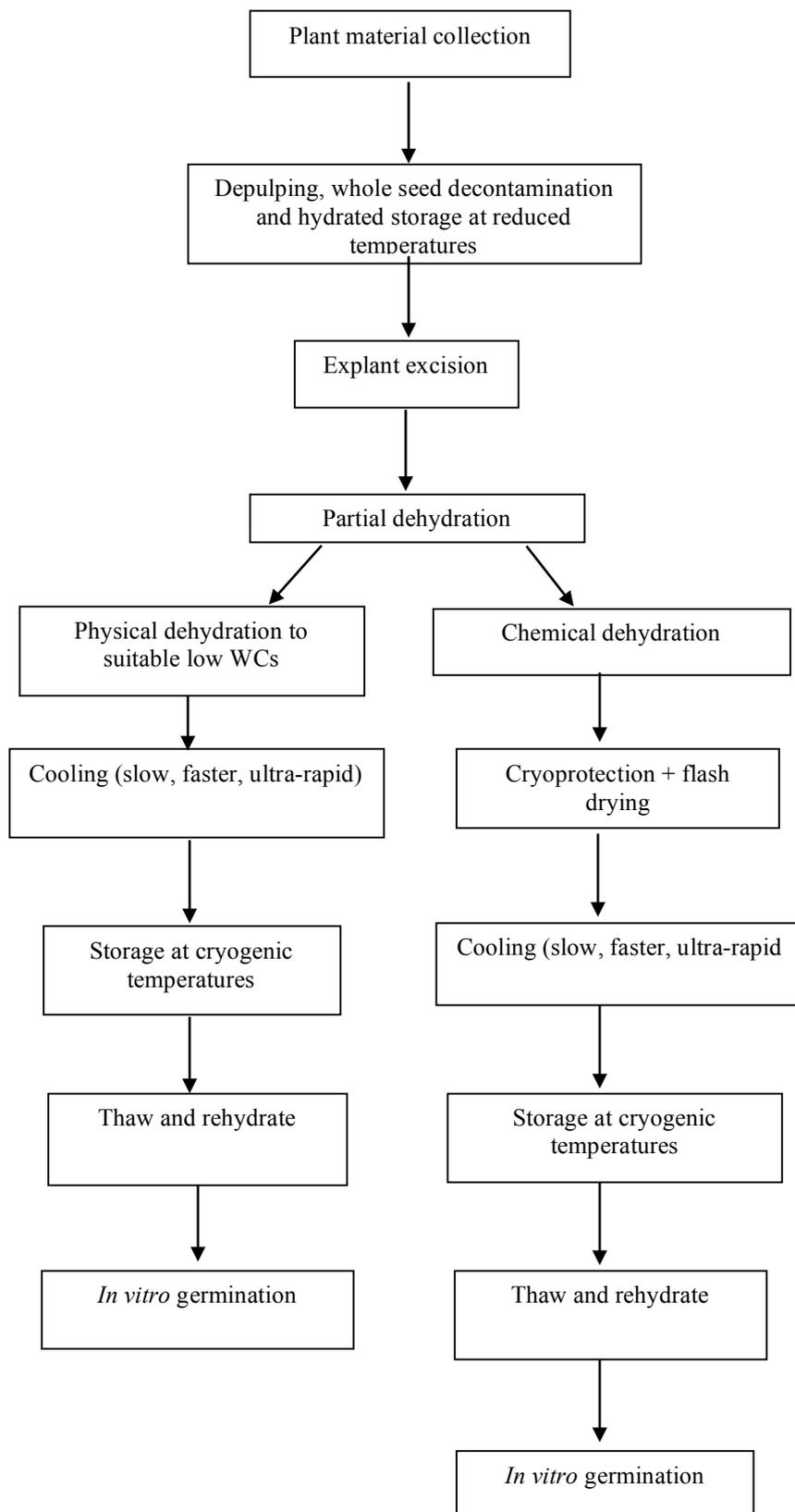
Figure 2.2: Schematic representation of the different steps involved in the cryopreservation protocol in the current study.

2.4.2.1 Explant selection

Most recalcitrant-seeded species produce seeds that are too large to be cryopreserved which is why embryonic axes excised from the seeds have been used as the explant of choice for most studies (Berjak *et al*., 2011b; Naidoo, 2012). However, when selecting an explant there are factors that need to be considered such as embryonic axis shedding WC, developmental stage and size. This is because the type and nature of the cells of explants determine whether the cells will withstand the stress incurred during freezing (Shibli *et al*., 2006). Zygotic embryos/embryonic axes have been selected as the explant for many species (Berjak *et al*., 1995; Goveia *et al*., 2004; Sershen *et al*., 2007; Naidoo *et al*., 2011; 2016; Kistnasamy *et al*., 2011; Ballesteros *et al*., 2014; Ngobese *et al*., 2014; Sershen *et al*., 2016) as the embryos not only carry the complete genetic information of a species but also constitute only a small fraction of the total mass and volume of the seed, theoretically facilitating rapid dehydration and cooling (Berjak and Pammenter, 2008), which are pre-requisites for successful cryopreservation (Chandel *et al*., 1995). One of the most important reason for their use is that they have the potential to develop into full plants (Pammenter and Berjak, 2014) without further tissue culture manipulations to induce root and shoot development. Excision of the embryonic axes can damage the plant tissue but this can be reduced in some cases through removal of the embryonic axis without cutting the cotyledonary body which surrounds the embryo, or by leaving about 2 mm segments of the cotyledon (Pammenter *et al*., 2011). Furthermore, not all recalcitrant seeds have embryonic axes that are small enough for cryostorage though some seeds have axes that are too large to be successfully dehydrated and frozen, and in such instances alternate explants need to be used (Varghese *et al*., 2009; Berjak and Pammenter, 2014).

It has been observed that *in vitro* germination of seeds followed by the excision of shoot apices/meristems from the germinating seedlings serve as an alternative choice of explants (Varghese *et al*., 2009; Berjak and Pammenter, 2014). Extracting alternate explants such as shoot apices/meristems from growing seed cultures offers the same genetic diversity as seeds, making them ideal alternative explants (Shibli *et al*., 2006; Berjak and Pammenter, 2014). However, excising explants either directly from the seeds or from *in vitro* germinated cultures can lead to excision damage which consequently results in an inability of shoots to develop into whole plants (e.g. *T. dregeana* - Goveia *et al*., 2004; *Landolphia kirkii* - Pammenter *et al*., 2011). This damage is said to be largely a consequence of the over-production of reactive oxygen species (ROS) upon excision (explained further in section 2.5) [Roach *et al.,* 2008; Pammenter *et al*., 2011; Varghese, *et al*., 2011].

2.4.2.2 Dehydration

As alluded to in section 2.3.2, one of the major problems with recalcitrant seed cryopreservation is their large size and high shedding WCs. Furthermore, whilst seed-derived explants such as embryonic axes can serve as smaller explants they are desiccation sensitive and cannot be dried to WCs amenable to cryopreservation and even with partial drying are still susceptible to lethal ice crystal damage during

cooling and rewarming (Wesley-Smith *et al*., 1991; 2001; 2014; 2015). Due to various reasons including their size, desiccation sensitivity, chemical composition of the embryos, topography of the shoot and root meristems and non-uniform distribution of water across explant tissues (Ballesteros *et al*., 2014), it is difficult to dehydrate explants from recalcitrant seeds to WCs amenable for cryopreservation of desiccation sensitive plant tissues, *ca*. 0.25-0.40 g g^{-1} (Wesley-Smith *et al*., 1992; Volk and Walters, 2006; Pammenter and Berjak, 2014). When dried to such low WCs, all or almost of the bulk water is lost from the tissues and the potential for ice crystal formation is reduced. Zygotic embryos/embryonic axes of many recalcitrant-seeded species can be rapidly dehydrated to WCs in this range (Pammenter *et al*., 1998; Ballesteros *et al*., 2014; Sershen *et al*., 2016).

In order to achieve the required WCs at which axes can be exposed to LN with a possibility of survival, explants are usually partially dehydrated through air drying by sterile airflow in a laminar airflow cabinet (Normah *et al*., 1986; Shibli *et al*., 2006) or by placing them over silica gel (Panis and Lambardi, 2006). More rapid drying can be achieved by using a flash drier which involves the insertion of a nylon gauze across a PVC pipe, mounting of a cooling fan in the middle, directing the airflow towards the gauze and placing the assemblage in a jar containing activated silica (Wesley-Smith *et al*., 2001). Flash drying is the most commonly used drying method for explants of recalcitrant seeds as it allows them to survive to lower WCs than conventional drying methods (Pammenter *et al*., 1998; Berjak and Pammenter, 2008; Varghese *et al*., 2011). Slow drying allows for aqueous-based oxidative damage to accumulate, as it takes a longer time for desirable WCs to be reached; metabolism-linked damage eventually resulting in cellular death and loss of viability at times even at high WCs (Pammenter *et al*., 1998; Wesley-Smith *et al*., 2001; Liang and Sun, 2002; Varghese *et al*., 2011; Berjak and Pammenter, 2013 Pammenter and Berjak, 2014). Slow drying involves placing plant material in plastic weighing boats, which were allowed to float over saturated salt solutions poured in glass Petri dishes (Wesley-Smith *et al*., 2001). In other studies, slow drying in embryonic axes from seeds was achieved by placing whole seeds in silica gel (Pammenter *et al*., 1998) or on table top at ambient conditions (Varghese *et al*., 2011) for few days. Rapid drying however avoids a prolonged accumulation of stress imposed during drying by reducing the time spent at intermediate WCs (Pammenter *et al*., 1998; Varghese *et al*., 2011; Sershen *et al*., 2016). There are therefore three types of damages that can occur due to drying *viz.,* physical damage, metabolism-linked damage and desiccation damage *sensu stricto* (Pammenter and Berjak, 1999; 2014). According to Pammenter and Berjak (2014), the physical damage occurs at high WCs and is associated with a reduced cellular volume. Metabolism-linked damage on the other hand occurs at intermediate WCs due to the dehydration of metabolically active tissues (Pammenter and Berjak, 2014). Lastly, desiccation damage *sensu stricto* is the type of damage that occurs at low water WCs as a consequence of the removal of structure associated water from macromolecules and membranous surfaces (Walters *et al*., 2001; Pammenter and Berjak, 1999; Berjak and Pammenter, 2008; Pammenter and Berjak, 2014).

2.4.2.3 Cryoprotection

Dehydration of recalcitrant explants can also be achieved by using cryoprotecting agents. Cryoprotectants are defined as chemical substances used to pretreat tissues prior to freezing, that concentrates the intracellular contents, allowing it to solidify without formation of ice crystals upon cryogen exposure (Engelmann, 2011b). Thus CPs, have the ability to decrease the WC so as to avoid the formation of lethal ice crystals (Sakai *et al*., 1991) and they also aid in stabilizing the cell structure during desiccation and cooling (Crowe and Carpenter, 1998; Bryant *et al*., 2001; Volk and Walters, 2006). There are two classes of cryoprotectants used for plant material: penetrating and non-penetrating CPs. The most commonly used penetrating cryoprotectants are colligative additives that increase the solute concentration; these include glycerol and DMSO, methanol and smaller molecular weight glycols (Mazur, 1984; Benson, 2008; Berjak and Pammenter, 2013). Glycerol is used because of its role of acting as an antifreeze agent, by reducing the extracellular solute concentration and loss of water (Benson, 2008). However, it is toxic to the cells and exposing plant material to CP mixtures containing glycerol for longer periods can be deleterious (Fahy, 1986; Fahy *et al*., 1990; Volk and Walters, 2006; Volk *et al*., 2006). Even though DMSO is known to be toxic to some plant material, it is the most preferred CP since it rapidly penetrates into cells (Panis and Lambardi, 2006). The penetrating CPs have an ability to enhance the cells viscosity, inhibiting ice nucleation and promoting glass formation (Mazur, 1984; 2004); they also reduce the efflux of water during cooling from the cytoplasm (Benson, 2008). Non-penetrating CPs such as sucrose and polyethylene glycol are generally used for chemical dehydration, i.e. the osmotic removal of potential freezable water from cells, thus causing an increase in solute concentration (Panis and Lambardi, 2006; Berjak and Pammenter, 2014).

Generally, cryoprotection of plant material is done through the use of an individual or mixture of cryoprotectants, comprised of both penetrating and non-penetrating CPs (Panis and Lambardi, 2006; Berjak and Pammenter, 2013). A single CP (such as DMSO) can be used effectively in isolation, however, a CP mixture of cryoprotectants such as DMSO, glycerol and sucrose can be even more effective than using DMSO independently (Shibli *et al*., 2006). Sucrose is used in cryoprotectant mixtures because it provides a colligative action of small molecules that aid in depressing the freezing point (Bachiri *et al.,* 1995; Shatanawi *et al.,* 1999; Shibli *et al*., 2006). The cryoprotection mixture can either be prepared with water only or in a culture medium and the latter is more effective option (Shibli *et al*., 2006). With the culture medium option, the pH of the medium is adjusted to the standard medium pH, and then filter sterilized, chilled and incubated with the plant material for approximately 1 hour (Withers, 1991; Shibli *et al*., 2006). In some cases, success has been achieved through partially dehydrating explants with CPs and subsequently physically drying with a flash dryer; thus the two methods of dehydration can be used in combination (Sershen *et al*., 2007).

2.4.2.4 Cooling

Exposing hydrated plant material to the cryogen results in lethal ice crystal formation, thus it is imperative that formation of ice crystals is avoided. This is an essential paradigm in cryopreservation and means to avoid ice crystal damage have been explored through manipulation of the cooling/warming rates, exposing tissues to the CPs and desiccating explants to suitably low WCs (Sakai and Engelmann, 2007; Benson, 2008; Wesley-Smith *et al*., 2014). Prior to cooling, it is of paramount importance that the right sized explants are successfully dehydrated and cryoprotected so as to prevent intracellular nucleation events from occurring because they lead to the formation of damaging ice crystals (Pammenter and Berjak, 2014; Wesley-Smith *et al*., 2014; 2015). There are different cooling rates that can be implemented so as to achieve survival after exposure to LN; these are slow, fast and rapid cooling (Shibli *et al*., 2006; Wesley-Smith *et al*., 2014). During *slow cooling*, which is mainly for enabling the process of protective dehydration, the exposure of explants to ultra-low temperatures is prolonged and this may have deleterious effects due to excessive cellular dehydration and formation of large ice crystals (Wesley-Smith *et al*., 2004; Shibli *et al*., 2006; Wesley-Smith *et al*., 2014). During slow cooling (for example 0.5°C-2.0°C min^{-1}, cooled down to -40°C and followed by immersion in LN), extracellular ice forms causing the intracellular water to be super cooled, thus causing a potential gradient between the exterior and interior of the cell (Mazur, 1984; 2004; Berjak and Pammenter, 2014; Pammenter and Berjak, 2014). This gradient will cause the intracellular water to move out of the cell to the exterior, thus leading to an increased concentration of intracellular contents which causes a decrease in the freezing point (Pammenter and Berjak, 2014). Slow cooling is sometimes referred to as 'equilibrium cooling' because the slow cooling process causes an almost equilibrium state of chemical potential across the membrane (Pammenter and Berjak, 2014). A few studies have reported success of slow cooled plant material, such as post-cryo survival of *L. kirkii* cooled at 1°C min^{-1} to -70°C (Kistnasamy *et al*. 2011).

Rapid cooling is achieved by plunging preconditioned explants into LN or nitrogen slush, (Withers, 1991; Shibli *et al*., 2006; Varghese and Naithani, 2008; Varghese *et al*., 2009). The concept of rapid cooling is similar to that of rapid dehydration in that, the stress imposed by cooling is applied more rapidly than damage (Berjak and Pammenter, 2013; Pammenter and Berjak, 2014). There are three different techniques that can be used in order to achieve rapid cooling, *viz*., ultra-rapid cooling, faster cooling and slowest of the rapid cooling (Berjak and Pammenter, 2014). Ultra-rapid cooling is achieved by direct plunging of explants into nitrogen slush [LN sub-cooled under vacuum to -210°C (Echlin, 1992)], (Sershen *et al*., 2007; Varghese *et al*., 2009) and subsequent introduction into LN (Berjak and Pammenter, 2014) and this was the cooling rate employed in this study. Faster cooling is achieved by enclosing explants in aluminum foil envelopes before plunging into LN, and the slowest of the rapid cooling techniques entails enclosing explants in cryovials and directly plunging them into LN (Berjak and Pammenter, 2014). Most cryopreservation studies, especially those involving zygotic

embryos and embryonic axes of recalcitrant seed recommend the use of rapid cooling as the cooling method for cryostorage (often placing naked axis in cryovials and directly plunging in LN). This is because ice crystal formation and growth is likely to occur at temperature ranges that are between 0 to -80°C, thus explants are more susceptible to ice crystal damage the longer they reside in this temperature range (Moor, 1973; Pammenter and Berjak, 2014). In a study by Gonzalez-Arnao *et al*. (1998) for example, survival of sugarcane shoot apices was achieved after both slow and rapid cooling, however, there was a higher percentage of survival after rapid cooling (Ortíz and Fe, 1999). A review by Berjak and Pammenter (2014), also suggests that rapid cooling is considered as the best option for successful cryopreservation of recalcitrant seed material.

2.4.2.5 Rewarming and rehydration

Even though cooling is a critical step for cryopreservation, the retrieval of plant material from cryostorage is just as important. Cryopreservation of recalcitrant seeded explants can be considered successful only once the plant material has been successfully retrieved and grown into mature plants (Pammenter and Berjak, 2014). This can be achieved through rapidly warming the cooled material. Warming plant material can lead to the reformation of ice crystals which are as damaging as the ice crystals formed during cooling (Shibli *et al*., 2006; Pammenter and Berjak, 2014). In order to minimise recrystallization, warming must be done rapidly (Pammenter and Berjak, 2014). Rapid warming is achieved by placing cooled axes in a pre-warmed solution set at 40°C (Pammenter and Berjak, 1999; Berjak and Mycock, 2004; Sahlibi *et al*., 2006). Studies show that use of CaMg solution (made up of 0.5 μM CaCl$_2$.2H$_2$O and 0.5 mM MgCl$_2$.6H$_2$O) (Mycock, 1999; Berjak and Mycock, 2004) and cathodic water (CW) solution generated by electrolysis of CaMg solution with (Naidoo, 2012; Naidoo *et al*., 2016) or without (Berjak *et al*. 2011b) ascorbic acid (AsA) promote survival of cryo-stored material. The rewarming solution used for the current study was cathodic water with ascorbic acid (CW + AsA).

Rewarming and rehydration are generally carried out in the dark so as to avoid production of ROS (Touchell and Walters, 2000; Berjak and Pammenter, 2014). After rapid rewarming, the plant material is usually fully rehydrated with the same solution (at room temperature) in the dark (Berjak and Pammenter, 2014). In a study by Berjak *et al*. (2011b), *Strychnos gerrardii* axes showed 70% post-cryo shoot production only when they were rewarmed and rehydrated in CW. In cases where a vitrification solution is used (PVS2) prior to cooling, an unloading solution containing basal culture medium supplemented with 1.2 M sucrose should be used (Berjak and Pammenter, 2014) during thawing.

2.4.2.6 *In vitro* regeneration

Explants used for cryopreservation are generally prone to fungal and bacterial contamination and could compromise the survival during regeneration after retrieval (Berjak and Pammenter, 2014). Explants that have been rewarmed and rehydrated are decontaminated using chemical decontaminants, dilute concentrations of sodium or calcium hypochlorite, sodium dichloro-isocynaruate and others, depending on the species (Sershen *et al*., 2007; Gebashe, 2015). After decontamination the explants are rinsed with solutions such as CaMg solution (Sershen, *et al*., 2007) or sterile distilled water (Berjak *et al*., 2011b) to both remove any cryoprotectants that cause deplasmolytic injury to cells (Shibli *et al*., 2006) and to prevent the extended activity of the decontaminant.

It is important that the axes are cultured on a medium that is suitable for facilitating survival or else, even if the explants tolerate drying and exposure to LN temperatures, post-cryo survival could be compromised (Naidoo, 2017; Naidoo *et al*., 2016). Solid media (rather than liquid media), containing plant growth regulators have been shown to promote survival after cryogen exposure (Engelmann, 2000; Shibli *et al*., 2006). It is also of paramount importance that cryo-retrieved explants are not immediately exposed to light and are kept in the dark until signs of survival are observed, so as to avoid photo-oxidative stress during regeneration that may hinder survival (Touchell and Walters, 2000; Kaczmarczyk *et al*. 2012; Berjak and Pammenter, 2014).

2.5. Oxidative stress preceding and following cryostorage

Each procedural step of cryopreservation may result in the production of ROS (Roach *et al*., 2008; Whitaker *et al*., 2010; Berjak *et al*., 2011b). Halliwell, (2006) described free radicals as any chemical species that are capable of existing independently and are formed by adding a single electron to a non-radical or when a covalent bond is broken (Benson and Bremner, 2004; Kaczmarczyk *et al*., 2012). Thus, when an increase in the formation of active oxygen occurs this can either result in oxidative damage in plant tissues or as an activation of the defense and repair mechanisms, because not all ROS can be damaging (Smirnoff, 1993). The formation of free radicals has for example been observed to occur during tissue culture, explant excision (Goveia, 2007; Roach *et al*., 2008; Berjak *et al*., 2011b), CP treatments (Sershen *et al*., 2012b), physical dehydration (Varghese *et al*., 2011; Sershen *et al*., 2016), as well as during cooling and rewarming (Sershen *et al*., 2012b). The first step in the cryo-protocol is excision of the explant which has been widely reported to result in an oxidative burst in embryonic axes of sweet chestnut (*Castanea sativa;* Roach *et al*., 2008), *T. dregeana* (Whitaker *et al*., 2010; Pammenter *et al*., 2011) and *S. gerrardii* (Goveia, 2007; Berjak *et al*., 2011b). According to Wang *et al*. (2007), the rapid generation and release of ROS is termed as the oxidative burst.

There are different types of ROS that can be produced in explants during the steps of cryopreservation which are highly reactive and damaging to the cells (Benson, 1990; Varghese and Naithani, 2008; Berjak *et al*., 2011b; Kaczmarczyk *et al*., 2012). The commonly produced ROS include superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$)

and singlet oxygen ($^1O_2$), to mention a few (Smirnoff, 1993; Halliwell, 2006; Berjak *et al*., 2011b; Kaczmarczyk *et al*., 2012). According to Smirnoff (1993), these are forms of oxygen that are more reactive than ground state oxygen and can impose damage to the cells. However, it should be remembered that ROS have a dual role in plants; they are involved in intracellular signalling as well as intracellular destruction – this appears to apply to seed tissues (Bailly, 2004; Bailly *et al*., 2008; Berjak and Pammenter, 2008; Whitaker *et al*., 2010; Moothoo-Padayachie *et al*., 2016). They are mostly produced during cellular metabolism as the by-product of the process. Superoxide and $^1O_2$ are examples of ROS that are generated as by-products of the electron chain from metabolism and photosynthesis (Kaczmarczyk *et al*., 2012).

Reactive oxygen species being higly reactive, are generally associated with oxidative damage to proteins, lipids and nucleic acids (Smirnoff, 1993; Halliwell, 2006; Berjak and Pammenter, 2008; Xin *et al*., 2010; Kaczmarczyk *et al*., 2012). Superoxide when produced on its own is not highly reactive and damaging, however, a subsequent formation of H_2O_2 and $OH^\bullet$ which are more reactive can bring about the most damage to cellular components (Smirnoff, 1993; Halliwell and Gutteridge, 2007). Hydroxyl radical and hydroperoxyl on the other hand are produced during the Fenton's reaction and the formation of hydroxyl is associated with lipid peroxidation in membranes (Berjak and Pammenter, 2008; Kaczmarczyk *et al*., 2012). Due to lipid peroxidation, membranes lose their fluidity allowing phospholipids to be exchanged between the two halves of the bilayer (Halliwell, 2006). This increases the leakage of the membrane, causing the protein membranes to be damaged, as well as inactivation of receptors, enzymes and ion chanels (Halliwell, 2006). One of the major problems with recalcitrant seed explants is their inability to produce shoots after cryogen exposure, and this has been associated with the production of free radicals (Goveia, 2007; Berjak *et al*., 2011b).

In studies on *T. emetica* where embryonic axes were used, survival was also limited to root production only, with no shoots forming and this was suggested to be due to an oxidative burst at the axis-cotyledon junction during excision (Goveia, 2007). According to Sershen *et al*. (2012b), the two procedural steps that are major contributors towards oxidative stress are dehydration and exposure to the cryogen. Metabolism-linked damage which results due to dehydration of the plant material to desirable WCs are suggested to be associated with unregulated ROS production (Berjak and Pammenter, 2008; Sershen *et al*., 2012b). The manifestation of oxidative stress in recalcitrant seed tissues during cryopreservation has been shown through the use of biomarkers such as conjugated dienes, hydroperoxides and malondialdehyde (Benson and Bremner, 2004; Sershen *et al*., 2016). The production of ROS therefore needs to be regulated during the various procedural steps of cryopreservation of recalcitrant seed material so as to achieve survival after each cryo-procedure and this can be obtained through the use of antioxidants. In the current study however, none of the biochemical analysis were done, but however their past research, knowledge and understanding was used in explaining the trends of the current study.

2.6. Amelioration of reactive oxygen species

As highlighted before, ROS production is only damaging when left uncontrolled which can result in oxidative stress that damages cellular macromolecules and eventually can lead to cell death. As a consequence, ROS levels needs to be regulated and plant tissues need to be protected against oxidative stress. The regulation of ROS and protection from oxidative stress is dependent on the antioxidant system (Varghese and Naithani, 2008; Xin *et al.*, 2010; Berjak *et al.*, 2011b). Antioxidants can be categorized into two major groups, *viz.* enzymes that catalytically remove ROS and non-enzymatic antioxidants that are oxidised to protect more important molecules of the cells through quenching ROS (Halliwell and Gutteridge, 2007; Kaczmarczyk *et al.*, 2012). Examples of enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and many more (Kranner and Birtić, 2005; Gill and Tuteja, 2010; Xin *et al.*, 2010; Berjak *et al.*, 2011b). An example of non-enzymatic antioxidant is glutathione (GSH), which acts as a buffer in cells (Moothoo-Padayachee *et al.*, 2016) as well as interacts with ROS resulting in a conversion of the ROS to its oxidised form (Smirnoff, 1993; Kranner *et al.*, 2006; Xin *et al.*, 2010). Another example of an important water soluble antioxidant is ascorbic acid (AsA), and its role is to scavenge ROS by quenching free radicals (Kaczmarczyk *et al.*, 2012).

Cathodic water (CW) has also been shown to have antioxidant properties, and has been reported to play a role in scavenging $^{\bullet}O_2^-$ and H_2O_2 (Berjak *et al.*, 2011b). However, Hanaoka (2001), suggested that the scavenging properties of CW, more especially towards $^{\bullet}O_2^-$ have been shown to be enhanced when the CW is incorporated with AsA (Naidoo, 2012). This is common practice in the laboratory[2] in which this study was conducted, where a solution of CW and AsA is used to ameliorate ROS production before and after cryogen exposure. The incorporation of cryoprotectants such as glycerol (Sershen *et al.*, 2007; Sershen *et al.*, 2012b) and DMSO have also been reported to play a role in scavenging ROS (Naidoo *et al.*, 2011; Naidoo *et al.*, 2016). The role of DMSO in counteracting production of ROS was documented in a study by Benson and Withers (1987), where it was used for scavenging $OH^{\bullet}$ during cryopreservation of plant and algal specimens. This was also shown in a study by Naidoo *et al.* (2011), where excised pre-cultured embryonic axes of *T. dregeana* were soaked for 30 min in DMSO and managed to produce shoots after selected steps of cryopreservation. The two studies therefore confirmed that cryoprotectants can play a role in ROS scavenging. However, it is important to note that in recalcitrant seeds the antioxidant system becomes dysfunctional due to dehydration which subsequently leads to an accumulation of ROS and loss of viability (Pammenter and Berjak, 1999; Xin *et al.*, 2010). Thus, in order to achieve survival after cryopreservation, there needs to be a balance

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between the production of ROS and its scavenging system. In the current study the CW+AsA solution was used to ameliorate the production of ROS after dehydration and cooling as a rehydrating solution.

2.7. Species investigated

The present study focused on three recalcitrant plant species that are indigenous to South Africa, *viz*. *Ammocharis coranica*, *Trichilia emetica* and *Castanospermum australe*. Although zygotic embryos/embryonic axes have been reported to be the most ideal explants for cryopreservation, previous attempts to cryopreserve zygotic embryos/embryonic axes of these species have been largely unsuccessful (Goveia, 2007; Ballesteros *et al.*, 2014). Hence, this study investigated the potential use of shoot apices excised from the shoot pole of the embryonic axes (embryonic shoot apices) as explants for the cryopreservation of their seed-derived germplasm.

2.7.1 *Ammocharis coranica*

Ammocharis coranica (Ker.Gawl.) Herb., is a summer bulbous plant from the family Amaryllidaceae, belonging to an African genus comprised of five species (Snijman and Linder, 1996; Koorbanally *et al.*, 2000). Amaryllidaceae is mostly found in the tropics and is also well represented in South Africa and in the Andean region (Bastida *et al.*, 2011). *A. coranica* is one of the most widespread species of this genus and is mostly found in the summer-rainfall region of Southern Africa (Koorbanally *et al.*, 2000). It is commonly known as Karoo Lily and Inchotho in isiZulu. It is harvested in South Africa for its medicinal purposes as it is known to contain alkaloid and triterpenoids (Louw *et al.*, 2002). *A. coranica* is famously known for its treatment of witchcraft illnesses (Pooley, 1998), and for having bulbs that can be boiled to obtain the thick paste used for repairing cracks in clay pots (Machocho *et al.*, 1999). The biochemicals of *Ammocharis* obtainable after boiling of the bulbs are also used as enemas for cleansing blood (Louw *et al.*, 2002).

Most of the members of the Amaryllidaceae are harvested for their medicinal preparations (Bastida *et al.*, 2011) and this has placed them under threat. In studies by von Fintel (2006), Sershen *et al.* (2008) and Ngobese *et al.* (2014), it was highlighted that most amaryllids have recalcitrant seeds and thus cannot be stored for long periods of time. The only solution for their long term conservation is to cryostore their seed germplasm, however, attempts to cryopreserve the germplasm of these species were largely unsuccessful (von Fintel, 2006). Limited success in cryopreserving embryos of *A. coranica* was reported by Ngobese *et al.* (2014), wherein 30% of embryos of *A. coranica* that were cryoprotected with glycerol managed to produce seedlings after cooling. The limited success reported with the previous attempts to cryostore the germplasm of *A. coranica* has made it imperative to use alternative explants for successful cryopreservation of this species.

2.7.2 *Castanospermum australe*

Castanospermum australe A. Cunn. is an Australian native tree from the family Fabaceae (Molyneux *et al*., 1990). It is now found growing in the subtropical regions in South Africa and Sri Lanka (Delahaie *et al*., 2013) and is commonly referred to as Moreton Bay chestnut or Black bean (Molyneux *et al*., 1990). This tree has large chestnut-like recalcitrant seeds and large glossy dark green leaves which are toxic to cattle (Australian National Botanic Gardens, 2002). The seeds of *C. australe* contain alkaloids which have been shown to have anti-HIV properties (Australian National Botanic Gardens, 2002). This is because the seeds contain castanospermine which has a potent α- and β- glucoside inhibitory activity that has an ability to inhibit replication of retroviruses (Molyneux *et al*., 1990). *C. australe* is also widely harvested for its timber. Seeds of *C. australe* have been documented to be desiccation sensitive thus making it difficult to store for longer periods (Han *et al*. 1997; Hill and Edwards, 2010). Mature seeds are large and shed at relatively high WCs, of about 1.94 ± 0.41 g g^{-1} DW (Delahaie *et al*., 2013). The mature seeds also have large embryonic axes, however, excision of embryonic axes more especially removal of the cotyledonary body has been shown to lead to wound-induced damage which causes a failure in shoot production (Naidoo *et al*., 2011; Ballesteros *et al*., 2014). Hence, Ballesteros *et al*. (2014) used excised embryonic axes with segments of the cotyledon attached; this gave 40% survival after cooling, but with only shoot production. In the current study, embryonic shoot apices were used as potential explants for cryopreservation and germplasm storage of *C. australe*.

2.7.3 *Trichilia emetica*

Trichilia emetica Vahl. is an evergreen tropical tree from the Meliaceae family, with a smooth dark grey-brown bark and dark glossy leaves (Komane *et al*., 2011). It is commonly known as Natal-Mahogany, taken from the family Mahogany, and Umathunzini in isiZulu (Boon and Pooley, 2010). The genus name *Trichilia* is derived from the Greek name "*tricho*" meaning 3-lobed fruit and the specific epithet emetica refers to its emetic properties (Komane *et al*., 2011). Of the 20 *Trichilia* species that have been identified to date in the Southern Africa region, *T. emetica* is mostly confined to Limpopo and KwaZulu-Natal (Komane *et al*., 2011). *Trichilia emetica* is edible, by both animals and humans and it also has medicinal uses. The oil extracted from the seeds is used for curing rheumatism and broken bones; the powder obtained from the bark is used as an emetic for stomach and intestinal related illnesses (Boon and Pooley, 2010).

The seeds of *T. emetica* are recalcitrant, as they are shed at high WCs (2.82 ± 0.50 g g^{-1} wmb), are metabolically active with an ongoing active metabolism after axis excision (Kioko *et al*., 2006). The trees of *Trichilia* produce a lot of seeds, however, the mature seeds do not last for long during the seeding season, due to fungal contamination and loss of viability when dehydrated (Kioko *et al*., 2006). For these reasons, it is difficult to store seeds of *T. emetica*, thus, in order to conserve the seeds, the germplasm needs to be cryopreserved. Attempts have been made to cryopreserve the embryonic axes of *T. emetica* with little [no production of shoots (Goveia, 2007)] or no survival at all (Roach *et al*.,

2008). However, in a study by Varghese *et al*. (2009), where shoot tips were used as explants for the germplasm conservation, 71% survival with the production of shoots was achieved. Gebashe (2015) also reported 68% survival with 40% shoot production when shoot apices of *T. emetica* were exposed to LN. However, the shoot tips had to be excised from *in vitro* grown shoots; moreover, the vigour of the shoots produced from the cryopreserved shoot tips were compromised. These findings led to the choice of embryonic shoot apices as explants for the cryopreservation of this species in the current study.

CHAPTER 3: MATERIALS AND METHODS

3.1 Seed collection and storage

Fruits of *Ammocharis coranica* were harvested from Cathcart, Eastern Cape, South Africa (32° 18' S 27° 08' E). The fruits were hand harvested, cleaned by removal of the fruit pulp and the seeds were extracted and wrapped in paper towel. The paper towel with seeds was kept in a card box and couriered on the same day to the Plant Germplasm and Conservation Research laboratory, School of Life Sciences, UKZN, Westville, Durban. Upon arrival, the seeds were removed from the paper towel, dusted with a fungicide, Benomyl 500 WP (active ingredient, benzimidizole; Villa Protection, South Africa), wrapped with slightly moistened paper towel and placed in brown paper bags. The paper bags were then stored in a 6^oC cold room until the cotyledonary body had protruded (signs of germination) from the seeds ($\pm$ 2 weeks). The seeds were allowed to germinate so as to allow the cotyledonary body which holds both the shoot and root meristem to protrude from the seed (Sershen, 2006; Ngobese, 2013), making it easy for the excision of the explant, refer to Figure 3.1.
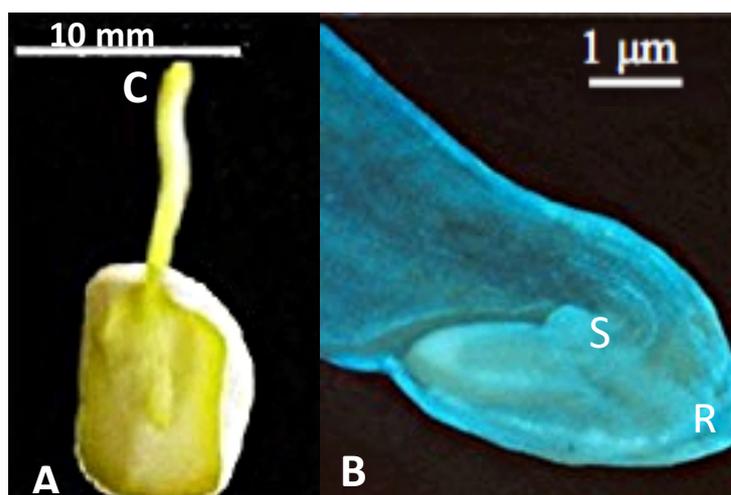
Figure 3.1: *Ammocharis coranica* seed (**A**) showing the protrusion of the cotyledonary body **(c)** and the tip of the cotyledonary body **(B)** showing where the root meristem **(r)** and shoot meristem **(s)** are located. (Picture taken from Ngobese, 2013).

Fruits of *Trichilia emetica* were harvested from trees at Mtunzini, 140km north of Durban, KwaZulu-Natal (28° 57' S 31° 45' E). Mature fruits were collected from the trees and some from the ground, and taken to the laboratory in Durban (Figure 3.2). The seeds were removed from the fruits and the damaged and fungi-contaminated fruits were discarded. The aril and the seed coat were then peeled off. Once all seeds were cleaned in this manner, they were decontaminated by treating with 1% (v/v) sodium hypochlorite (NaOCl) containing a few drops of Tween 20$^®$ (wetting agent) for 20 min on a shaker (Labcon, Instrulab CC, Maraisburg, South Africa) at 100 rpm. These seeds were then treated

with an anti-fungal 'cocktail' made up of 0.5ml L^{-1} Early Impact (active ingredients, triazole and benzimidizole; Zeneca Agrochemicals, South Africa) and 2.5ml L^{-1} Previcur N (active ingredient, propamocarb-HC; AgrEvo, South Africa) for 120 min on a shaker (Labcon, Instrulab CC, Maraisburg, South Africa) at 100 rpm. These decontamination treatments have been shown to effectively reduce fungal proliferation in stored recalcitrant seeds (Calistru *et al.*, 2000; Goveia *et al.*, 2004; Myeza, 2005). Lastly, the seeds were rinsed three times with sterile distilled water and placed on paper towel and dried overnight back to their original batch fresh weight. The seeds were then prepared for hydrated storage as described in section 3.2.

Figure 3.2: Mature fruits of *Trichilia emetica* **(A)** immediately after harvest and sorting out of seeds from the fruits at the laboratory **(B)**.

Mature pods of *Castanospermum australe* (Figure 3.3), which were identified based on their brown colour, were collected from trees growing in Pietermaritzburg, KwaZulu-Natal, South Africa (29° 37' S 30° 23' E). Upon arrival at the laboratory, the pods were sprayed with 70% ethanol, placed on trays and stored in a 16°C cold room.

Figure 3.3: Mature pods of *Castanospermum australe* showing the seeds inside the pods. Besides the open pod, a seed without seed coat, excised embryonic axis and excised embryonic shoot apice can be seen.

3.2 Hydrated storage

Plastic buckets (5L) and plastic mesh grids were used for hydrated storage of the seeds. Prior to storage, the buckets and grids were sterilized by soaking in 1% NaOCl overnight and subsequently were sprayed with 70% ethanol. The decontaminated seeds of *T. emetica* were dusted with fungicidal powder, Benomyl 500 WP, and placed as a monolayer on the sterilized mesh grids. The mesh grids were then suspended about 100 mm above the base of the sterilized buckets. The base of the bucket was lined with a paper towel moistened with 1% NaOCl while the lid of the bucket was lined with dry paper towel to prevent condensate from dripping onto the seeds. The sealed buckets were stored in a cold room at $16^{o}C$. The buckets were constantly monitored for fungal contamination and the paper towel was kept moist to maintain the hydrated conditions.

3.3 Excision of explants

Embryonic shoot apices were used as explants in this study. The manner in which explants were excised differed between the species, due to the difference in the development of the seeds. In *A. coranica*, the root and shoot tips are found at the tip of the extended cotyledonary body as shown in Figure 3.1 above (Sershen, 2006; Ngobese, 2013); the seeds were hence allowed to develop in storage until the cotyledonary body had protruded out. The elongated cotyledonary body was excised using a 10 pt. sterile blade, leaving 2 mm from the root and shoot tip (Figure 3.4 C). This represented the primary explant. In *A. coranica*, the shoot and root tips lie in close proximity to each other (Figure 3.1) which makes it necessary to allow the primary explant, consisting of shoot and root tips, to develop before excising the shoot tips to facilitate shoot tip growth away from the root tip. The primary explant was surface decontaminated (described below in 3.5.1) and cultured in Petri dishes on full strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The primary explants were allowed to develop to form full plantlets (with roots and shoots). Shoot apices (secondary explants) were excised from these plantlets, inside a laminar air-flow (J10 Tech Lab. Companion, BC-01E; Korea) using a dissecting microscope and an 11pt. sterile scalpel. Water content and germinability of these excised shoot apices were determined (described in sections 3.4 and 3.5.1 respectively). Embryonic shoot apices of *T. emetica* and *C. australe* were excised 2 mm from the embryonic axis tip (Figure 3.4 A and B). The shoot apices were excised from the embryonic axis using a 10 pt. sterile scalpel. The apices were used to determine the WC (described in section 3.4) and their ability to form complete plantlets (described in section 3.5). These were then used as explants for further studies.
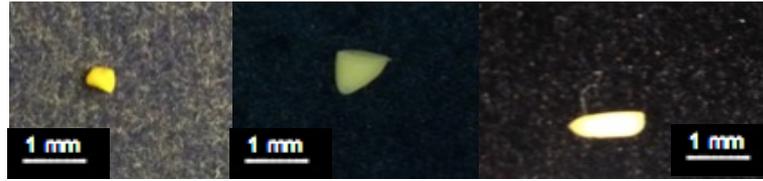
Figure 3.4: Shoot apices excised from embryos of *T. emetica* **(A)** *C. australe* **(B)** and the extended cotyledonary body excised from an embryo of *Ammocharis coranica* **(C)**.

3.4 Gravimetric determination of water content

Water content of freshly excised embryonic shoot apices (control; n=10) and apices subjected to different procedural stages of cryopreservation (treatments; n=10) were determined gravimetrically by weighing the shoot apices before and after oven-drying at 80°C for 48h. The shoot apices were individually placed on aluminum foil boats and weighed using a six-place balance (Mettler MT5, Germany). Water content was expressed on a dry mass basis (g H_2O per g dry mass; g g^{-1}).

3.5 *In vitro* plantlet formation

The shoot apices of the three species were used to determine the ability of untreated embryonic shoot apices to develop into plantlets prior to the cryo-procedure. After each stage of the cryo-procedure (excision, dehydration, cryoprotection, cooling and thawing) shoot apices of *T. emetica* and *C. australe* were surface decontaminated and assessed for viability (n=15). However, viability for shoot apices of *A. coranica* was only assessed after initial excision and was not investigated for any further cryopreservation stages used in this study. Ten explants per drying interval were used for water content determination, for each of three drying curves. For viability assessment, 15 explants were used in each of three replicates per treatment.

3.5.1 Decontamination and *in vitro* culture of shoot apices

The primary explant of *A. coranica* was surface decontaminated with 1% (v/v) NaOCl containing a few drops of Tween 20® for 5 min and rinsed three times with sterile distilled water. The explants were then blotted on sterile filter paper and carefully placing the cut surface onto full strength MS medium. Once the explants had developed into full plantlets, shoot apices were excised (as explained in 3.3) and immediately cultured on full strength MS medium containing sucrose (30 g L^{-1}) and agar (8 g L^{-1}) supplemented with 1 mg L^{-1} 6-benzylaminopurine (BAP) at a pH of 5.6-5.8 to promote shoot production. Following production and elongation of shoots, the explants were transferred to culture tubes (1 per culture tube) containing full strength MS medium supplemented with 0.1 mg L^{-1} Indole-3-butyric acid (IBA) to promote root production.

Shoot apices of *T. emetica* were decontaminated with a 5000 ppm (0.05% w/v) sodium dichloro-isocynaruate (NaDCC) solution for 5 min and rinsed three times with sterile CaMg. The shoot

apices were then blotted on a sterile filter paper and placed on full strength MS medium supplemented with 1 mg L^{-1} BAP to promote shoot production. The explants were cultured with the cut surface touching the medium to enable easy absorption of nutrients from the medium. Once shoots had developed and elongated they were transferred to culture tubes with full strength MS medium supplemented with 0.1 mg L^{-1} IBA to promote root production.

Shoot apices of *C. australe* were decontaminated with 1% (w/v) Amoxicillin® solution for 5 min and rinsed once with sterile distilled water. Axes were further decontaminated with 1% calcium hypochlorite (w/v) for 5 min and rinsed 3 times with sterile CaMg solution. The shoot apices were then blotted on a sterile filter paper and cultured on full strength MS medium supplemented with 1 mg L^{-1} BAP and 0.1 mg L^{-1} gibberellic acid (GA_3) to promote shoot production. Here also, it was ensured that the cut surfaces of the explants touched the surface of the medium. Once shoots developed and subsequently elongated, they were transferred to culture tubes with full strength MS medium supplemented with 0.1 mg L^{-1} IBA to promote root production. The *in vitro* germination medium used for the three species was determined through conducting preliminary studies with various media so as to select the most suitable medium that promotes shoot and root production for each species.

The cultures were incubated in a growth room under cool fluorescent light (light intensity of $52\mu Es^{-1} m^{-2}$ produced by fluorescent tubes [Philips TL-D De Luxe Pro; 58W/965]) for 8 to 9 weeks for shoot production and 5 to 6 weeks for root production, being exposed to a 12 h day-12 h night regime. The shoot apices that were dehydrated, cryoprotected and cooled were initially incubated in the dark so as to minimize the risk of photo-oxidative-induced free radical damage, for 12 h day- 12 h night regime until there were signs of survival (see 3.10 and 3.11 below). Thereafter, they were transferred to the growth room for the exposure to light as explained above.

3.6 Generation of electrolyzed water (cathodic water)

An autoclaved calcium magnesium (CaMg) solution containing the electrolytes $0.5\mu M$ $CaCl_2.2H_2O$ and $0.5mM$ $MgCl_2. 6H_2O$ (Mycock, 1999) was electrolysed at room temperature for an hour using a Bio-Rad Powerpac (BioRad, Hercules, California) set at 60 V (Figure 3.5). The apparatus consisted of two 250ml glass beakers, each containing 200ml of the CaMg solution, with an anode in one beaker and a cathode in the other. To complete the circuit, an agar-based salt bridge containing saturated potassium chloride (0.3g agar and 3g KCl dissolved in 10ml distilled water) was used. Electrolysis for an hour resulted in cathodic water in the beaker with the cathode immersed in it. Once cathodic water was prepared, 1 g of ascorbic acid (AsA) was added to 100 ml of cathodic water to yield 1% (w/v) solution of AsA in cathodic water (cathodic water + AsA [CW+AsA]) which was used for rehydration of explants (Naidoo, 2012).

Figure 3.5: Electrolysis apparatus for generating cathodic water.

3.7 Desiccation

The experiments on desiccation and subsequent cryopreservation studies were done only on two recalcitrant-seeded species: *C. australe* and *T. emetica* due to the unavailability of seeds of *A. coranica*. The excised shoot apices (n=25 per drying interval) were rapidly dehydrated using a flash drier (Berjak *et al*., 1990; Pammenter *et al*., 2002) for various intervals. After each drying interval shoot apices were sampled to assess WC (n=10) and viability (n=15). The drying intervals used were 0, 15, 30, 45, 60, 75, and 90, 105 min for *C. australe* and up to120 min in intervals of 15 min for *T. emetica*.

A flash drying curve reflecting viability in relation to WC at various drying times was generated in order to determine the shortest period of drying which corresponds with acceptable viability retention. Shoot apices that have been dried to suitable WCs were used for further experiments. Prior to *in vitro* germination (see 3.5 above), dried axes were rehydrated in a solution of CW+AsA (described in section 3.6) for 30 min in the dark and decontaminated (see 3.5.1 above).

3.8 Pre-culture and Cryoprotection

Excised shoot apices of *T. emetica* and *C. australe* were pre-cultured on solid full strength MS medium containing 0.4 M sucrose and 0.2 M glycerol for 2 days and 3 days respectively (modified from Varghese *et al*., 2009). The cultures were kept in the dark at room temperature.

The pre-cultured shoot apices of *T. emetica* (n=15) were osmoprotected by placing them in 2 mL polypropylene cryovials containing a loading solution (2.0 M glycerol + 0.4 M sucrose) for 15 min and thereafter treated with chilled PVS2 [30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO] in liquid MS medium incorporated with 0.4 M sucrose (Sakai *et al*., 1990; Varghese *et al*., 2009). Since PVS2 is toxic to plant tissues, exposure to the solution was done in a sequential manner by treating the axis with 50% PVS2 solution for 5 min followed by 100% PVS2 for 10 min (modified from Varghese *et al*., 2009). Immediately after PVS2 treatment, the shoot apices were cooled, as described in section 3.9.

Shoot apices of *C. australe* (n=15) were subjected to three cryoprotection treatments, either with aqueous glycerol alone or with a combination of glycerol and sucrose and lastly PVS2. The pre-cultured shoot apices were immersed in a 5% (w/v) solution of glycerol followed by 10% (w/v) glycerol (Sershen *et al*., 2007), each for 30 min (CP1=cryoprotection treatment 1) and 5% glycerol + [0.5 M] sucrose followed by 10% glycerol + [1 M] sucrose, each for 30 min (CP2=cryoprotection treatment 2). After cryoprotection, the shoot apices were rapidly dehydrated using a flash drier for different intervals and thereafter cooled at different cooling rates as described in section 3.9. Shoot apices that were cryoprotected with PVS2 were subjected to cooling without flash drying.

3.9 Cryopreservation

Embryonic shoot apices of *C. australe* (n=15) were subjected to cryopreservation immediately after flash drying, after cryoprotection followed by flash drying and after treatment with PVS2 while apices of *T. emetica* were cryopreserved immediately flash drying, and after exposure to PVS2. Given below are two flow diagrams showing specific treatments during cryopreservation of *T. emetica* (Figure 3.6). *C. australe* and (Figure 3.7)
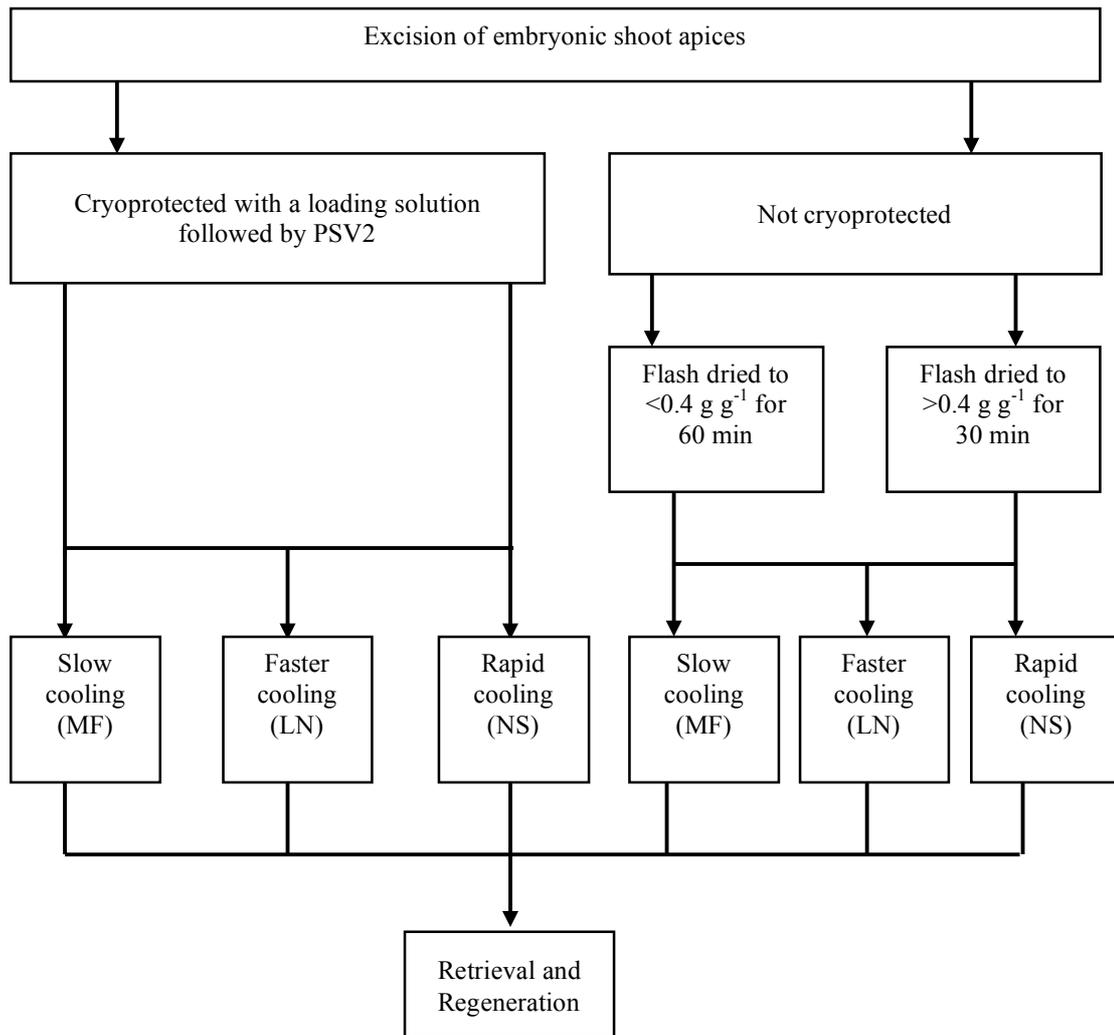
Figure 3.6: Schematic diagram showing different treatments involved in the cryo-procedure of *T. emetica*. After each cooling treatment the embryonic shoot apices were regenerated in the established *in vitro* germination medium. MF refers to Mr. Frosty®, LN refers to direct liquid nitrogen and NS refers to nitrogen slush
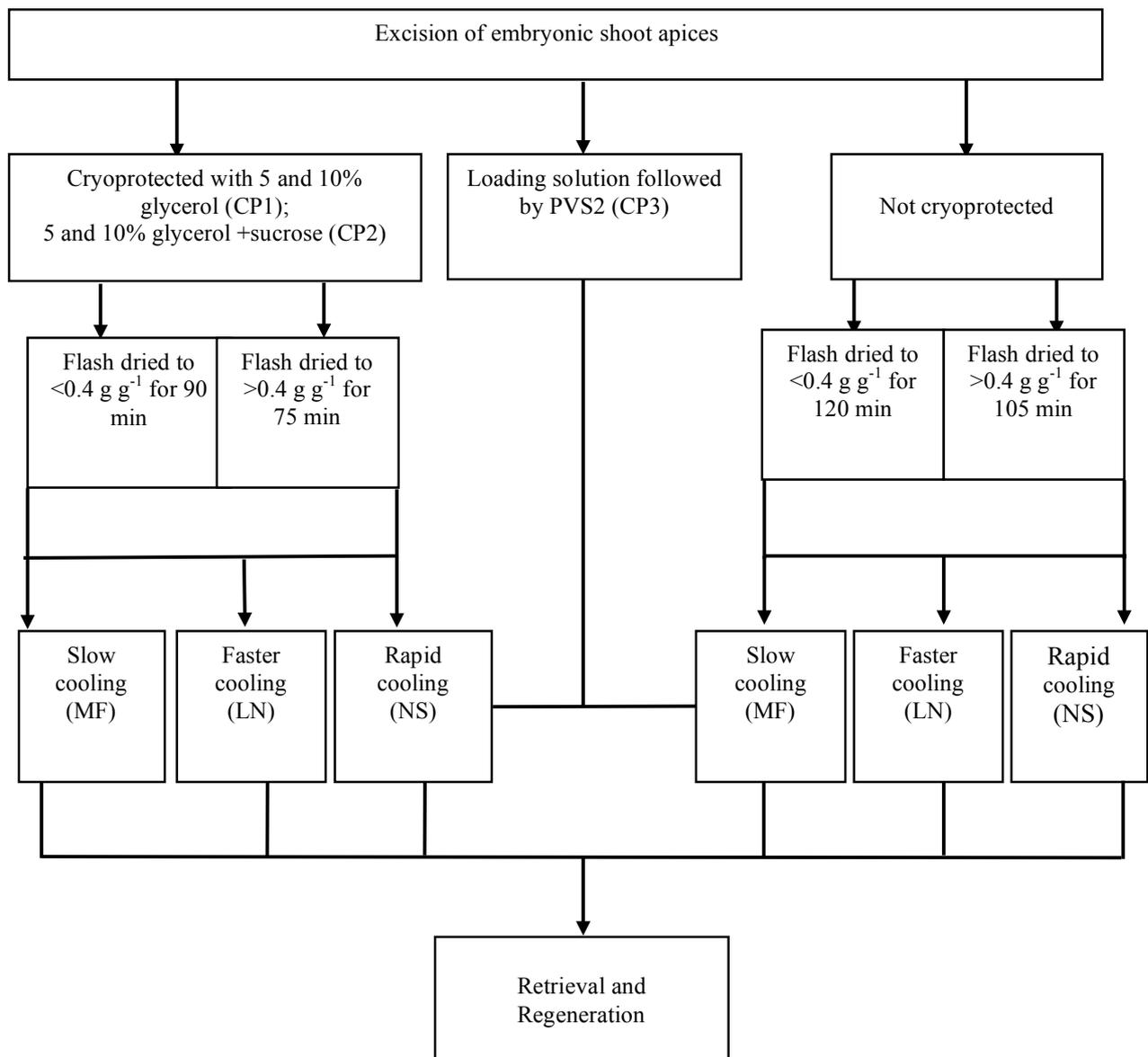
Figure 3.7: Schematic diagram showing different treatments involved in the cryo-procedure of *C. australe*. After each cooling treatment the embryonic shoot apices were regenerated in the established *in vitro* germination medium.

3.9.1 Cooling rates

The shoot apices were subjected to three cooling rates namely, slow, faster and rapid cooling. For slow cooling, the shoot apices were enclosed in 2 mL polypropylene cryovials (Greiner[TM]), with 5 axes in each cryovial, and cooled at $1^{o}C$ min[-1] using Mr. Frosty® (Nalgene[TM], USA) to an intermediate temperature of $-40^{o}C$ in a $-80°C$ deep freezer. Thereafter, the cryovials were mounted onto chilled aluminum cryo-canes and immediately plunged into liquid nitrogen (LN) at $-196^{o}C$.

For faster cooling, the shoot apices were placed in cryovials (5 axes in each cryovial) and mounted onto pre-chilled aluminum cryo-canes. The cryo-canes with the cryovials were then plunged into LN, attaining a cooling rate of approximately -200°C min^{-1} (Vertucci, 1989; Varghese *et al.*, 2009).

For rapid cooling, the treated shoot apices were plunged into nitrogen slush (LN sub-cooled to -210°C [Echlin, 1992]). The shoot apices were then transferred under LN into LN-containing cryovials, mounted onto cryo-canes and immersed into LN. For all the cooling treatments, the cryo-canes with the cryovials were stored in LN for at least 24 h.

3.10 Thawing (Re-warming) and Rehydration

Upon retrieval from LN, the shoot apices of both *C. australe* and *T. emetica* were thawed in a solution of CW+AsA at 40°C for 2 min, rehydrated in a similar solution at 25°C in the dark for 30 min. Thereafter the shoot apices were decontaminated and recovered *in vitro* (described in section 3.5). For treatments where shoot apices were cooled while immersed in PVS2 solution, the cryovials were thawed in a water bath at 40°C for 2 min. The PVS2 solution was then decanted and replaced with an unloading solution (1.2 M sucrose) at 25°C for 10 to 15 min (Varghese *et al.*, 2009). The shoot apices were then transferred into a solution of CW+AsA and kept in the dark for 30 min.

3.11 Survival and shoot production

After each stage of the cryo-protocol, explants were cultured and kept in the dark until the first signs of survival were observed, and monitored on a weekly basis, with constant sub-culturing in the event of contamination, until shoot production occurred. Survival was scored as greening of the explants and opening of the leaf primordia. For each species, shoot apices (n=15) were cultured on their respective shoot initiation media (Section 3.5) to assess survival after each cryo-stage. The number of shoot apices showing shoot elongation and organized growth were recorded and expressed as shoot production percentage.

3.12 Statistical analysis

Statistical analyses of data were performed using SPSS statistical software program version 24 (SPSS Inc., Chicago, Illinois, USA). The data was initially tested for normality using a 1-sample Kolmogorov Smirnov test. To analyse the percentage survival and shoot production, data was arcsine transformed to satisfy the assumptions of normality and analysis of variance (ANOVA) was performed to detect significant inter-treatment differences prior to cooling. Multiple comparison of the different treatments was performed using the Tukey's post-hoc test at 5% level of significance.

CHAPTER 4: RESULTS

The main aim of this study was to investigate the potential use of embryonic shoot apices as explants for cryopreservation of few recalcitrant-seeded species in which whole zygotic embryos or embryonic axes are unsuitable for this purpose *viz.*, *Ammocharis coranica*, *Trichilia emetica* and *Castanospermum australe*. The first part of this chapter presents results on the potential of the embryonic shoot apices of all three species to produce full plantlets prior to the subjection to the sequential cryopreservation steps for possible cryopreservation. The second part of the chapter reports on the responses of *C. australe* and *T. emetica* embryonic shoot apices to the procedural steps involved in cryopreservation: excision, partial dehydration (physical drying and cryoprotection), cooling, rewarming and regeneration.

4.1 Initial water content and *in vitro* germination of embryonic shoot apices of *A. coranica*, *C. australe* and *T. emetica*.

Table 4.1: Post-harvest initial WC (g g^{-1}) of shoot apices, and percentage root and shoot production from shoot apices excised from the embryonic axes of *A. coranica*, *C. australe* and *T. emetica*. Values represent mean± SD (n=10).

Plant species	Water content (g g^{-1})	Shoot production (%)	Root production (%)
A. coranica	4.51±0.91	100	100
C. australe	1.79±0.13	100	100
T. emetica	1.34±0.34	100	100

The high WCs of the shoot apices in Table 4.1 are typical of embryonic tissues in recalcitrant seeds. When the explants were *in vitro* germinated in specific media reported below, 100% root and shoot production was observed in all three species. The WC of *A. coranica* apices was particularly higher (*ca.* 4.51 g g^{-1}) compared with the other two species (*ca.*1.79 for *C. australe* and *ca.*1.34 g g^{-1} for *T. emetica*) probably because shoot apices were excised only after allowing the whole zygotic embryos (primary explant), which exhibited a WC of *ca.* 3.83 g g^{-1} (Figure 4.1), to germinate *in vitro* for 9 days; the shoot apices (secondary explant) were then excised from the primary explant. The WC for shoot apices of *A. coranica* were thus estimated for *in vitro* germinated shoot apices, while for *C. australe* and *T. emetica* WC was calculated using shoot apices excised directly from the embryos of non-germinated seeds.

Figure 4.1: The different developmental stages of *A. coranica* showing the protrusion and excision of the cotyledonary body which holds both the root and shoot meristem (Primary explant).

In *A. coranica*, the shoot pole lies in close proximity with the root, thus making it impossible to excise the shoot apex directly from the embryonic axes. Hence, it was essential that the primary explant be germinated in this species to be able to excise the shoot apex. Figure 4.1 shows how the primary axis (extended cotyledonary body excised 2 mm away from the tip) was first germinated so as to excise the embryonic shoot apices of *A. coranica* (secondary explant). Once the shoot apices had been excised from the primary explant they were used for various preliminary studies. One such study was to determine the most suitable *in vitro* growth media supplemented with various growth regulators for 100% shoot and root production from explants (Table 4.2). It is noteworthy to mention that it was essential that the preliminary studies on media selection for *A. coranica* were performed as there are limited studies on cryopreservation of this species, where *in vitro* germination was done. The different media used to test the germination of secondary explants of *A. coranica* were:

1. Full strength Murashige and Skoog medium (MS) with 0.8% Agar, 3% sucrose, supplemented with 1 mg L^{-1} trans-zeatin to induce shoot production and 0.1 mg L^{-1} IBA (Indole-3-butyric acid) to induce root production (Medium 1).

2. Full strength MS medium with 0.8% Agar, 3% sucrose, supplemented with 1 mg L^{-1} BAP (6-benzylaminopurine) to induce shoot production and 0.1 mg L^{-1} IBA to induce root production (Medium 2).

3. Full strength Woody Plant Medium (WPM) with 0.8% Agar, 3% sucrose, supplemented with 1 mg L^{-1} trans-zeatin to induce shoot production and 0.1 mg L^{-1} IBA to induce root production (Medium 3).

4. Full strength WPM with 0.8% Agar, 3% sucrose, supplemented with 1 mg L^{-1} BAP to induce shoot production and 0.1 mg L^{-1} IBA to induce root production (Medium 4).

Table 4.2: The effects of various *in vitro* growth media on shoot and root production of excised shoot apices of *A. coranica*. The (*) represent the medium that was selected. Values represent means only for percentage survival and mean± SD for shoot and root length (n= 3 replicates of 15 explants).

Growth medium	No. of days in culture	Survival (%)	Shoot production (%)	Shoot length (mm)	Root production (%)	Root length (mm)
1. MS + 1 mg L^{-1} trans-zeatin → 0.1 mg L^{-1} IBA	25	100	85	0±0	100	2±0.82
	50	90	70	3.7±0.83	90	6±0.33
	75	90	70	4.4±0.92	90	12±0.75
*2. MS + 1 mg L^{-1} BAP → 0.1 mg L^{-1} IBA	25	100	100	2±0.85	100	2±0.67
	50	100	100	6±0.82	100	6.6±1.71
	75	100	100	11.3±1.68	100	14±1.63
3. WPM + 1 mg L^{-1} trans-zeatin → 0.1 mg L^{-1} IBA	25	100	70	0±0	100	1.5±0.55
	50	85	60	2±1.15	85	3±0.65
	75	85	60	3.5±0.75	85	4±0.94
4. WPM + 1 mg L^{-1} BAP → 0.1 mg L^{-1} IBA	25	100	80	0±0	100	1.5±0.71
	50	75	75	2±0.74	75	3.7±0.74
	75	75	75	3±0.67	75	5±0.65

Survival = greening of explant and opening of leaf primordia

Shoot production = shoot extension for 8 mm

Results in Table 4.2 shows that the most suitable germination medium for maximum root and shoot production in *A. coranica* shoot apices was Medium 2, as it was shown to result in the highest shoot and root production, with 100% survival (also shown on figure 4.2 below). Additionally, medium 2 appears to be ideal for the development of plants from embryonic shoot apices as tissues in this medium had the longest shoot and root length as opposed to medium 1, 3 and 4. With regards to all the four germination media used for the preliminary studies, medium 3 seemed to be the medium with the least overall survival, whilst medium 2 gave the optimum results.

Similar studies were done for the selection of a suitable medium that would result in high survival and maximum shoot and root production for *T. emetica* and *C. australe* embryonic shoot apices. The medium selection studies used for *T. emetica* were based on a study by Varghese *et al.* (2009) and Gebashe (2015) on shoot meristems of *T. emetica*. The different medium used were:

1. Full strength Murashige and Skoog medium (MS) with 0.8% Agar, 3% sucrose, supplemented with 0.05 mg L^{-1} BAP and 0.1 mg L^{-1} GA$_3$ to induce shoot production and 0.1 mg L^{-1} IBA to induce root production (Medium 1).

2. Full strength Woody Plant Medium (WPM) with 0.8% Agar, 3% sucrose, supplemented with 0.05 mg L^{-1} BAP and 0.1 mg L^{-1} GA$_3$ to induce shoot production and 0.1 mg L^{-1} IBA to induce root production (Medium 2).

3. Full strength MS medium with 0.8% Agar, 3% sucrose, supplemented with 1 mg L^{-1} BAP to induce shoot production and 0.1 mg L^{-1} IBA to induce root production (Medium 3).

Table 4.3: The effects of various germination medium on the *in vitro* shoot and root production from excised shoot apices from embryonic axes of *T. emetica*. The (*) represent the medium that was selected. Values represent means only for percentage survival and mean± SD for shoot and root length (n= 3 replicates of 15 explants).

Growth medium	No. of days in culture	Survival (%)	Shoot production (%)	Shoot length (mm)	Root production (%)	Root length (mm)
1. MS + 0.05 mg L^{-1} BAP + 0.1 mg L^{-1} GA$_3$ → 0.1 mg L^{-1} IBA	30	97	50	0±0	97	2±0.68
	60	90	35	2±58	90	4±0.75
	90	83	30	2±1.68	83	10±1.60
2. WPM + 0.05 mg L^{-1} BAP + 0.1 mg L^{-1} GA$_3$ → 0.1 mg L^{-1} IBA	30	95	30	0±0	95	2±0.54
	60	82	20	3±0.67	82	6±1.05
	90	80	20	3±0.60	80	10±0.90
*3. MS + 1 mg L^{-1} BAP → 0.1 mg L^{-1} IBA	30	100	100	2±0.62	100	3±0.58
	60	100	100	5±1.69	100	8±0.76
	90	100	100	6±1.63	100	16±2.57

Survival = greening of explant and opening of leaf primordia

Shoot production = shoot extension for 8 mm

Results presented above (Table 4.3) shows that medium 3 (MS + 1 mg L^{-1} BAP + 0.1 mg L^{-1} IBA) was the optimum medium to be used for regeneration of *T. emetica* embryonic shoot apices. Medium 3 maintained the highest (100%) percentage survival, and root and shoot production of explants throughout the germination period (Figure 4.3), while medium 1 and 2 experienced a reduction in percentage survival with increased time in culture. The shoot and root length of explants in medium 1 and 2 was the least as opposed to medium 3, which also was able to form shoots and roots within the first 30 days.

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With regards to the germination of *C. australe* embryonic shoot apices, the various media chosen for testing included medium used in a study by Ballesteros *et al.* (2014) and the previously selected medium for *A. coranica* and *T. emetica* (above). The different medium used were:

1. Full strength Murashige and Skoog medium (MS) with 0.8% Agar, 3% sucrose without any plant growth regulators being supplemented (Medium 1).

2. Full strength Murashige and Skoog medium (MS) with 0.8% Agar, 3% sucrose, supplemented with 1 mg L^{-1} BAP to induce shoot production and 0.1 mg L^{-1} IBA to induce root production (Medium 2).

3. Full strength Murashige and Skoog medium (MS) with 0.8% Agar, 3% sucrose, supplemented with 1 mg L^{-1} BAP and 0.1 mg L^{-1} GA$_3$ to induce shoot production and 0.1 mg L^{-1} IBA to induce root production (Medium 3).

Table 4.4: The effects of various *in vitro* germination medium on shoot and root production of excised shoot apices from embryonic axes of *C. australe*. The (*) represent the medium that was selected. Values represent means only for percentage survival and mean± SD for shoot and root length (n= 3 replicates of 15 explants).

Growth medium	No. of days in culture	Survival (%)	Shoot production (%)	Shoot length (mm)	Root production (%)	Root length (mm)
1. MS only	20	92	15	0±0	90	2±0.71
	40	85	27	1.5±0.81	83	4.5±1.22
	60	80	30	1.5±1.08	80	6±0.91
2. MS + 1 mg L^{-1} BAP → 0.1 mg L^{-1} IBA	20	95	64	0±0	95	2.5±0.80
	40	90	70	2±1.76	90	6±1.29
	60	90	73	3±0.76	86	11.5±2.00
*3. MS + 1 mg L^{-1} BAP + 0.1 mg L^{-1} GA$_3$ → 0.1 mg L^{-1} IBA	20	100	86	2±0.69	100	3±1.23
	40	100	97	8±2.12	100	10±3.51
	60	100	100	13±2.62	100	18±2.83

Survival = greening of explant and opening of leaf primordia

Shoot production = shoot extension for 8 mm

Results presented above (Table 4.4) shows that explants grown on medium 3 exhibited the highest percentage survival (100%) and mean shoot (13 mm) and root (18 mm) length after 60 days in

culture. Thus, medium 3 (MS + 1 mg L^{-1} BAP + 0.1 mg L^{-1} GA$_3$ + 0.1 mg L^{-1} IBA) was selected as the germination medium for all *C. australe* trials that were carried out in the current study. It is important to note that all the different media tested for *C. australe* explants managed to achieve more than 90% survival, but medium 1 and 2 did not have elongated shoots in the first 30 days. The incorporation of GA$_3$ in the medium therefore enhanced the shoots to elongate. Thus, the medium that was selected was medium 3 as it had overall good results for survival, shoot formation and root formation (Figure 4.4).

Shown below are images of plantlets that developed after *in vitro* growth in the most suitable medium for each species (as shown in Tables 4.2, 4.3 and 4.4). Explants that exhibited shoot elongation were transferred onto root induction medium (MS medium incorporated with 0.1 mg L^{-1} IBA). From these results it is evident that the embryonic shoot apices excised from different recalcitrant-seeded plant species were capable of developing into full plantlets, and thus can be used as potential explants for further cryopreservation studies.
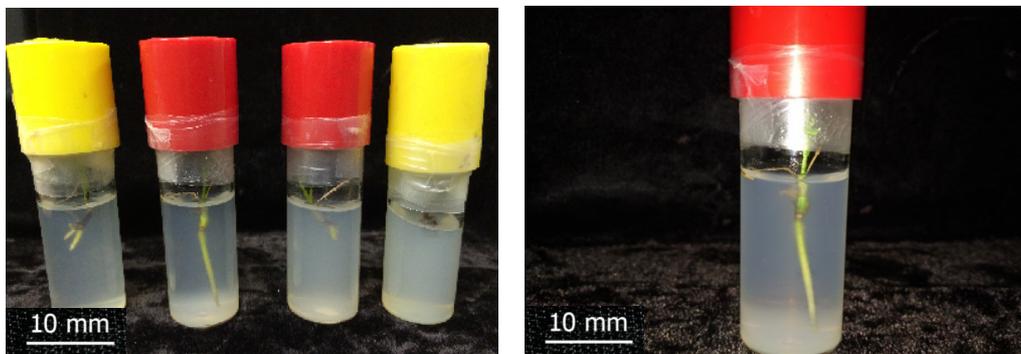
Figure 4.2: Seedling development from freshly excised embryonic shoot apices of *A. coranica* after 12 weeks of germination in MS medium supplemented with 1 mg L^{-1} BAP and 0.1 mg L^{-1} IBA.
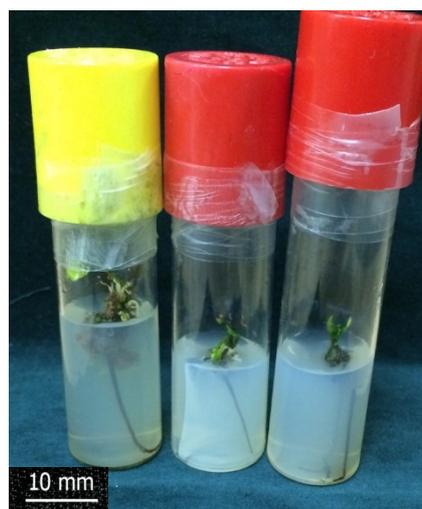
Figure 4.3: Seedling development 14 weeks of freshly excised embryonic shoot apices of *T. emetica* germinated in MS medium supplemented with 1 mg L^{-1} BAP followed by MS medium supplemented with 0.1 mg L^{-1} IBA.

Figure 4.4: Seedling development of *C. australe* after 10 weeks in culture, generated from freshly excised embryonic shoot apices in MS medium supplemented with 1 mg L^{-1} BAP + 0.1 mg L^{-1} gibberellic acid (GA$_3$) followed by MS medium supplemented with 0.1 mg L^{-1} IBA.

4.2 The response of various procedural steps of cryopreservation on the ability of excised *T. emetica* and *C. australe* embryonic shoot apices to produce plantlets.

Before embryonic shoot apices could be subjected to cryopreservation, it was essential to establish the appropriate dehydration time to achieve suitably low WCs (±0.40 g g^{-1}) for cryopreservation. This was done by sampling shoot apices after various intervals of flash drying (FD) for WC; at each interval a sub-sample of explants were also rehydrated and recovered by *in vitro* culture on the optimum growth medium to assess percentage seedling production. Similarly, WC and % seedling production was assessed after cryoprotection and the combination of cryoprotection and FD. After dehydration, and cryoprotection + dehydration, shoot apices with WCs in the range of < and > *ca.* 0.40 g g^{-1} (after Sershen *et al*., 2012b) were selected for exposure to different cooling treatments. Subsequently, after each cooling treatment, the shoot apices were thawed and regenerated to assess viability after exposure to the cryogen.

4.2.1 Response of explants to rapid dehydration pre-treatment

Embryonic shoot apices of *T. emetica* maintained a high capacity to survive as the explant WC declined from *ca.* 1.34 (control) to *ca.* 0.51 g g^{-1} after 30 min FD, with percentage survival (denoted as greening of explant and opening of leaf primordia) only declining from 100 to 95%. This drying interval was hence selected for trials in the range of explants with WC > *ca.* 0.40 g g^{-1}. Flash drying for 45 min reduced the WC to *ca.* 0.37 g g^{-1} with 80% seedling production (Figure 4.5). As percentage seedling production was still high after 45 min FD drying, this drying time was selected for trials in the range of < *ca.* 0.40 g g^{-1}. Upon further dehydration % seedling production decreased to 45% at *ca.* 0.33 g g^{-1} after 60 min FD and 0% at WC of *ca.* 0.22 g g^{-1} after 105 min FD (Figure 4.5).
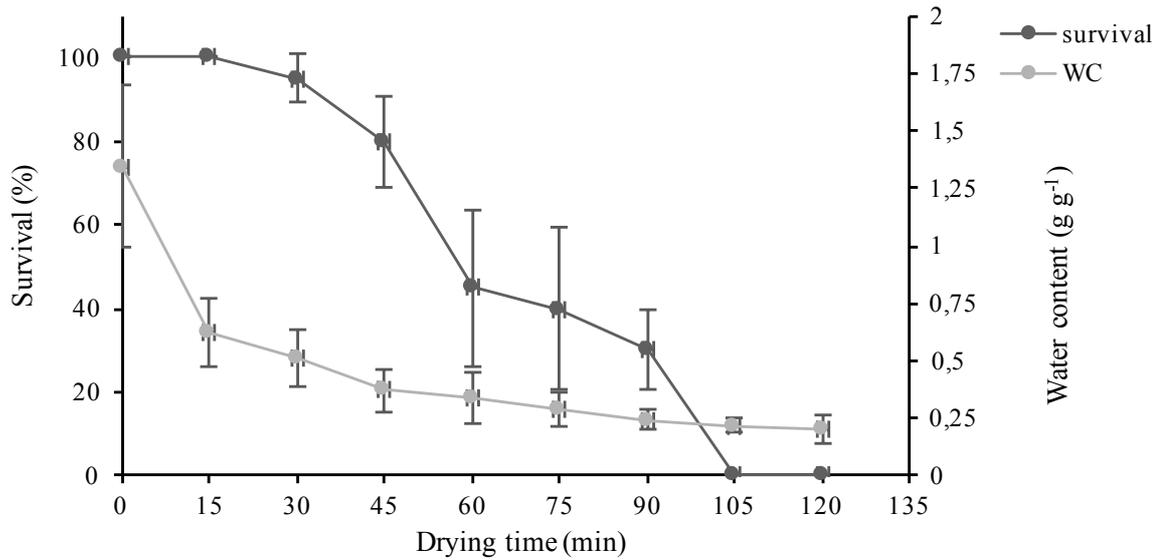
Figure 4.5: Effect of flash drying on survival (% greening of explant and opening of leaf primordia) and water content (g g^{-1}) of embryonic shoot apices of *T. emetica*. Values are means ± SD (n= 15 for germination and for WC, n= 10).

Embryonic shoot apices of *C. australe* maintained high germinability from the onset at a mean WC of *ca.* 1.79 g g^{-1} with 100% survival to WC of *ca.* 0.76 g g^{-1} after 45 min FD, still maintaining 100% survival (Figure 4.6). Further dehydration for up to 75 min lowered the WC (*ca.* 0.58 g g^{-1}) but not drastically the survival, and still maintaining a high survival (83%). Flash drying for 105 min reduced the mean WC to *ca.* 0.40 g g^{-1}, but still maintaining 70% survival. This interval was therefore selected for trials that are in the range of > *ca.* 0.40 g g^{-1}. Flash drying for 120 min lowered the mean WC to *ca.* 0.37 g g^{-1} and the survival to 60%; this interval was selected for trials in the range of < *ca.* 0.40 g g^{-1}.
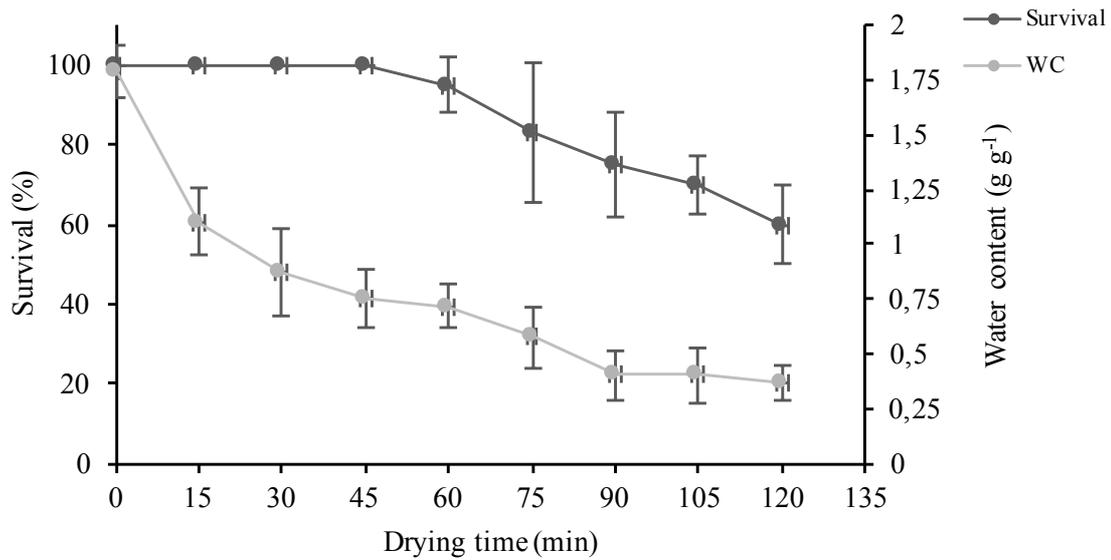
Figure 4.6: Effects of different drying intervals on survival (% greening of explants and opening of leaf primordia) and water content (g g^{-1}) of freshly excised embryonic shoot apices of *C. australe*. Values are means± SD (n= 15 for germination and for WC, n= 10).

Opening of leaf primordia
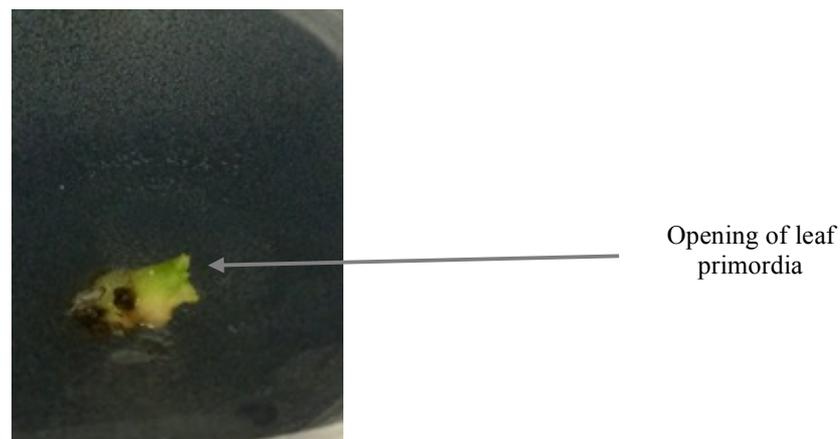
Figure 4.7: Shoot apex of *Castanospermum australe* showing greening and opening of a leaf primordia which were used as signs of survival after flash drying, cryoprotection and cooling.

4.2.2 Response of explants to cryoprotection

Embryonic shoot apices of *C. australe* and *T. emetica* were treated with cryoprotectants (CPs) so as to dehydrate the explants and increase their cytoplasmic viscosity before cooling. When shoot apices of *C. australe* were treated with cryoprotectants (5 and 10% glycerol [CP1]; 5 and 10% glycerol + sucrose where it was 0.5 M and 1 M for sucrose [CP2]), for 30 min in a 5% solution followed by 30 min in a 10% cryoprotectant solution, axis WC declined from *ca*. 1.79 to *ca*. 1.46 g g^{-1} after treatment with 5 and 10% glycerol (Figure 4.8) and to *ca*. 1.17 g g^{-1} after treatment with 5 and 10% glycerol + sucrose (Figure 4.9). After exposure of explants to CP1 followed by FD, there was a substantial reduction in

WC. For example, after CP1 + 60 min FD, though explant WC reduced to *ca.* 0.41 g g^{-1}, 70% of these explants retained the ability to produce seedlings (Figure 4.8). Hence, CP1 + 60 min FD was selected for cryopreservation trials at > *ca.* 0.40 g g^{-1} range. Further dehydration for 90 min lowered explant WC to *ca.* 0.37 g g^{-1}, with seedling production of 55% and this treatment combination was also selected for cryopreservation trials at < *ca.* 0.40 g g^{-1} range. Similarly, exposing explants to CP2 followed by a 90 min FD reduced explant WC to *ca.* 0.45 g g^{-1} and resulted in 65% seedling production. This interval was also selected for cryopreservation trials at > *ca.* 0.40 g g^{-1} range. Upon further dehydration after treatment with CP2 (for 105 min FD), the WC reduced to *ca.* 0.35 g g^{-1} and exhibited 40% seedling production; this treatment combination was selected for cryopreservation trials at the < *ca.* 0.40 g g^{-1} range. As mentioned above, survival of the pre-treated explants was displayed by greening of the explant and opening of the leaf primordia (Figure 4.7), which later led to full seedling production.

Freshly excised embryonic shoot apices of *C. australe* were also cryoprotected with pre-chilled PVS2 (30% [w/v] glycerol, 15% [w/v] ethylene glycol and 15% [w/v] DMSO) in liquid MS medium incorporated with 0.4 M sucrose (CP3). However, this CP treatment was only done prior to cooling with Mr. Frosty$^{®}$; no WC and percentage survival trials were done otherwise.
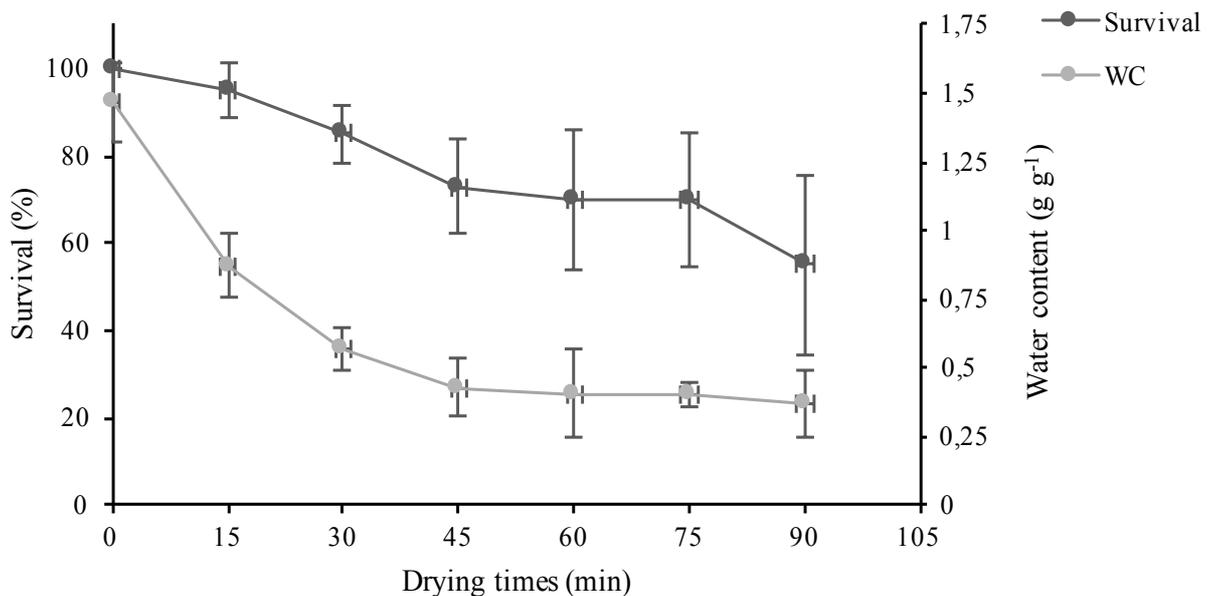

Figure 4.8: Percentage survival [(%) greening of explant and opening of leaf primordia] and water content (g g^{-1}) of embryonic shoot apices of *C. australe* treated with 5 and 10% glycerol and subsequently flash dried for different intervals. Values are means± SD (n= 15 for germination and for WC, n= 10).
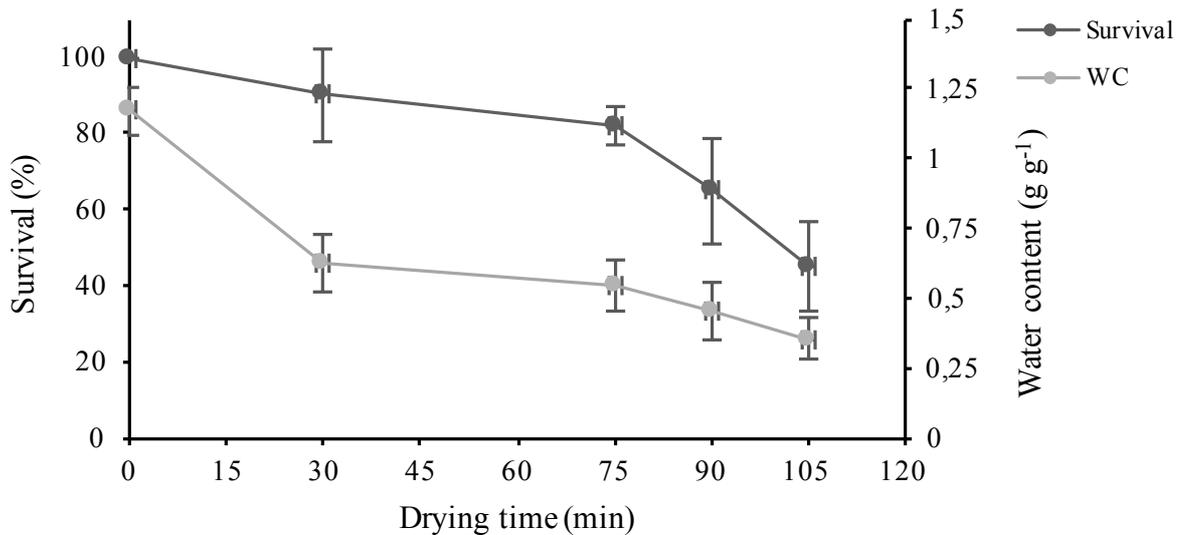
Figure 4.9: Percentage survival (%) and water content (g g^{-1}) of embryonic shoot apices of *C. australe* after treatment with 5 and 10% glycerol + sucrose CP, and subsequently flash dried for different intervals. Values are means ± SD (n= 15 for germination and n=10 for WC).

From the above experiments of *C. australe*, the following treatments were selected for cryopreservation to assess their cryo-survival after retrieval from LN:

- Explants that were flash dried for 105 (> *ca.* 0.4 g g^{-1}) and 120 min (< *ca.* 0.4 g g^{-1})
- Explants treated with CP1 and flash dried for 60 (> *ca.* 0.4 g g^{-1}) and 90 min (< *ca.* 0.4 g g^{-1}).
- Explants treated with CP2 and flash dried for 90 (> *ca.* 0.4 g g^{-1}) and 105 min (< *ca.* 0.4 g g^{-1}).
- Freshly excised explants that were treated with pre-chilled PVS2 (CP3).

These treatments were selected on the basis that the WC of the explants exhibited > 40% survival (as explained above) after the WC was reduced to levels considered suitably low for successful cryopreservation of recalcitrant seed germplasm. Given below (Figure 4.10) is a summative figure of WC and survival across the treatments after preconditioning.
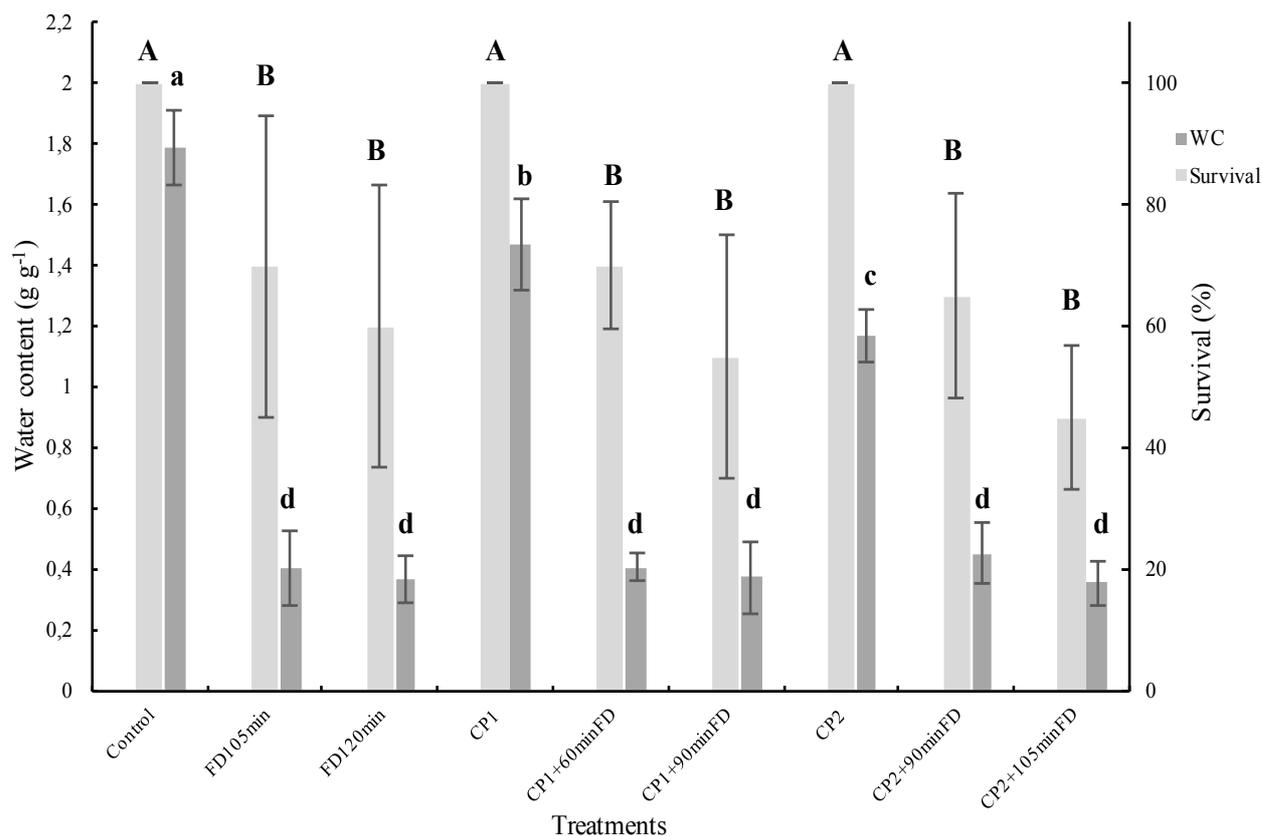
Figure 4.10: Water content (g g^{-1}) and survival (%) of embryonic shoot apices of *C. australe* before (control) and after flash drying (FD), after CP and CP+FD treatments. CP1= 5 and 10% v/v glycerol and CP2= 5 and 10% v/v glycerol + sucrose solution. The letters above the bars denote significant differences (p<0.05) in WC and survival between treatments (One-Way ANOVA). Values are means± SD (n= 15 for germination and for WC, n= 10).

Statistical analysis revealed that there was a significant difference in WC between freshly excised explants, flash dried explants and explants treated with CP. However, there was no significant difference in survival between these treatments (control, CP1 and CP2; Figure 4.10). A significant difference in WC as well as survival was also seen between freshly excised and explant that were only dried, thus showing the impact of dehydration on the WC of plant tissues and the survival. From the above figures it is also shown that CPs played a role in the dehydration of explants. However, it is worthwhile to note that germinability was affected after CP + FD, as survival rapidly reduced when shoot apices were cryoprotected and subsequently flash dried. Thus, subjecting explants to CPs and subsequently flash drying drastically reduced WC and overall survival of shoot apices.
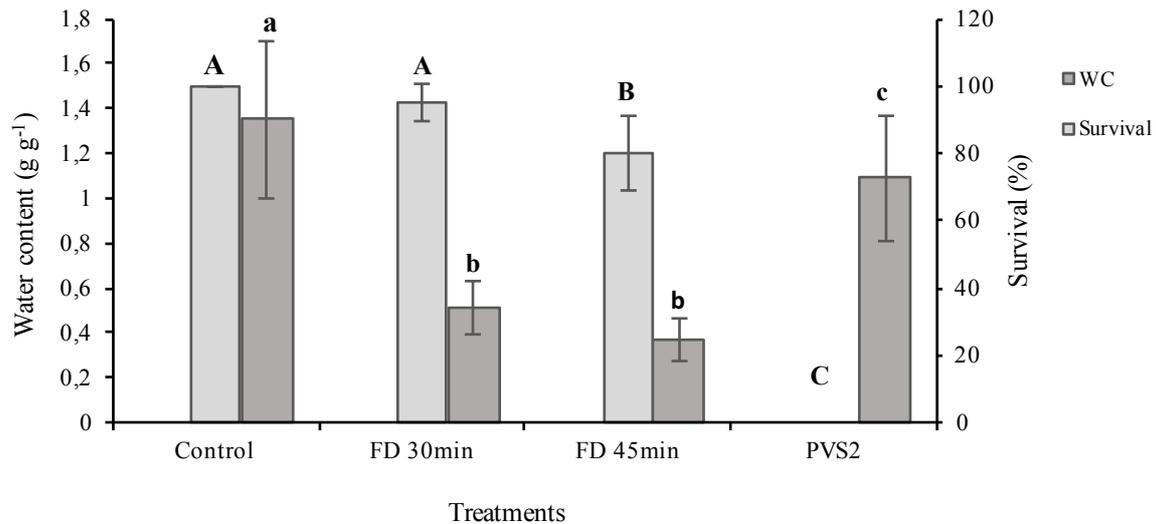
Figure 4.11: Water content (mean ± SD) and survival (%) of embryonic shoot apices of *T. emetica* before (control) and after flash drying (FD), and cryoprotection with PVS2. The letters above the bars denote significant differences (p<0.05) in WC and survival between treatments (One-Way ANOVA). Values are means ± SD (n= 15 for germination and for WC, n= 10).

Similar selection treatments of explants for exposure to different cooling treatments were done for *T. emetica*. The pre-treatments were also selected on the basis that the WC of the explants was reducible to concentrations suitable for LN exposure. The CP used for *T. emetica* was pre-chilled PVS2 as this cryoprotectant has been documented to be effective on shoot meristems (Varghese *et al.,* 2009; Gebashe, 2015). Prior to PVS2 treatment, the explants were pre-cultured in MS medium containing 0.4 M sucrose and 0.2 M glycerol for 2 days. In the current study none of the explants survived treatment with PVS2, even before exposure to LN (Figure 4.11). Results from the statistical analyses showed that there was a significant difference in WC between freshly excised (*ca.* 1.35 g g^{-1}) and explants that were flash dried (*ca.* 0.57 to *ca.* 0.31 g g^{-1}) as well as treated with PVS2 (*ca.* 1.09 g g^{-1}). There was no significant difference in survival between freshly excised apices and apices dried for 30 min, but there was are significant difference between freshly excised apices and those that were dried for 45 min (Figure 4.11). The figures above show that the cryoprotectant played a role in the dehydration of explants, but drastically affecting the germinability of the explant. Treatment with PVS2 did not result in any survival, thus making it significantly different from the other treatments.

Listed below are the treatments selected for testing for cryo-survival of *T. emetica* embryonic shoot apices:

- o Explants that were flash dried for 30 min (> *ca.* 0.4 g g^{-1})
- o Explants that were flash dried for 45 min (< *ca.* 0.4 g g^{-1}).
- o Freshly excised explants that were treated with pre-chilled PVS2 (CP3).

4.2.3 Post-cryo survival

After each of the selected pre-treatments mentioned above, the plant material was cooled using the following techniques:

- o   Slow cooling (cooling with Mr. Frosty®)
- o   Faster cooling (direct liquid nitrogen immersion)
- o   Ultra-rapid cooling (cooling in nitrogen slush - NS)

In the current study, embryonic shoot apices of *T. emetica* and *C. australe* did not respond well to cryogen exposure, irrespective of the cooling method used. Embryonic shoot apices of *T. emetica* were subjected to the three cooling treatments after dehydration with flash drying for 30 and 45 min and treatment with [CP3 (PVS2) – Figure 4.11]. Explants of *T. emetica* did not survive any of the cooling treatments (Table 4.5). The results obtained after cooling shoot apices of *T. emetica* were also influenced by the reduction of survival prior to cooling.

Table 4.5: Survival of embryonic shoot apices of *T. emetica* before and after cryogen exposure (Slow cooling). Values are mean± SD of n= 3 replicates of 15 explants each.

Treatment	Survival before LN (%)	Survival after LN (%)
2days pre-culture	80±7.36	0
2days pre-culture + PVS2	0	0
30 min FD	95±6	0
45 min FD	80±11	0

Survival = greening of explants and opening of leaf primordia

Negligibly low levels of post-cryo survival were achieved for explants of *C. australe* (Table 4.6). From the three cooling treatments that were used, the only treatment that resulted in post-cryo-survival in *C. australe* after cryopreservation was the direct immersion in LN (faster cooling [Table 4.6; Figure 4.12; 4.13]). Embryonic shoot apices that were flash dried for 105 min and not treated with any CPs exhibited the best post-cryo survival, with an overall percentage survival of 16% depicted by greening of the explant and opening of the leaf primordia. It is noteworthy to emphasize that this preconditioning treatment and cooling rate are the only treatments that resulted in the highest survival percentage of embryonic shoot apices in the current study. Shoot apices that were preconditioned with cryoprotectants responded more poorly to cryogen exposure. Only 8% of explants treated with CP1+ 90 min FD and 1% of explants treated with CP1 + 60 min FD survived fast cooling. None of the shoot apices that were treated with CP2 and CP3 survived any of the cooling rates (Table 4.4; Figure 4.13).

Table 4.6: Percentage survival and shoot production (mean± SD) of pre-treated explants of *C. australe* after different cooling treatments. The (*) represent the cooling treatment that resulted in survival and shoot production.

	Mean survival (%)			Mean shoot production (%)		
Dehydration treatments	Slow cooling	Fast cooling	Ultra-rapid cooling	Slow cooling	Fast cooling	Ultra-rapid cooling
*FD + 105 min	0±0	16±13	0±0	0±0	10±10	0±0
FD + 120 min	0±0	0±0	0±0	0±0	0±0	0±0
*CP1 + 60 min	0±0	1±1	0±0	0±0	1±0	0±0
*CP1 + 90 min	0±0	8±7	0±0	0±0	6±6	0±0
CP2 + 90 min	0±0	0±0	0±0	0±0	0±0	0±0
CP2 + 105 min	0±0	0±0	0±0	0±0	0±0	0±0
CP3	0±0	0±0	0±0	0±0	0±0	0±0

Survival = greening and opening of the leaf primordia

Shoot production = shoot extension for 8 mm

Shown below are images of explants from the different cooling treatments that were a success after cryopreservation (Figure 4.12). Success after recovery from LN was based on greening of the explant and opening of the leaf primordia. Figures 4.12 and 4.13 are examples from some of the treatments used in the study, showing explants that were treated with CP1 + 90 min FD + NS; 105 min FD + LN; PVS2 + LN; PVS2 + NS; CP1 + 60 min + NS and 120 min FD + LN.
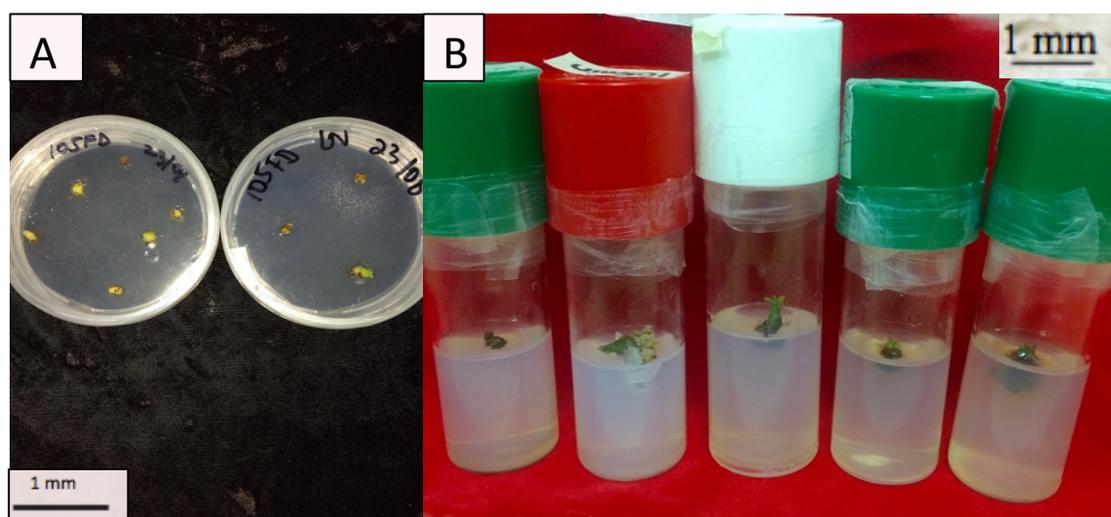
Figure 4.12: Post-cryo survival (greening of explants and opening of leaf primordia) and shoot production of embryonic shoot apices of *C. australe* after 3 (A) and 6 (B) months of retrieval from liquid nitrogen. The cooling rate used was fast cooling.
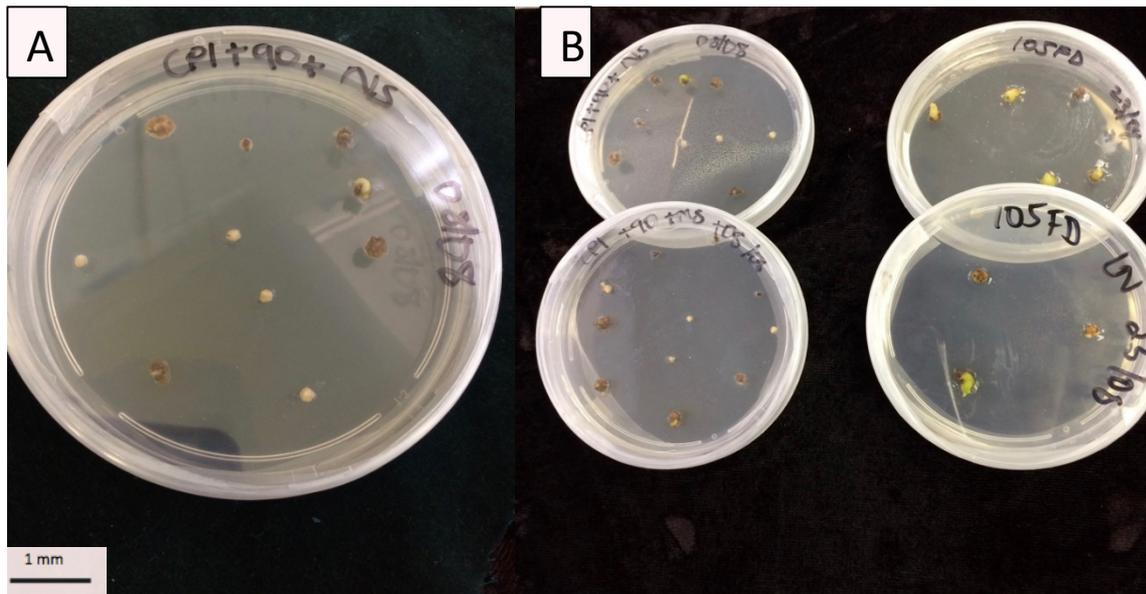
Figure 4.13: Post-cryo survival of embryonic shoot apices of *C. australe* that were cooled using ultra-rapid cooling (A) and fast cooling (B). Figure A and B shows explants that survived (greening of explants and opening of leaf primordia) and those that did not survive (cream-white and brown explants) cryopreservation, taken 9 weeks after retrieval from liquid nitrogen.

CHAPTER 5: DISCUSSION

Cryopreservation is considered as the only viable option for the long-term germplasm conservation of many plant species that produce desiccation sensitive seeds (Berjak and Pammenter, 2013). However, developing successful cryostorage protocols for many such species is very challenging for various reasons (Berjak *et al*., 2011b; Berjak and Pammenter, 2013). The development of successful cryopreservation protocols is of paramount importance to maintain genetic stability of plant germplasm during long term storage (Kaczmarczyk *et al*., 2012). Successful cryopreservation is characterised by the continued survival and growth of an explant into a fully functional plantlet *in vitro*. Survival is influenced by individual and cumulative stresses imposed on explants during cryopreservation i.e., during excision, cryoprotection, rapid dehydration, cooling and rewarming. Each of these stages are often accompanied by unregulated production of ROS (Berjak *et al*., 2011b), coupled with the depletion of endogenous antioxidants which are known to aid in ameliorating ROS production (Varghese *et al*., 2011; Naidoo, 2012; Sershen *et al*., 2012b; Sershen *et al*., 2016). An imbalanced redox state often compromises the survivability of explant after cryopreservation (Sershen *et al*., 2012b; Naidoo *et al*., 2016; Sershen *et al*., 2016). Success in cryopreservation is also largely influenced by the explant selection which encompasses the topography and variation in anatomical structure that may have an impact on the stress imposed on the explant (Ballesteros *et al*., 2014). Once selected, the explants must be physically and/or osmotically dehydrated to suitably low WCs that will limit the formation of lethal ice crystals during exposure to the cryogen and recrystallisation during subsequent rewarming (Berjak and Pammenter, 2008; Wesley-Smith *et al*., 1992; 2015).

In the present study, embryonic shoot apices excised from the embryonic axes of three recalcitrant-seeded species were assessed on their ability to generate full plantlets. The selection of shoot apices as explants was aimed at reducing the explant size that will be small enough for cryopreservation and ensuring uniform dehydration during the procedural steps of cryopreservation. The findings of the present study are interpreted in relation to reports made on embryonic axes, zygotic embryos and vegetative shoot tips/meristems. It is noteworthy to mention that in a study by Chmielarz *et al*. (2011), embryonic shoot apices of *Q. robur*, which were referred to as plumules, were successfully cryopreserved. This was the first known report where shoot apical meristems excised from the embryo (prior to *in vitro* germination) were used as explants for cryopreservation of a recalcitrant-seeded species. The explants used in the current study are similar to the explants used in the above study. The only difference lies in the explant size: where shoot apices of 0.5 – 1.0 mm were used in the study by Chmielarz *et al*. (2011), while 2 mm explants were used in the current study.

5.1 Development of plantlets from embryonic shoot apices of *A. coranica*, *C. australe* and *T. emetica* after excision

As mentioned above, before the selected explants were exposed to cryopreservation, it was essential to determine whether they could develop into full plantlets. The explant size contributes towards achieving success after cryopreservation (Mycock *et al*., 1995), as this may affect the efficiency of drying and cooling. Most cryopreservation studies on recalcitrant seed germplasm have focused on the use of embryonic axes or zygotic embryos (Chandel *et al*.,1995; Sershen *et al*., 2007; Xin *et al*., 2010). However, those explants in recalcitrant seeds of many species are large, necessitating longer drying time and facilitating slower cooling rates, which most often compromises post-cryo survival, hence the need for alternative explants (Pence, 1991; Goveia *et al*., 2004; Perán *et al*., 2006; Varghese *et al*., 2009; Gebashe, 2015). Previous studies have shown that cryopreservation of whole embryonic axes may result in limited to no survival (Chmielarz, 1997), as well as survival of roots only as exemplified in species such as *T. dregeana* (Kioko *et al*., 1998) and *E. capensis* (Perán *et al*., 2006). Shoot apices excised from the embryo, which confer reduced explant size, were selected as an alternative explant based on the reduced survival obtained in other studies where whole embryos were used.

The shoot apex has advantages over zygotic embryos in its significantly reduced mass and uniformity of tissue type, which may confer a faster and more even drying (Naidoo *et al*., 2011; Engelmann, 2012) and cooling rate (Wesley-Smith *et al*., 1992). In the current study, after excision and *in vitro* germination, the embryonic shoot apices exhibited complete survival (greening of explant and opening of leaf primordia) on shoot and root initiation medium (shoot and root development; Figures 4.2, 4.3 and 4.4). The ability of shoot apices to develop into complete plantlets support suitability of explant selection. The high percentage survival was obtained after subjecting the explants to different preliminary studies specific to each species Table 4.2, 4.3 and 4.4). While embryonic shoot apices of *A. coranica* and *T. dregeana* developed into full plantlets after *in vitro* germination in full strength MS medium with 0.8% Agar, 3% sucrose, supplemented with 1 mg L^{-1} BAP for shoot production and 0.1 mg L^{-1} IBA for root production, shoot apices of *C. australe* developed into full plantlets after *in vitro* germination in full strength MS medium with 0.8% Agar, 3% sucrose, supplemented with 1 mg L^{-1} BAP and 0.1 mg L^{-1} GA$_3$ for shoot production and 0.1 mg L^{-1} IBA for root production.

The shoot apices were obtained by excision from the zygotic embryo, an act which has previously been reported to induce the initial damage on the explant (Goveia *et al*., 2004; Roach *et al*., 2008; Whitaker *et al*., 2010; Berjak *et al*., 2011b). Excision has been reported to cause the primary wound in explants which may precondition the explants to accumulative damage as the explant is passed through the various cryopreservation stages (Berjak *et al*., 2011b; Pammenter *et al*., 2011b). The shoot meristem region of the embryo in many recalcitrant-seeded species seem to be particularly susceptible to excision damage due to its proximity to the point of excision (Ballesteros *et al*., 2014), and this often results in the lack of shoot development (Kioko *et al*., 1998; Goveia *et al*., 2004; Berjak *et al*., 2011b; Ballesteros *et al*., 2014). Previous studies on various recalcitrant species such as *T. dregeana*, *T. emetica*

(Goveia *et al*., 2004; Kioko, 2003) and *E. capensis* (Perán *et al*., 2006) (Hajari, 2011; Hajari *et al*., 2011) have shown a lack of shoot production after excision (Naidoo *et al*., 2011). Inhibited shoot development has been partially attributed to the oxidative burst associated with excision damage that occurs when surrounding tissue is removed from the axes (Goveia *et al*., 2004; Berjak *et al*., 2011b Pammenter *et al*., 2011b). This was also reported in a study by Roach *et al*. (2008) where axes of *Castanea sativa* were used for the understanding of the stresses that follow wounding and dehydration. It was shown that wounding damage leads to the excessive production of superoxide which affects shoot development. In the current study however, the act of excision did not inhibit shoot production as anticipated, as explants of the three species managed to develop and elongate shoots (Figures 4.2, 4.3 and 4.4). The shoot apices were excised in such a way that damage to the shoot tip is limited. Present results show that apices excised from the shoot region of the embryo imposed minimal damage to the shoot tip which promoted higher shoot production *in vitro.*

The initial WC ranges of the three species (*ca*. 1.3 to *ca*. 4.5 g g^{-1} [Table 4.1]) used were similar to the initial WCs of shoot meristems and zygotic explants from most recalcitrant-seeded species (e.g. *T. emetica* [Varghese *et al*., 2009]; various amaryllid species [Sershen *et al*., 2007] and *C. australe* [Ballesteros *et al*., 2014]). This shows that the amount of water in the explants though high despite the explant being maximally reduced in size, may not hinder cryopreservation. It has been reported that explants excised from the embryo are more likely to produce mature plants than other plant tissues such as callus tissues (Goldberg *et al.*, 1994; Kaczmarczyk *et al*. 2012). This is because they are comprised of an embryonic axis consisting of shoot and/or root meristem (West and Harada, 1993; Goldberg *et al*., 1994). Though some studies have reported on the use of root tips as explants for cryopreservation, storing roots in the cryogen in such studies have been shown to be mainly for their future use for medicinal purposes (Yoshimatsu *et al*., 1996; Jung *et al*., 2001; Oh *et al*., 2009). Those authors reported on storage of adventitious roots in LN without the aim of generating full plantlets after retrieval from the cryogen but using these explants for the extraction of secondary metabolites when required.

The embryonic shoot apices in this study were excised from the shoot pole of the embryo to reduce the size of the explant for cryopreservation as well as to ensure shoot development which is limited when embryonic axes are used as explants. Selecting an explant which is excised from the shoot pole implies that it will be comprised of apical meristems which are capable of resulting in shoot production when *in vitro* germinated in suitable medium (Kaczmarczyk *et al*., 2012). Achieving full plantlet formation from shoot apices excised from the embryo therefore implied that these explants can be used for the procedural stages of the cryopreservation protocol.

5.2 Impact of dehydration on water content and survival of *T. emetica* embryonic shoot apices
It has been suggested that the drying characteristics of the different tissues of an explant are determined by the embryo morphology, anatomy and size (Ballesteros *et al*., 2014). Dehydration by means of flash drying reduces explant WC as rapidly as possible, promoting post-cryo survival and this was shown

with the use of zygotic embryos (Pammenter *et al*., 2002; Sershen *et al*., 2012a) and embryonic axes (Sershen *et al*., 2007). The rate of water loss is variable depending on the physical and chemical characteristics of the different species being investigated (Sershen *et al*. 2012a; Ballesteros *et al*., 2014). Rapid as opposed to slow drying curtails the time for the accumulation of damage consequent upon aqueous-based deleterious reactions that occur at intermediate WCs during drying (Varghese *et al*., 2011). In a study by Sershen *et al*. (2012a), zygotic embryos of *Amaryllis belladonna* that had undergone flash drying (> *ca*.0.4 g g^{-1}) showed some abnormalities in the nuclear structure suggesting derangement of intra-nuclear organisation. In that study it was also shown that flash drying for longer intervals (< *ca*. 0.4 g g^{-1}) may result in vacuolation, a phenomenon associated with an increase in solute concentration in vacuoles due to drying (Farrant *et al*., 1989; Wesley-Smith *et al*., 2001b; Walters *et al*. 2001; Sershen *et al*., 2012a) which ascertains the fact that the faster embryonic tissues can be dried, the better it is for survival before (Varghese *et al*., 2011) and after cryopreservation (Ballesteros *et al*., 2014; Sershen *et al*., 2016). When embryonic shoot apices of *T. emetica* were flash-dried, they lost water rapidly, reaching values in the region of > and < *ca*. 0.4 g g^{-1} after 30 to 60 min (Figure 4.5), but this was accompanied by a decline in survival from 95 to 45% respectively (Figure 4.5). After 60 min of drying (*ca*. 0.33 g g^{-1}), apices of *T. emetica* drastically lost viability and ultimately exhibited no survival after 105 (*ca*. 0.22 g g^{-1}) and 120 min (*ca*. 0.21 g g^{-1}) of drying (Figure 4.5). Loss of viability commensurate with tissue dehydration is common in desiccation sensitive germplasm and may be caused by aqueous-based, metabolism-linked and ROS-mediated damage (Berjak *et al*., 1990; Pammenter *et al*. 1991, Walters *et al*., 2001; Ballesteros *et al*., 2014). Pammenter and Berjak (1999) also suggested that loss in viability which accompanies longer drying intervals could be attributed to physical damage following the removal of water; that is, shrinkage of cells. The reduction in survival during dehydration could therefore result from a combination of physical (Xin *et al*., 2010) and metabolic damage (Varghese and Naithani, 2002; Varghese *et al*., 2011). However, Walters *et al*. (2001) suggested that even though dehydration results in several stresses that are imposed on cells, the methods to distinguish them and determining factors that limit survival due to dehydration have not yet been established. In light of the established oxidative damage that occurs during the successive stages of cryopreservation, the present study aimed to circumvent unregulated ROS generation by using cathodic water with 1% ascorbic acid, a solution which has been shown to have antioxidant properties (Berjak *et al*., 2011b; Gebashe, 2015; Naidoo *et al*., 2016). However, this solution was only used after dehydration and after cooling as a rewarming solution.

The decline in viability with dehydration is a common trend that has been observed in previous studies on recalcitrant seed germplasm (Sershen *et al*., 2008). Pammenter *et al*. (1991) worked on embryonic axes of *Landolphia kirkii*, and reported the drastic reduction in the axis WC from 1.50 to *ca*. 0.32 g g^{-1} within 30 min commensurate with an approximately 50% decline in viability. The embryonic shoot apices of *T. emetica* displayed a moderate reduction in viability, from 100 to 80% survival after 45 min of flash drying to *ca*. 0.37 g g^{-1}. However, in a study by Kioko *et al*. (2006) embryonic axes of

T. emetica reached *ca.* 0.42 g g^{-1} after 60 min of flash drying and 85% of these explants subsequently survived. This therefore suggests that explants used in the current study took a shorter dehydration time to lose water and reach almost similar WCs achieved in Kioko *et al.* (2006). This may be due to the reduction in the explant size in the current study. According to Kioko *et al.* (2006), the shoot tip loses water much more rapidly than the root tip, thus explaining why the embryonic shoot apices of *T. emetica* lost water more rapidly than whole embryonic axes. It is noteworthy to mention that rapid loss in WC (beyond 45 min of FD) had a drastic negative impact on viability of the shoot apices in this study. Where embryonic axes were used, 80% survival with 25% shoot formation was obtained after 90 min of FD with WC of *ca.* 0.3 g g^{-1} (Kioko *et al.*, 2006), while the survival of embryonic shoot apices used in the current study reduced to 30% survival after 90 min of FD with *ca.* 0.24 g g^{-1} (Figure 4.5). Overall survival in Kioko *et al.* (2006) was denoted by the elongation of the radical beyond 5 mm and shoot development by the appearance of the first pair of leaves, while in the current study it was denoted by greening of the explant and opening of the leaf primordia, indicating development of the shoot.

Sensitivity to dehydration in recalcitrant tissue may be better understood via ultrastructural investigation of the cells. Previous ultrastructural studies on rapidly dried embryonic axes of *T. emetica* indicated that when these axes were rapidly dried to *ca.* 0.3 g g^{-1} the root meristems retained their ultrastructural integrity (Wesley-Smith *et al.*, 2001; Kioko *et al.*, 2006). Furthermore, Kioko *et al.* (2006) reported that the shoot meristem of these axes showed some ultrastructural abnormalities, due to the shoot pole drying much quicker than the root meristem. As mentioned above those authors reported that only 25% of axes dried to *ca.* 0.3 g g^{-1} produced shoots. The present study therefore reports on the improvement of shoot production as around 60 min FD shoot apices managed to dry to WCs around *ca.* 0.33 g g^{-1} with 45% survival which also included shoot production.

As alluded to above, studies on recalcitrant-seeded species such as *T. emetica* (Goveia *et al.*, 2004), *C. sativa* (Roach *et al.*, 2008), *T. dregeana* (Varghese *et al.*, 2011) and *S. gerrardii* (Berjak *et al.*, 2011b) have reported a burst in ROS upon excision. This ROS production due to excision is exacerbated by dehydration and at unregulated concentrations may be toxic to cells, and may result in necrosis and cell death (Halliwell, 2006; Varghese *et al.*, 2011). This may explain the decline in viability of *T. emetica* explants seen in Figure 4.5, where viability declined with dehydration. In other studies, a relationship was shown between WC, accumulation of ROS, electrolyte leakage and survival (Varghese *et al.*, 2011; Sershen *et al.*, 2016), as it was noticed that low WC values result in high electrolyte leakage due to dehydration, thus resulting in a decrease in survival (Chaitanya and Naithani, 1994; Chandel *et al.*, 1995; Wesley-Smith *et al.*, 2001; Liang and Sun 2002; Varghese and Naithani, 2002; Sershen *et al.*, 2016). Even though biochemical analyses were not performed in the present study, the results obtained here may be linked to excision and dehydration-induced oxidative stress, based on previous studies. In this study, the response of *T. emetica* embryonic shoot apices to dehydration was poor when compared to other recalcitrant species, as irrespective of rapid dehydration, they lost viability. That said, the

visible opening of the leaf primordia indicated development of the shoot which is promising as shoot development from excised embryonic explants has been reported to be a persistent problem.

Based on previous cryopreservation studies on zygotic embryos, it has been suggested that explants with WCs ranging between 0.25 – 0.40 g g^{-1} are more suitable for successful cryopreservation (Wesley-Smith *et al*., 2001; Sershen *et al*., 2007; Berjak *et al*., 2011b; Ballesteros *et al.*, 2014). However, percentage survival associated with this range of WC must be at least 60% and above before cryogen exposure in order to assure survival after cryopreservation (Sershen *et al*., 2012a; Sershen *et al*., 2012b). The percentage survival of shoot apices in this study declined severely when dried beyond *ca.* 0.37 g g^{-1} reaching to 0% survival at *ca.* 0.21 g g^{-1}. The loss in viability in both *T. emetica* an*d T. dregeana* embryonic tissues has been documented to be highly influenced by excision damage which becomes exacerbated with drying (Goveia *et al*., 2004; Naidoo *et al*., 2011; Pammenter *et al*., 2011; Naidoo, 2017). During the duration of this study it was noted that the seeds of *T. emetica* were accumulating fungal contamination while in hydrated storage despite the previously used sterilization protocol for this species. Due to this further cryopreservation studies could not be carried out. However, the explants were exposed to cooling (no survival was obtained) after PVS2 treatment to ensure study completion and to confirm that the explant was not suitable for the remaining stages of cryopreservation.

5.3 Impact of dehydration on water content and survival of *C. australe* explants

The seeds of *C. australe* are generally larger than those of the other two species used in the present study and subsequently the shoot apices excised from them are much larger. This may explain why the response of *C. australe* explants to flash drying was different to that of *T. emetica*. Shoot apices of *C. australe* took much longer (105 – 120 min) to reach WCs (*ca.* 0.40 – *ca.* 0.37 g g^{-1}) amenable for cryopreservation. However, despite this relatively slower drying rate, high levels of viability were maintained throughout the drying intervals in *C. australe*, with 60% survival after 120 min of flash drying to a WC of *ca.* 0.37 g g^{-1} (Figure 4.6). This could imply that *C. australe* is more tolerant to desiccation than *T. emetica*, since high percentage survival (60% at *ca.* 0.37 g g^{-1}, Figure 4.6) was maintained in the suitable WC ranges for cryopreservation in *C. australe* explants and lower percentages (45% at *ca.* 0.33 g g^{-1}) were obtained in *T. emetica* (Figure 4.5).

It has been suggested that tissues that are more desiccation tolerant have protective substances that aid in reducing dehydration induced damaged imposed onto the tissues (Bewley and Oliver, 1992; Han *et al*., 1997). These are substances such as sugars that protect the tissues during dehydration by replacing water and stabilizing membranes, and protective proteins such as dehydrins (Crowe *et al*., 1992; Han *et al*., 1997). In a study by Koster and Leopold (1998), it was also reported that sugars such as sucrose confer desiccation in tissues, as it was noticed that loss in sugars results in loss in desiccation tolerance. *Castanospermum australe* is a recalcitrant-seeded species that has been observed to produce dehydrin-related polypeptides that aid in desiccation tolerance (Farrant *et al*., 1996; Han *et al*., 1997). Dehydrins aid in increasing desiccation tolerance by acting with compatible solutes (such as glycerol

and sucrose) in serving as structural stabilizers of macromolecules during water loss (Close, 1996; Han *et al*., 1997). This explains how shoot apices of *C. australe* may be better equipped to survive dehydration damage. It was also shown that seeds of *T. dregeana*, which belong to the same family as *T. emetica* (both being tropical species), did not contain dehydrin-related proteins (Han *et al*., 1997). It is envisaged that *T. emetica* might also lack dehydrins and this would have contributed to the drastic loss in viability shown above (5.2)

5.4 The effect of cryoprotection on water content and survival of embryonic shoot apices of *T. emetica* and *C. australe*.

Dehydration of plant material can be achieved through physical or chemical dehydration, with the latter involving the use of cryoprotectants (CPs). Cryoprotectants dehydrate tissues by replacing the water with the CP solution, allowing the explants to withstand cryogen exposure through vitrification (Benson, 2008). Vitrification during cooling is dependent on the WC achieved during dehydration, hence the use of CPs is accompanied by drying so as to aid in the reduction of WC to suitable levels amenable for successful cooling. The use of CPs is essential when working with explants such as shoot apices or meristems, as they concentrate the cell contents forming a viscous solution that prevents formation of ice crystals (Engelmann, 2011b; Berjak and Pammenter, 2013). The CPs that were used in the present study were glycerol, sucrose and PVS2. According to Benson (2008), effective cryoprotection occurs when penetrative cryoprotectants are used at non-toxic concentrations that allow for dehydration of cells without causing osmotic damage. It is well documented that cryoprotectants have the capacity to reduce WC in plant material such as in zygotic embryonic axes of *T. dregeana* (Kioko *et al*., 1998), *T. emetica* (Varghese et al., 2009), *E. capensis* (Hajari *et al.*, 2011). Treatment of explants of *T. emetica* with PVS2, which contains a penetrating cryoprotectant (DMSO), however, was not beneficial to the explants which was contrary to what was expected. It is assumed that DMSO, a component of PVS2, would have been toxic to the tissues, as the tissues used were fairly smaller than usual. PVS2 has been previously shown to be beneficial for cryopreservation of *T. emetica* shoot tips obtained from *in vitro* grown shoots (Varghese *et al*., 2009). In a recent study by Thabethe (2017), it was reported that PVS2 is effective at reducing the concentration of water in embryonic axes of *T. emetica* to levels suitable for cryopreservation, while retaining viability (68% survival after slow cooling). The embryonic shoot apices of *C. australe* also did not survive exposure to PVS2 (section 4.2.2) thus implying that PVS2 treatment as done in this study is toxic for embryonic shoot apices.

The other CP solutions used in this study (CP1 [5 and 10% glycerol] and CP2 [5 and 10% glycerol + sucrose]) were only tested with *C. australe* explants. Those CPs were relatively inefficient in dehydrating explants of *C. australe* prior to flash drying, only managing to reduce WC from *ca.* 1.79 g g^{-1} to *ca.* 1.46 g g^{-1} after exposure to CP1 (5 and 10% glycerol; Figure 4.8) and *ca.* 1.17 g g^{-1} after CP2 (5 and 10% glycerol + sucrose; Figure 4.9). Similarly, viability was not compromised after the use of the cryoprotectants only, as they retained 100% survival after treatment with the CPs. In a study by

Varghese *et al*. (2009), similar observations were obtained where vegetative shoot tips of *T. emetica* maintained 100% viability after exposure to 5 and 10% of glycerol + sucrose. However, rapid dehydration of the explants of *C. australe* following treatment with the cryoprotectant solutions reduced the WC of the explants with a consequent reduction in viability (Figure 4.9 and 4.10). This trend is similar to that reported by Kistnasamy *et al*. (2011) for recalcitrant embryonic axes of *L. kirkii,* where the axes lost viability after cryoprotection. The reduction in survival following CP may be attributed by the fact that CPs can be toxic to plant material (Villalobos and Engelmann, 1995; Gonzalez-Arnao *et al*., 2008).

A comparison of CP1 and CP2 in one of the previous studies by Varghese *et al*. (2009) showed that CP1 was more effective at retaining viability of vegetative shoot tips of *T. emetica* before cryogen exposure than CP2. However, in the current study there was no significant difference in percentage survival between shoot apices treated with CP1 and those treated with CP2 (Figure 10).

5.5 Impact of different cooling rates on the survival of embryonic shoot apices of *T. emetica* and *C. australe*

Embryonic shoot apices of *C. australe* could be successfully cryopreserved, though with low percentage of survival (Table 4.6). However, axes of *T. emetica* did not show any signs of survival after cooling. Full plantlet development after exposure to cooling is rare and is inhibited by cumulative metabolic and physical damage incurred throughout the cryopreservation protocol (Fuller, 2004; Berjak *et al*., 2011b). Despite pre-treatment of the embryonic shoot apices in the current study with different CPs, and subsequently drying these as rapidly as possibly to WCs considered to be suitably low for successful cryopreservation (Wesley-Smith *et al*., 2001; Berjak *et al*., 2011b; Ballesteros *et al.*, 2014), post-cryo survival after recovery was fairly low with a maximum of only 16% for *C. australe* and complete death for *T. emetica*. It is worthwhile to mention that in *T. emetica*, viability was reduced to 30% after 90 min of flash drying (Figure 4.5) and was completely lost after further drying for 120 min, while in *C. australe,* it was retained to 60% after 120 min (Figure 4.6) thus implying that the extreme reduction in viability after cooling and rewarming could either be due to the cumulative stress imposed on the shoot apices or the shoot apices were cooling sensitive. Although three cooling rates were employed: slow, fast and rapid cooling, survival was only achieved with fast cooling of the cryoprotected (with CP1) and flash dried embryonic shoot apices.

Treating embryonic shoot apices with cryoprotectants prior to liquid nitrogen exposure was not as beneficial as expected. This could be due to the biophysical and chemical stress imposed on the tissues (Benson, 2008) during the various steps of cryopreservation, as well as sensitivity towards cooling. Among the three cryoprotectants that were used in the study, the only CP treatment that resulted in survival after exposure to LN was CP1. CP1 contained glycerol, and glycerol is known as a colligative cryoprotectant that ameliorates the damaging effects of excessive cell volume change and toxic solutions. According to Ballesteros *et al*. (2014), glycerol was reported to aid in stabilizing the

membrane of *C. australe* during rehydration, thus enhancing survival after cryopreservation. This might have also been the case in the current study, as survival of the cooled explants was obtained with explants treated with 5 and 10% glycerol. None of the explants treated with CP2 survived cooling, this could possibly be because glycerol is less toxic when used in isolation more especially during short term exposure to the cells (Fuller, 2004), unlike when it is used in solution with another cryoprotectant, where the toxicity has been reported to be more severe (Volk and Walters, 2006).

The overall survival percentage of embryonic shoot apices of *C. australe* was relatively low (0-16%) as compared to the survival of shoot apices of *Q. robur* (Chmielarz *et al.*, 2011). This could be explained by the differences in the explant size: Chmielarz *et al.* (2011) used smaller explants (0.5 – 1.0 mm) while the explants were 2.0 mm in the current study. In a study by Vidal *et al.* (2005) where shoot tips of different sizes were used, it was shown that *in vitro* germinating shoot tips of 0.5 – 1.0 mm resulted in a significantly higher final shoot length than that of shoot tips of 2.0 mm. This implies that further reduction in the size of the embryonic shoot apices of *C. australe* could have improved survival after cooling.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Cryopreservation is a very important area of investigation in cryobiology, as it deals with conservation of genetic resources. To date, the germplasm of many plant species have been successfully cryopreserved following optimisation of the procedural steps, some of which are investigated in this study. However, successful cryopreservation is not guaranteed as plant species respond differently to the cumulative stresses induced by the cryo-procedure. This is particularly true for the seed-derived germplasm of recalcitrant-seeded species. The present study reported the first attempt to cryopreserve the embryonic shoot apices of *T. emetica* and *C. australe*, two recalcitrant-seeded species for which previous attempts at cryopreserving their zygotic germplasm have been unsuccessful (Kioko *et al*., 1998; Kioko, 2003; Goveia, 2007). Furthermore, it reports the successful generation of fully functional plantlets from embryonic shoot apices of the three selected recalcitrant seeded species *viz. A. coranica, T. emetica* and *C. australe*. Embryonic shoot apices from all the three recalcitrant species studied could develop into complete plantlets (Figures 4.2 to 4.4) on suitable medium. The advantages of using these explants for cryopreservation are two-fold: (i) embryonic tissue is used at a size considered to be ideal for cryopreservation, and (ii) the removal of the root tips ensure more uniform dehydration of the explant thus reducing damage to the shoot meristem. Thus, embryonic shoot apices appear to be suitable explants for cryopreservation of genetic resources. It may however, be noted that the term 'embryonic shoot apex' was loosely used in the case of *A. coranica* as the shoot tips (secondary explants) were excised from plantlets obtained from cotyledonary bodies (primary explants) cultured *in vitro*. The selection of the secondary explants was justified by the difficulty in excising the shoot tips due to its proximity to the root tips (Figures 3.1 and 3.4). Nevertheless, this justification might be contested.

This study also looked at responses of the apices of *T. emetica* and *C. australe* to cumulative stresses during physical dehydration and cryoprotection, two important procedural steps of cryopreservation. During the dehydration of the explants, both species exhibited a decline in viability as the apices lost water. Apices of *T. emetica* were more sensitive to water loss compared with *C. australe*; apices of *T. emetica* showed a drastic loss of viability as the explants were dehydrated beyond 45 min of FD. Cryopreservation of dehydrated apices of *T. emetica* was unsuccessful. Due to previous studies reporting successful use of PVS2 for cryoprotection, the apices of *T. emetica* were treated with PVS2 and exposed to three cooling rates. Although the explants survived exposure to PVS2 in those studies, none of the shoot apices survived cooling (Table 4.5), thus suggesting that the explants used could not withstand the stresses imposed during cryopreservation. This may have been influenced by the seed size during collection which was fairly small, making the excised explants even smaller. *Castanospermum australe* retained sufficiently high viability (45 – 70%) after pre-treating the explants with cryoprotectants followed by flash drying. These apices survived exposure to LN although the survival (greening of explant and opening of leaf primordia) was quite low (16% survival) after cryopreservation.

The overall results of the present study give a clear indication of how shoot apices from recalcitrant-seeded embryos respond differently to the individual steps of cryopreservation; the different species used required methods unique to each so as to obtain desirable WC ranges and acceptable viability. Even though the success rate was low and could only be obtained for one species (*C. australe*), the use of embryonic shoot apices looks promising for future studies on cryopreservation. The results obtained from this study supports the suggestion made by Chmielarz *et al*. (2011), that plumules (shoot apices excided from the embryo) appear to be more suitable explants for cryopreservation than whole embryonic axes. However, there is a need to further standardize the various stages of cryopreservation (e.g. introduction of a pre-treatment stage after excision [Chmielarz *et al*., 2011]). Studies on ultrastructure and biochemical events during the procedural steps of cryopreservation may give further insight into the possible reasons for the poor post-cryo survival achieved in this study. Lastly, it is worth mentioning that obtaining cryopreservation protocols that ensure long-term storage of plant genetic resources would be economically beneficial since cryopreservation is a cost-effective way of conserving plant germplasm. Cryopreservation has also been shown to be a safe method for long-term storage of genetic resources, that utilizes less space (Sakai, 2000; Berjak and Pammenter, 2004). A successful cryopreservation protocol for *A. coranica* and *T. emetica* would assure a continued availability of these species that are presently harvested for medicinal purposes. A successful cryopreservation protocol for *C. australe* will ensure the availability of planting material for this species, which is widely harvested for its timber.

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