

**Studies on the cryopreservation of shoot apices
from recalcitrant-seeded *Trichilia emetica* Vahl. and
Trichilia dregeana Sond.**

FIKISIWE CYNTHIA GEBASHE

Submitted in fulfilment of the requirements for the degree of
MASTER OF APPLIED SCIENCES IN BIOTECHNOLOGY

in the Faculty of Applied Sciences

Durban University of Technology

Steve Biko Campus

Durban

South Africa

February 2015

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

Signature:-..... Name: ...Prof. P. Berjak.....Date: ...Deceased....

Signature:-.....Name: ...Ms A. Makhathini.....Date: ...26/03/2015....

Signature:-.....Name: ...Dr. D. Varghese.....Date: ...26/03/2015....

Preface

The experimental work contained in this Master's dissertation has been carried out at the Plant Germplasm Conservation Research Laboratory at the School of Life Sciences (formerly known as the School of Biological and Conservation Sciences), of the University of KwaZulu-Natal, on the Durban-Westville campus. Supervision was provided by Professor P. Berjak and Dr D. Varghese (UKZN), and Ms A. Makhathini (DUT).

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

.....

Fikisile Gebashe

March 2015

Declaration

I, Fikisiwe Gebashe, declare that:

1. The research reported in this dissertation, except where otherwise indicated, and is my original research.
2. This dissertation has not been submitted for any degree or examination at any other university.
3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
 - a. their words have been re-written but the general information attributed to them has been referenced;
 - b. where their exact words have been used, then their writing has been placed inside quotation marks, and referenced.
5. This dissertation does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and in the reference sections.

.....

Fikisile Gebashe

March 2015

Acknowledgements

I would like to thank God for giving me the strength, ability, resources and opportunity to complete this Masters' dissertation.

Also I would like to thank my supervisors Professor P. Berjak, Dr D. Varghese and Miss A. Makhathini, for their support, extensively and timeously going through my dissertation, especially Dr D. Varghese. Gratitude is also due to Professor P. Berjak and Professor N.W. Pammenter for welcoming me into the research group and for their financial support throughout this Master's degree.

Aneliswa Makhathini for listening, caring and wanting the best for me; thank you.

Dr D. Varghese and Dr B. Varghese for your kindness and for taking the time and going through the document till the end, I will forever be grateful.

To the Plant Germplasm Conservation Research Group as a whole, your kindness and support is highly appreciated, especially to Miss Nomali Ngobese.

Special gratitude to the staff of the Microscope and Microanalysis Unit, in particular Mr. Vishal Bharuth whose help is kindly acknowledged.

My loving family, my mother (Nokubonga Gebashe) and my sister (Ntombizethu Gebashe) for their continuous support, love and prayers.

To the friendships that I have made and bonds that will never be forgotten: Anathi Nkayi, Candyce Areington, Madonna Vezi, Minoli Appalasamy and Nireshnee Naicker.

Dedication

To God.

“‘For I know the plans I have for you’, declares the Lord, ‘plans to prosper you and not to harm you, plans to give you a hope and a future’ ” – Jeremiah 29:11

Abstract

In contrast to orthodox seeds, recalcitrant seeds are short-lived, shed at relatively high water contents (WCs), and are desiccation sensitive. Presently, the only option for long-term conservation of genetic resources of such plant species is by cryostorage in liquid nitrogen (LN; -196°C) or in the vapour phase over LN (at -150°C to -160°C). A number of cryopreservation protocols applied for recalcitrant zygotic embryos or embryonic axes of tropical/sub-tropical species have reported survival as either root or shoot development or callus formation, with no shoot or root production after cryopreservation. This is a consequence of the challenges encountered when optimising the WC for successful cryopreservation across species. Other shortcomings may also be the formation of ice or the sensitivity to desiccation resulting in lethal damage or poor re-growth. However, for successful cryopreservation, a normal plantlet with a shoot and a root needs to be obtained post-cryo.

Specimens required for successful cryopreservation must be small; therefore embryonic axes excised from seeds have been often used as the explants of choice. However, in some cases, excised embryonic axes of mature recalcitrant seeds are too large to be cryopreserved, or, even if small, may be adversely affected by excision, dehydration and/or immersion in LN, thus failing to produce plantlets after cryopreservation. As a result, in such cases, there is a need to develop explants alternative to zygotic axes such as buds derived from *in vitro* shoots, shoot meristems, or shoot apices and somatic embryos. These alternative explants must have a high capacity for plantlet formation before and after cryopreservation. The present study aimed to successfully cryopreserve shoot apices of *Trichilia emetica* and *T. dregeana*, tropical recalcitrant-seeded tree species, and monitor the responses or effects of some of the procedural steps involved in cryopreservation on the survival and shoot production from these shoot apices. The main foci of the investigation were to produce vigorous plantlets after

cryopreservation and ultimately develop a protocol for the successful cryopreservation of germplasm of these species. Furthermore, this study reports on a number of factors that may affect survival after cryopreservation, viz. WC of the explants, PVS2 treatment, production of reactive oxygen species (ROS) and levels of endogenous total aqueous antioxidants (TAA) during the various steps of cryopreservation. The effects of the various steps of cryopreservation on the ultra-structure of the shoot apices were also observed. Cathodic protection (by using highly reducing cathodic water; CW) of the explants was attempted to improve vigour and shoot production from the surviving shoot apices after cryopreservation as cathodic water has been reported to ameliorate the excessive burst of ROS, which often accompanies the stresses imposed by the procedural steps of cryopreservation. Experiments were also performed to optimise the medium for vigorous shoot formation from the shoot apices.

Shoot apices of *T. emetica* in this study had an initial WC of *ca.* 2.2 g g⁻¹ dry weight (DW) upon excision. Although the WC of the shoot apices decreased slightly after cryoprotection with PVS2, it did not result in sufficient dehydration before cooling. Upon retrieval from LN, 68% of the shoot apices survived and 40% of those produced shoots. Treatment of shoot apices with CW did not improve the survival or shoot production from the apices following cryo-retrieval. This could be a direct consequence of increase in WC of the shoot apices following CW treatment.

Water content is not the only factor affecting successful cryopreservation; the production of ROS and the level of antioxidants may also have an impact on regrowth after cryogen exposure. Rapid changes in temperature when the samples are cryo-stored and then rewarmed result in an increase in ROS production, which could have affected the shoot production. More importantly the antioxidant activity showed a rapid decrease during recovery,

especially in the CW treated shoot apices, which might have also led to the poor survival and shoot production from the shoot apices.

Ultrastructural observations showed the injurious effects of PVS2 treatment typified by derangement of plastids, development of numerous small vesicles along the cell membrane and abnormalities in the structure of the nuclear envelope in the shoot apical cells both before and after cryogen exposure. Following cryo-retrieval, the meristem cells were extensively deteriorated – indicating non-survival, however, some shoot apices had areas of surviving cells which might have led to 40% shoot production after cryopreservation.

Based on the studies on optimising medium composition for shoot formation from the apices, woody plant medium (WPM) with 1 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA was found to be the best medium which gave a higher shoot production of 67 – 70% before cryopreservation compared with only 18 – 20% shoot formation on media used previously. Therefore, this medium was used as the recovery medium. Encapsulation-dehydration of the shoot apices and the use of PVS3 instead of PVS2 for cryoprotection were also employed in an attempt to improve the survival and shoot production after post-cryo, but both methods did not result in any shoot production although 92% and 90% of the shoot apices survived cryogen immersion, respectively.

While the shoot apices of *T. emetica* resulted in 40% shoot production following retrieval from LN and recovery on WPM with 1 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA, attempts to further improve the shoot production were not successful. The results of this study suggest that the shoot apices used were possibly not sufficiently developed, and with the commensurately high WC, proved to be unsuitable explants for germplasm conservation of *T. emetica*. The injurious effects of PVS2 treatment both before and after cryogen exposure as observed from the ultra-structural studies provide a clue to the repeated failure to cryopreserve embryonic

axes of many tropical recalcitrant-seeded species after treatment with PVS2. Maintaining mother material in culture for longer durations before explant excision in order to allow better development of the axillary buds and render the cytosol more concentrated, and optimising the exposure duration to loading solution and concentration of sucrose in the loading solution might however, provide sufficient dehydration tolerance to PVS2 leading to successful vitrification up on cooling.

Table of Contents

Preface.....	i
Declaration.....	ii
Acknowledgements.....	iii
Abstract.....	v
List of Figures	xii
List of Tables	xv
Abbreviations and Symbols	xvi
Units of Measurement.....	xviii
1. INTRODUCTION	1
2. LITERATURE REVIEW	5
2.1 Conservation of Biodiversity	5
2.2 <i>Ex situ</i> conservation	8
2.3 Seeds	8
2.3.1 Post-harvest behaviour and storage	10
2.4 <i>In vitro</i> preservation.....	13
2.4.1 Cryopreservation.....	15
2.4.2 Cryopreservation techniques.....	16
2.5 Advantages of cryopreservation	19
2.6 Factors affecting cryopreservation.....	19
2.7 Oxidative stress accompanying procedural steps for, and after, cryopreservation...	25
2.8 Cathodic protection.....	27
2.9 <i>In vitro</i> regeneration.....	28
2.9.1 Culture conditions	28
2.10 Recalcitrant-seeded species studied.....	28
2.10.1 <i>Trichilia emetica</i> Vahl.	28

2.10.2	<i>Trichilia dregeana</i> Sond.	30
2.11	AIM AND OBJECTIVES	33
2.11.1	Aim	33
2.11.2	Objectives	33
3.	MATERIALS AND METHODS	34
3.1	Seed collection and storage	34
3.2	Seed processing	34
3.3	Hydrated storage	35
3.4	Cryopreservation of shoot apices	35
3.4.1	Production and excision of shoot apices	35
3.4.2	Pre-culture and cryoprotection (PVS2) of shoot apices	36
3.4.3	Water content determination	36
3.4.4	Survival and shoot production prior to cooling	36
3.4.5	Cooling	37
3.4.6	Rewarming (Thawing)	37
3.4.7	Recovery	38
3.5	Use of cathodic water in the cryopreservation of shoot apices	38
3.5.1	Generation of cathodic water	38
3.5.2	Treatment of shoot apices with cathodic water (CW)	39
3.6	Biochemical estimations	40
3.6.1	Estimation of extracellular superoxide production	40
3.6.2	Estimation of total aqueous antioxidant activity	41
3.7	Methods to improve shoot production and post-cryo vigour of shoots obtained from cryopreserved shoot apices	43
3.7.1	Media manipulation studies	43
3.7.2	Cryopreservation of shoot apices using PVS3 as the cryoprotectant	44

3.7.3	Cryopreservation of shoot apices using encapsulation- dehydration technique	45
3.8	Ultra-structural studies.....	46
3.9	Statistical Analysis.....	46
4.	RESULTS AND DISCUSSION	48
4.1	<i>In vitro</i> germination of seeds of <i>Trichilia emetica</i> and <i>T. dregeana</i>	48
4.2	Explants for cryopreservation	48
4.3	Water content, survival and shoot production	50
4.4	Methods to improve shoot production from shoot apices.....	54
4.4.1	Type of medium.....	55
4.4.2	Treatment with cathodic water.....	58
4.4.3	Solid and liquid medium.....	60
4.5	Pre-conditioning of explants and cryoprotection.....	61
4.6	Cooling rates	64
4.7	Vitrification using PVS3 as the cryoprotectant	69
4.8	Oxidative stress during the procedural steps of cryopreservation	73
4.8.1	Production of extracellular superoxide	73
4.8.2	Total aqueous antioxidant activity	76
4.9	The effects of excision, cryoprotection (PVS2) and subsequent cryopreservation on the ultrastructure of <i>T. emetica</i> shoot apices.....	78
4.10	Encapsulation-dehydration as a method of cryopreservation	84
4.10.1	Dehydration of the encapsulated shoot apices	84
4.10.2	Cryopreservation.....	85
5.	CONCLUSION.....	87
6.	REFERENCES	90

List of Figures

Figure 1: Outline of approaches to germplasm conservation (from Goveia, 2007).	7
Figure 2: Schematic diagram of the various developmental stages of seed formation (diagram courtesy Prof. Berjak).....	11
Figure 3: Freshly harvested seeds (A) and fruits (B) of <i>T. emetica</i>	30
Figure 4: Three-lobed mature fruits and seeds of <i>T. dregeana</i>	32
Figure 5: Electrolysis apparatus used to generate cathodic water.	39
Figure 6 (A and B): Typical meristem cells of a shoot apex showing well organized cells. <i>P</i> , plastid; <i>V</i> , vacuole; <i>Cw</i> , cell wall; <i>Vs</i> , vesicle; <i>Pm</i> , plasma membrane; <i>N</i> , nucleus and <i>M</i> , mitochondria.....	49
Figure 7: Water content (7A), survival and shoot production (7B) from <i>T. emetica</i> shoot apices when treated with and without cathodic water during the different steps of cryopreservation using medium (WPM + 0.05 mg L ⁻¹ BAP + 0.1 mg L ⁻¹ IBA + 0.1 mg L ⁻¹ GA ₃) and procedure described in Varghese <i>et al.</i> (2009). Vertical bars in figures represent mean SD. Columns labelled with different letters are significantly different; <i>p</i> < 0.05 (uppercase letters for differences in WC, survival and shoot production across different steps (ANOVA) and lowercase between WC, survival and shoot production at each step (<i>t</i> test) with and without CW).	51
Figure 8: Micrographs showing the effects of sequential cryoprotection with PVS2 (50% followed by 100%; A and B) and 100% PVS2 (C and D) on the shoot apical meristem cells. <i>P</i> , plastid; <i>V</i> , vacuole; <i>Cw</i> , cell wall and <i>Nu</i> , nucleolus.	63
Figure 9: Survival and shoot production from excised shoot apices of <i>T. emetica</i> following excision and after cryopreservation. Apices were excised, pre-cultured, cryoprotected	

with PVS 2; cooled slowly and recovered on WPM with 1 mg L ⁻¹ BAP and 0.1 mg L ⁻¹ IBA. Letters above the graph represent significant differences in survival and shoot production $p < 0.05$ before and after cryogen exposure.....	66
Figure 10: Survival and shoot production of <i>T. emetica</i> shoot apices during the steps of cryopreservation. Apices were excised, pre-cultured, cryoprotected with PVS 2; cooled slowly and recovered on WPM with 1 mg L ⁻¹ BAP and 0.1 mg L ⁻¹ IBA. Letters above the graph represent significant differences in survival and shoot production $p < 0.05$ before and after cryogen exposure.....	67
Figure 11: Shoot formation from cryopreserved shoot apices of <i>T. emetica</i> (– CW) using slow cooling method, 3 months (A), 4 months (B) and 5 months (C) into recovery.	71
Figure 12: Shoot formation from cathodic water treated and cryopreserved shoot apices of <i>T. emetica</i> using slow cooling method, 3 months (A), 4 months (B) and 5 months (C) into recovery.	72
Figure 13: Production of extracellular superoxide in shoot apices of <i>T. emetica</i> during the steps of cryopreservation. Bars represent mean \pm SD, columns labelled with different letters are significantly different; $p < 0.05$ (uppercase letters for differences in $\cdot\text{O}_2^-$ across different steps and lowercase between $\cdot\text{O}_2^-$ at each step with and without CW).	74
Figure 14: Total aqueous antioxidant activity in shoot apices of <i>T. emetica</i> during the stages of cryopreservation. The numbers above columns represent survival after each step of cryopreservation. Vertical bars represent mean \pm SD, columns labelled with different letters are significantly different; $p < 0.05$ (uppercase letters for differences in TAA across different steps and lowercase between TAA at each step with and without CW).	76

- Figure 15 A-C: TEM micrographs showing ultrastructure of freshly (control) excised shoot meristematic cells of *T. emetica*. A-C Control meristematic cells: A over view of the whole cell, B and C show different organelles such as mitochondria, plastids and nucleus. *P* plastid, *V* vacuole, *Cw* cell wall, *Pm* plasma membrane, *NE* nuclear envelop, *Vs* vesicles, *Nu* nucleolus, *Hc* Heterochromatin81
- Figure 16 A-C: Micrographs of pre-cultured (0.3 M sucrose and 0.5 M glycerol) and cryoprotected (PVS2) shoot apices meristematic cells of *T. emetica*. An entire meristematic cell of a tunica layer, B plastid derangement following PVS2 treatment, C alteration of nuclear envelop. *P*, plastid; *V*, vacuole; *Cw*, cell wall; *Pm*, plasma membrane; *NE*, nuclear envelop; *Vs*, vesicles; *Nu*, nucleolus; *Hc*, Heterochromatin. ...82
- Figure 17 A-C: Ultrastructure of meristematic cells of *T. emetica* shoot apices after cryo-exposure. A showing small areas of surviving nearby cells after cryoprotection and also cryopreservation, B Total destruction of apical meristem cells after cryo-exposure. C further derangement of plastids and appearance of many small vesicles. *P*, plastid; *V*, vacuole; *Cw*, cell wall; *Pm*, plasma membrane; *NE*, nuclear envelop; *Vs*, vesicles; *Nu*, nucleolus; *Hc*, Heterochromatin.83
- Figure 18: Change in water content of encapsulated shoot apices of *T. emetica* up on slow drying in a laminar air flow chamber. The numbers above the points represents survival percentage after dehydration in the laminar flow at different time intervals. Bars represent mean WC \pm SD.84

List of Tables

Table 1: Physiological storage classes related to desiccation and temperature (Schmidt, 2000)	13
Table 2: Advantages and disadvantages of in vitro preservation (Humbeck <i>et al.</i> , 2008)	14
Table 3: Comparison of germination rate of <i>T. emetica</i> and <i>T. dregeana</i> seeds. <i>T. emetica</i> seeds were germinated only on half-strength MS medium	48
Table 4: The effects of media on survival and shoot production from shoot apices of <i>T. emetica</i> without CW treatment	55
Table 5: The effects of media on survival and shoot production from shoot apices of <i>T. emetica</i> treated with CW before culturing	56
Table 6: Summative results of survival and shoot production from shoot apices grown in different types of media	58
Table 7: Comparative study of cathodic water treatment methods on survival and shoot production from shoot apices	59
Table 8: Growth of excised shoot apices on solid and liquid WPM medium	60
Table 9: Growth of excised shoot apices on the solid and liquid MS medium	60
Table 10: Survival and shoot production following cryopreservation of shoot apices cryoprotected with PVS 2 and PVS 3	70
Table 11: Survival of encapsulated shoot apices of <i>T. emetica</i> after cryopreservation	85

Abbreviations and Symbols

ABTS	2, 2' –azino-bis-3-ethylbenzthiazoline-6-sulphonic acid diammonium salt
APX	ascorbate peroxidase
AsA	ascorbic acid
BAP	6-benzylaminopurine
CAT	catalase
Ca(OCl)₂	calcium hypochlorite
CaCl₂.2H₂O	calcium chloride dihydrate
CaMg	calcium magnesium solution
CW	cathodic water
DM	dry mass
dmb	dry mass basis
DMSO	dimethyl sulphoxide (Me ₂ SO)
EDTA	ethylene diaminetetraacetic acid
GA₃	gibberellic acid
Gly	glycerol
H₂O₂	hydrogen peroxide
IAA	indole acetic acid
IBA	indole butyric acid
KCl	potassium chloride
KH₂PO₄	potassium dihydrogen phosphate
K₂S₂O₈	potassium persulfate
LN	liquid nitrogen
MgCl₂.6H₂O	magnesium chloride hexahydrate
MS	Murashige and Skoog
MSH	MS with hormones

NaCl	sodium chloride
Na₂HPO₄	disodium hydrogen phosphate
NaOCl	sodium hypochlorite
NOX	NADPH oxidases
O₂	oxygen
¹O₂	singlet oxygen
O₃	activated/ triplet state oxygen
• O₂⁻	superoxide anion/radical
• OH	hydroxyl radical
PBS	phosphate buffer saline
PEG	polyethylglycol
PVP	polyvinylpyrrolidone
PVS2	plant vitrification solution 2
PVS3	plant vitrification solution 3
RH	relative humidity
ROOH	organic hydroperoxide
ROS	reactive oxygen species
SOD	superoxide dismutase
TAA	total aqueous antioxidants
TEM	transmission electron microscope
v/v	volume per volume
w/v	weight per volume
WC	water content
WA	water agar
WPM	woody plant medium

Units of Measurement

%	per cent
°C	degree Celsius
cm	centimetre
d	day(s)
g	gram(s)
g g⁻¹	g per g
g l⁻¹	g per litre
h	hour(s)
l	litre(s)
min	minute(s)
ml	milliliter(s)
mm	millimeter(s)
mM	millimolar
MPa	megaPascal(s)
ms	millisecond(s)
nm	nanometer(s)
s	second(s)
μ	micron(s)
μmol s⁻¹	micromoles per second

1. INTRODUCTION

The conservation and maintenance of plant biodiversity is of utmost importance globally to preserve valuable species from being extinct. Rapid increase in human population, a variety of human-induced processes such as deforestation, urbanization, changes in agricultural practices, climate change scenarios, changes in land-use and invasive species have had dramatic effects on plant diversity, which is reflected in the increase in the number of threatened and/or endangered species (Rao, 2004). Loss of plant diversity jeopardises resilience in ecosystems, including the ability to respond to the stresses brought about by climate change (Abbas and Quaiser, 2011), thereby diminishing possible re-establishment of species after disasters. Over exploitation of plant resources does not only contribute to this loss, but also limits the availability of basic raw materials, food, fuel and genetic variability which is very important in breeding programmes (Ohmiya *et al.*, 2003; FAO, 2010). Traditionally, plant genetic resources have been conserved using two methods: *in situ* and *ex situ* conservation. Seed storage (typically *ex situ* conservation) is considered to be one of the best long-term conservation of plant genetic material (Rao, 2004). The main responsibility of facilities preserving genetic stock in the form of seeds is to ensure that they are maintained for an indefinite period while retaining the highest possible viability (Vertucci and Roos, 1990). To achieve this, it is crucial to optimise the conditions for seed storage. In order for the seeds to be stored successfully for long periods, they must be desiccated to low WCs and exposed to lower temperatures. However, seeds of many species especially from tropical and sub-tropical areas are not desiccation tolerant and do not survive low temperatures (Lynons, 1973; Roberts, 1973; Graham and Patterson, 1982).

Orthodox seeds are desiccation-tolerant and are shed at very low WCs (generally $<0.1 \text{ g g}^{-1}$ DW). As long as they are in a good condition, these seeds will retain viability for predictably long periods in the dry state, and hence can be stored under low RH and low temperature

conditions (Roberts, 1973; Ellis and Roberts, 1980). Recalcitrant seeds, on the other hand are those that do not undergo maturation drying, the final phase of orthodox seed development, and hence are shed at a relatively high WC (*ca.* 1.0 – 6.0 g g⁻¹ DW; Ballesteros *et al.*, 2014). They are sensitive to desiccation and very often to chilling, necessitating its storage usually under high RH and ambient temperature conditions (Roberts, 1973; Farrant *et al.*, 1989; Berjak and Pammenter, 2000). As a consequence of being desiccation-sensitive and chilling-sensitive, the genetic resources of these seeds cannot be stored for significant periods using the conventional low RH / low temperature storage. However, short- to medium-term storage is possible at moderate temperatures under hydrated storage conditions at the shedding WC of the seeds, allowing for negligible or no water loss. These conditions are aimed at facilitating and/or promoting viability retention for a few weeks to months (King and Roberts, 1980).

Another noticeable feature of recalcitrant seeds that severely hampers their storability is that these seeds remain metabolically active as well as desiccation-sensitive at all developmental and post-harvest stages (Berjak *et al.*, 1989; Farrant *et al.*, 1989). Being metabolically active even after shedding from the parent plant, they tend to/can initiate germination while in storage. As germinated seeds (now seedlings) have a requirement for additional water (which is not provided in the storage containers) and as a result, the seedlings become water-stressed and increasingly debilitated (Berjak *et al.*, 1989). Often this is accompanied by an acute problem of fungal proliferation on and in the seeds/seedlings (Mycock and Berjak, 1990; Calistru *et al.*, 2000; Berjak and Pammenter, 2004) which adds to the difficulties of storing the germplasm of these seeds for any significant period. The only option for long-term conservation of the genetic resources of recalcitrant-seeded species is by cryostorage, generally in LN (-196 °C) or the vapour phase (-150 to -160 °C) above LN (Berjak *et al.*, 1996; Engelmann, 2011). However, most recalcitrant seeds cannot be cryostored as seeds, as

they are invariably too large to dry rapidly or to survive the exposure to ultra low temperatures. Hence, embryonic axes excised from such seeds are considered to be ideal for cryopreservation as they are usually small to undergo very rapid dehydration (which can be achieved via flash drying [Berjak *et al.*, 1990; Pammenter *et al.*, 2002]) to WCs which are amenable for cooling to LN temperatures without incurring lethal consequences.

For successful cryopreservation, the seeds/explants must be partially dehydrated, which appears to pose a challenge as these seeds are desiccation-sensitive. Under conditions of slow dehydration, most of these seeds/explants die at a WC of $\leq 0.8 \text{ g g}^{-1}$ (Berjak and Pammenter, 2004). However, it has been found that when the rate of dehydration is rapid, the recalcitrant seeds tend to tolerate a greater degree of dehydration than if dried slowly (Pammenter *et al.*, 1998; Varghese *et al.*, 2011). In several cases, however, embryonic axes of mature recalcitrant seeds are themselves large structures (e.g., species of the family Lecythidaceae [Berjak *et al.*, 1996] commonly typified by Brazilian nuts, and *Theobroma cacao* [cocoa] of the family Sterculiaceae [Pence, 1991]) and therefore cannot be used as explants for cryostorage. In other cases, even if the embryonic axes are small, the shoot meristems have been shown to be lethally affected by excision and/or dehydration and LN immersion (e.g. *Trichilia dregeana* [Goveia *et al.*, 2004], *Ekebergia capensis* [Perán *et al.*, 2006; Hajari *et al.*, 2011]) and survival after retrieval from LN is limited to root production only. In all such cases, there is an urgent need to develop alternative explants that can be appropriately dehydrated and exposed to cryogenic temperatures, while still retaining a high capacity for plantlet formation once retrieved from cryostorage.

Successful cryostorage necessitates the usage of the smallest explants that will develop into a complete plant as a large surface area to volume ratio of small explants will allow quick freezing simultaneously of the entire explant (Berjak and Pammenter, 2008; Wesley-Smith *et*

al., 2014). A smaller explant size also facilitates the rapid reduction of WC to levels that eliminate or minimize freezing damage (Wesley-Smith *et al.*, 1991; 2014, Ballesteros *et al.*, 2014). This makes shoot apices or shoot meristems ideal alternate sources of germplasm for cryopreservation under circumstances where little or no success has been achieved using embryonic axis, because they comprise a smaller number of relatively undifferentiated cells that are suggested to remain genetically stable during the regeneration process (Mroginski *et al.*, 1991), although this latter contention remains to be rigorously tested (Harding, 2004).

Contemplating the theoretical benefits of using shoot apices for cryopreservation and the practical success of cryo storage of shoot apices of many plant species (though mostly of temperate species), this proposed study aimed at developing suitable *in vitro* regeneration protocols for shoot apices of selected recalcitrant-seeded species. The project also aimed at developing protocols for the cryopreservation of these explants for the long-term *ex situ* conservation of these species and methods to improve the viability and growth of the plantlets recovered from cryostorage.

2. LITERATURE REVIEW

2.1 Conservation of Biodiversity

Conservation of biological diversity is of global importance as an increasing proportion of the world population is dependent on the genetic resources (Reed *et al.*, 2011). Such genetic resources are usually produced or available only in a few regions, thus increasing chances of overuse (Brush, 1991) and eventual extinction. Diversity is fundamental for human civilisation, therefore its conservation is imperative to sustain development and life on earth (SCBD, 2008). The Convention of Biological Diversity (CBD) defines biodiversity as ‘*the variety of life on earth*’, consisting of the variability within species (*viz.* plants, animals and microorganisms), among species and of ecosystems. Biodiversity also refers to the inter-relationships between living things, and between living things and their environment (SCBD, 2006; 2008). A few years back the CBD had undertaken a task of measuring the biodiversity, which is of considerable importance, especially with regards to the conservation of plant diversity. It has been reported by many agencies that the rate of loss of floral biodiversity appears to be particularly alarming especially in Africa (Sershen *et al.*, 2010) as a result of civil war, wild fires, invasive aliens, conversion of land for agriculture and silviculture, poor land management, urbanisation, non-sustainable harvesting for food, medicine, fuel and construction, overgrazing, displacement and loss of landraces, pests and diseases, pollution, and incomplete knowledge of the biology (e.g. reproductive and adaptive abilities) of many plant species (Geldenhuys, 2000). Hence, an estimation of about 60,000 known genetic resource were found to be threatened or in danger of extinction (Botanic Gardens Conservation International, 2010), because of overexploitation, unsustainable harvest of wild life (Kramer and Havens, 2009) and climate change (Jackson and Kennedy, 2009). Rao (2004) also reported on the decline of plant genetic resources that is caused by external

pressures, which results from the need to increase yield and quality of resources commercially.

To negate such an irreversible loss, a global strategy for plant conservation, viz. the Convention on Biological Diversity (2006), was developed to halt the loss of plant biodiversity systematically. This strategy involves understanding, documenting, conserving and generating awareness around plant biodiversity, implementing its sustainable use and building infrastructure and human capacity for the conservation of this diversity.

Conservation of genetic resources can be achieved by the execution of either *in situ* or *ex situ* (see Fig. 1 below) practices (Rao, 2004). *In situ* conservation involves maintaining plants in their indigenous environment such as nature reserves or national parks, thus perpetuating natural evolution of the species. In the context of *in situ* conservation there are a number of social and economic factors that need to be considered including the availability of a sufficient number of individuals to maintain viable populations, the availability of land, and maintenance costs of natural reserves (Berjak *et al.*, 1996). *Ex situ* conservation on the other hand involves conservation outside the native habitat and offers advantages of protecting plants from destruction, modification or deterioration that can be effected (Rao, 2004) by conserving the genetic resources away from their native habitats. Examples of *ex situ* conservation are maintenance in seed banks, fields, plantations, arboreta, gene banks and botanical gardens (Rao, 2004). A key requirement however for this type of conservation is the acquisition of genetically representative populations or samples. Ideally, for successful *ex situ* storage the diversity within a species should be determined before germplasm collection is carried out, so that the sample size accommodates for such conservation programmes.

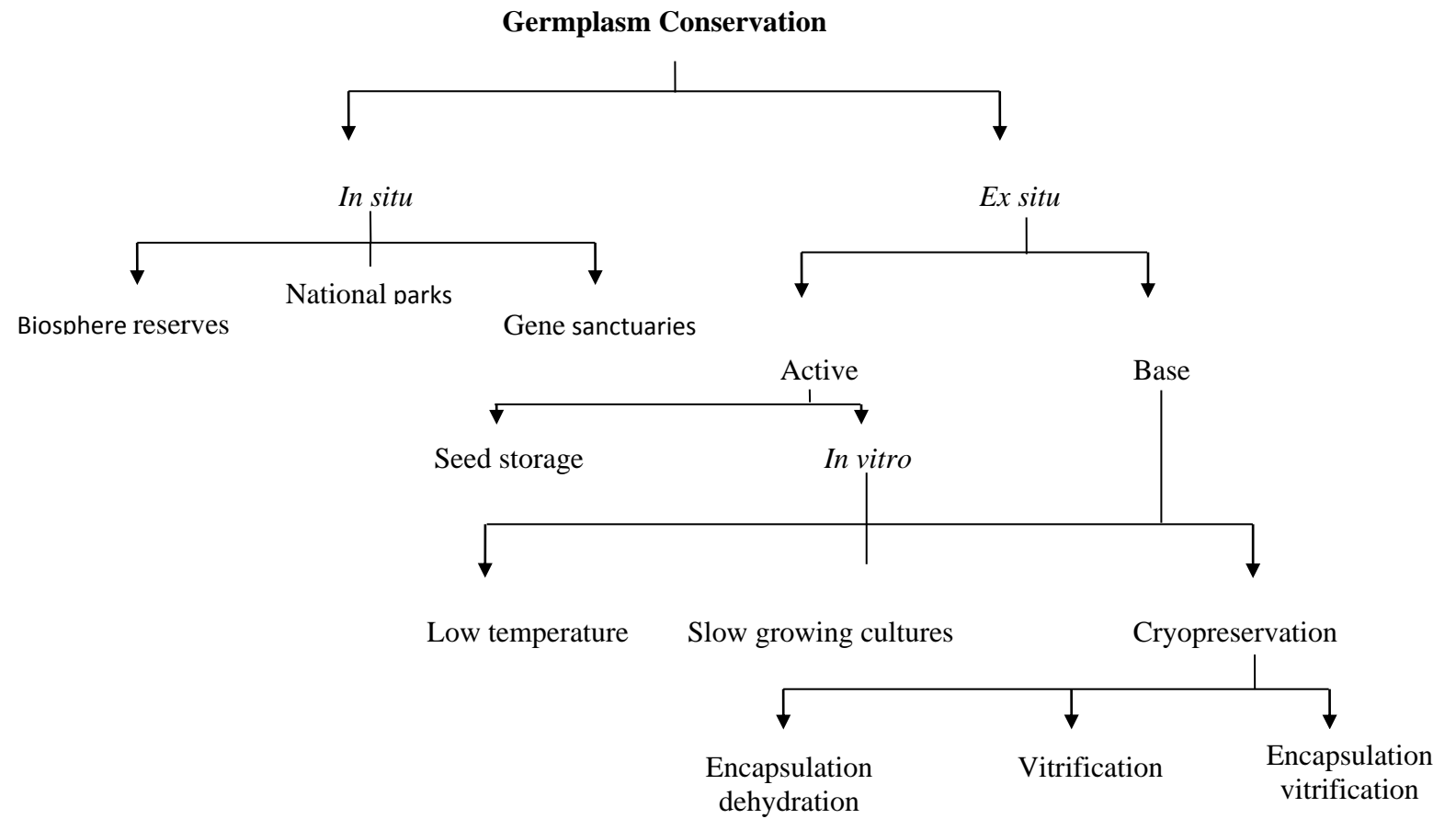


Figure 1: Outline of approaches to germplasm conservation (from Goveia, 2007).

2.2 *Ex situ* conservation

Ex situ conservation is the maintenance of plant gene resources under controlled conditions, i.e. removed from their natural habitats (Dhillon and Saxena, 2003) and cultivation in botanic gardens, arboreta and nurseries, and by seed storage or cryopreservation in gene banks and *in vitro* conservation (Paunescu, 2009). *Ex situ* have two types of storage strategies i.e. active collections which refer to available short-to medium-term stored samples and base collections referring to material preserved for long-term. Active collection may include seeds held in seed/gene banks and it is these seeds that are used for distribution (Linington, 2003). In seed/gene banks, base collections are complementary to active collections in that germplasm is stored in an environment free from vulnerable depletion by nature and by anthropods in the ecosystems (Li and Pritchard, 2009; Linington, 2003). Therefore base collections distribute disease free plants, thus minimising the cost of disease indexing (Lynch *et al.*, 2007) which is necessary in active collections. Seeds are the most common form of germplasm used in conserving higher plants, but not all plants can be conserved by means of seed storage (Vertucci and Roos, 1990). Such plants are vegetative propagated plants that are not bred from seeds; a number of commercial plants that are subject to incompatibility barriers and then there are seeds of many plant species that are desiccation or chilling sensitive (Lynch *et al.*, 2007). Therefore tissue culture has proven to be an option for conservation of such species and also *in vitro* conservation which enables the availability of genetic resource.

2.3 Seeds

A seed is formed from a fertilized mature ovule, and contains the embryo plus extra-embryonic nutritive tissue (endosperm) in some cases, enclosed in protective layers of the testa or seed coat (Schmidt, 2000). Traditionally seeds have been grouped into two main groups according to their physiological storage potential, either as orthodox or recalcitrant (Roberts, 1973; King and Roberts, 1980). Orthodox seeds (viz. *Zea mays*, *Triticum aestivum*,

Phaseolus vulgaris, *Cicer arietinum* etc.) undergo a period of drying during the final stage of their maturation and are shed at low WC which is in equilibrium with the prevailing RH. All orthodox seeds not only survive considerable desiccation (to around 5% on a wet mass basis or 0.053 g H₂O g⁻¹ dry mass; g g⁻¹) but can be stored for prolonged periods without loss of quality or viability under defined conditions of storage temperature and WC (Ellis and Roberts, 1980; Sutherland *et al.*, 2002). These seeds tolerate conditions such as drying and chilling, as a result long term germplasm conservation is facilitated. The longevity of such seeds can mainly be determined by seed moisture content and storage temperature, as life-span is predictable with decrease in temperature and moisture content (Ellis and Roberts, 1980).

Recalcitrant (non-orthodox) seeds are characterised by little, or no, maturation drying and are hence shed at high WCs and remain desiccation sensitive both during development and after they are shed (Farrant *et al.*, 1986; Berjak *et al.*, 1989; Pammenter and Berjak, 1999; Berjak and Pammenter, 2008). Some examples of tropical species that produce recalcitrant seeds are cacao (*Theobroma cacao*), rubber (*Hevea brasiliensis*), durian (*Durio zibethinus*) and jackfruit (*Artocarpus heterophyllus*); temperate recalcitrant species include oaks (e.g. *Quercus robur*), silver maple (*Acer saccharinum*) and horse chestnut (*Aesculus hippocastanum*) (Sutherland *et al.*, 2002). These seeds are highly susceptible to desiccation injury (Pammenter and Berjak, 1999), do not undergo cellular de-differentiation and metabolic shut-down during maturation but are shed in a state of incipient germinative metabolism (Whitaker *et al.*, 2010). Recalcitrant seeds rapidly lose their germination capacity as a result of desiccation (for example, within 8 d in *Shorea robusta*; Chaitanya and Naithani, 1994), and undergo deteriorative ultrastructural changes if dehydrated below a certain WC (Pammenter *et al.*, 1998) or even when stored at the shedding WC in the hydrated condition (Pukacka and Ratajczak, 2006). Deteriorative ultrastructural changes include extensive

vacuolation, degeneration of organelles such as mitochondria and plastids, the endomembrane system, and ultimately, cellular lysis (Kaczmarczyk, 2008; Quain *et al.*, 2009).

After shedding, normal cellular metabolism results in the seeds producing ROS at a controlled rate (Pukacka and Ratajczak, 2006; Tommasi *et al.*, 2006; Whitaker *et al.*, 2010) but their failure to retain a critical amount of water leads to metabolic damage typified by uncontrolled production of ROS (Cheeseman, 2007; Wang *et al.*, 2007; Suzuki *et al.*, 2012). Under these circumstances, protective anti-oxidative reactions are inadequate to remove ROS effectively (Pukacka and Ratajczak, 2006; Varghese *et al.*, 2011). Production of uncontrolled ROS damages many macromolecules including those of the membranes (i.e. protein and lipids; Halliwell and Gutteridge, 1984) and nucleic acids (Pan and Yau, 1991), the damage being followed by cell death (Halliwell and Gutteridge, 1984; Whitaker *et al.*, 2010). Loss of viability of recalcitrant seeds after shedding is not only brought about by ROS, but also by prevailing fungal proliferation during their storage (Calistru *et al.*, 2000; Berjak, 2005; Myeza, 2005).

2.3.1 Post-harvest behaviour and storage

In order for recalcitrant seeds to be successfully stored for the maximum possible periods, understanding their physiological behaviour after harvest and their requirements for successful storage is imperative. Sensitivity to desiccation (which is removal or loss of intracellular water) seems to be the ultimate contributing factor to the difficulty of storing recalcitrant seeds for extended periods (Berjak *et al.*, 1989). Desiccation tolerance refers to organisms or plants that survive dehydration to an overall WC ≤ 0.1 g g⁻¹ DW (Vertucci and Farrant, 1995). The acquisition of desiccation tolerance in orthodox seeds (which is essentially lacking in recalcitrant seeds) depends on various mechanisms that occur during

the maturation drying phase (Fig. 2) of seed development (Pammenter and Berjak, 1999; Berjak, 2006; Berjak and Pammenter, 2008).

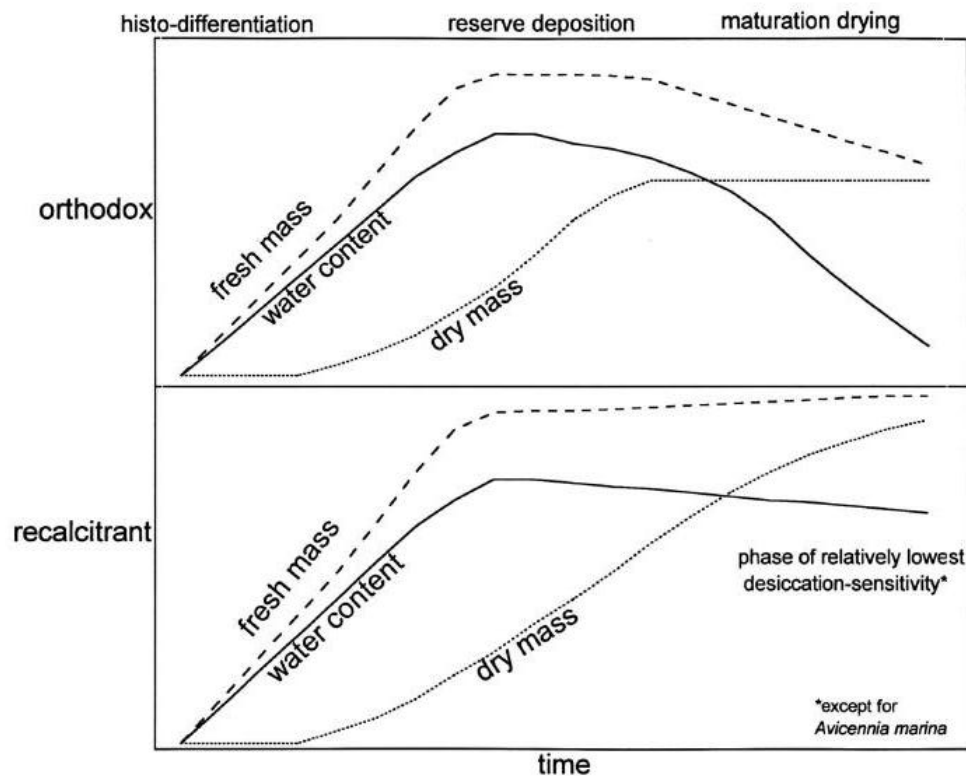


Figure 2: Schematic diagram of the various developmental stages of seed formation (diagram courtesy Prof. Berjak).

The mechanisms which render tolerance to desiccation tolerant seeds involve mechanical protection (such as vacuole reduction or filling with insoluble material, DNA conformational changes, cytoskeleton disassembly and reassembly) and metabolic protection (against uncontrolled ROS production which includes antioxidant activity and metabolic switch off). Below a WC of 0.3 g g^{-1} , the intracellular glassy (vitrified) state contributes towards the

retention of the ultrastructural integrity as the cells undergo dehydration (Berjak and Pammenter, 2008).

With the current rate of loss of plant diversity in the 21st century, and a realistic prediction of global climate change looming on the planet, storage of seeds has become inevitable for food security, for planting in the future, as breeding material or genetic resources for conservation. Seed storage is the most convenient *ex situ* conservation method for long term conservation of plant germplasm (Rao, 2004). The convenience is associated with the low costs and the fact that seeds present fewer problems than the other propagules that are usually used for *ex situ* conservation (Goveia, 2007). However, it must be noted that long-term storage is possible only for orthodox seeds, as noted above (in the Introduction). The longevity of seeds in storage is determined by initial seed quality, temperature of storage, moisture content of seeds and good storage conditions. Different strategies of conservation apply to different types of seeds, depending on their physiological response to desiccation and low temperatures. In contrast, recalcitrant seeds are sensitive to desiccation and those of many tropical species do not tolerate low temperatures (Berjak and Pammenter, 1999; Berjak and Mycock, 2004). Between the two seed categories, there is another category of seeds that are termed as intermediate; they can be dried to lower WCs but relatively higher than the orthodox seeds and are sensitive to low temperatures typically employed for orthodox seeds (Hong and Ellis, 1996; Schmidt, 2000). Based on the storage moisture and temperature requirements, seeds can be categorised as orthodox, intermediate, temperate recalcitrant and tropical recalcitrant (Table 1). According to their physiological storage potential, recalcitrant seeds require other methods of conservation than those used in conventional practices (Goveia, 2007).

Table 1: Physiological storage classes related to desiccation and temperature (Schmidt, 2000)

	Orthodox seeds	Intermediate seeds	Temperate recalcitrant seeds	Tropical recalcitrant seeds
Storage moisture (water) content	Low	Low	High	High
Storage temperature	Low	High	Low	High

2.4 *In vitro* preservation

In vitro culture techniques have been widely used for the germplasm storage and multiplication of several recalcitrant-seeded and vegetatively propagated species (Grout, 1990; Engelmann, 1997a; Humbeck *et al.*, 2008). Generally in plant tissue culture, cells, tissues or organs are excised from the parent plant, surface decontaminated and cultured on a growth medium (Lipavska and Vreugdenhil, 1996; Mandal *et al.*, 2000). These techniques provide the additional advantage because media used in tissue culture can be modified to induce different growth responses: for example, unorganized and undifferentiated callus or organized tissues and organs that can be used to generate plants. The advantages and disadvantages of *in vitro* preservation are given in Table 2.

Table 2: Advantages and disadvantages of *in vitro* preservation (Humbeck *et al.*, 2008)

ADVANTAGES	DISADVANTAGES
<ul style="list-style-type: none"> • Preserved material maintained under sterile conditions 	<ul style="list-style-type: none"> • Growth retardants could alter plant morphology and can induce DNA methylation.
<ul style="list-style-type: none"> • No risk of infestation and infection by insect activity 	<ul style="list-style-type: none"> • Somaclonal variations can occur
<ul style="list-style-type: none"> • No risk of damage by factors affecting the environment 	<ul style="list-style-type: none"> • Secondary infections can occur
<ul style="list-style-type: none"> • Less labour intensive than field collections 	<ul style="list-style-type: none"> • Mislabelling is a source of error that is detected in field gene banks.
<ul style="list-style-type: none"> • Material of species and varieties available throughout the year. 	

In vitro preservation can be divided into three approaches: cryopreservation, preservation of actively growing cultures and preservation of slow growth cultures (Scowcroft, 1984; Grout, 1990). Cryopreservation is the storage of plant material in the liquid/vapour phase of nitrogen (Engelmann, 1991; Rao, 2004). This is the only and most promising option for the long-term storage of germplasm of recalcitrant-seeded species. For medium- to short-term storage, the cultures are stored in their actively growing state or, more often, under slow growth conditions. The storage of actively growing cultures is achieved when plant germplasm is kept as sterile tissues or plantlets on nutrient media, whereas the slow growth involves storage of material under growth-limiting conditions (Shibli *et al.*, 2006). The growth rate of *in vitro* cultures can be minimised by several methods; these can either be chemical or physical. The chemical approaches include the use of growth retardants, incubation in reduced oxygen or agents which increase the osmotic potential of the media, while the physical approaches may involve subjecting the plants to low light intensities, low culture

temperature conditions, or modification of the nutrients in the medium (Engelmann, 1991; Rao, 2004; Shibli *et al.*, 2006; Goveia, 2007).

The ultimate aim of any *in vitro* conservation approach is the successful regeneration and propagation of genetically stable plantlets. In order to obtain healthy plantlets, organised cultures such as shoots are generally used for *in vitro* germplasm storage since undifferentiated tissue poses a higher risk of somaclonal variation (Rao, 2004; Shibli *et al.*, 2006 and Goveia, 2007).

2.4.1 Cryopreservation

Cryopreservation, in this context, refers to maintenance in the frozen state – i.e. storing plant germplasm at ultra-low temperatures, usually that of LN (-196 °C) or in the vapour above LN at -140°C to -160 °C (Kartha, 1981; Shibli *et al.*, 2006; Day *et al.*, 2008; Humbeck *et al.*, 2008; Hamilton *et al.*, 2009) to retain its viability for future initiation of plants (plant generation). Cryopreservation has proven to be the most promising method that ensures the safe and cost-effective long-term conservation of genetic resources of species that produce recalcitrant seeds or are vegetatively propagated (Sakai, 2000, Wesley-Smith, 2002; Berjak and Pammenter, 2004; Panis *et al.*, 2005). It can also be used as a tool to preserve endangered plant species, species with scarce seeds or seeds of doubtful quality and/or species with threatened imminent extinction (Touchell *et al.*, 2002; Ohmiya *et al.*, 2003; Mandal and Dixit-Sharma, 2007). At cryogenic temperatures, cellular division and metabolic processes in plant tissues are curtailed, thereby allowing unlimited period of time for storage of germplasm without alteration and modification (Kartha, 1985; Engelmann, 1991; Mycock *et al.*, 2004; Benson, 2004). Cryopreservation is not only low-maintenance and cost effective (Engelmann, 2004), but it also avoid problems of increased ploidy, mutations, biosynthesis capacity and undesirable phenotypes (Kartha, 1985; Blackesley *et al.*, 1996). Even though

cryopreservation shows potential as one of the long-term conservation methods, there is still a spectrum of problems that lead to unsuccessful recovery of plantlets from explants (Berjak *et al.*, 2011). However, a number of factors need to be considered for successful cryopreservation to be achieved and these will be elucidated in detail later in this chapter.

2.4.2 Cryopreservation techniques

Cryopreservation is based on the classical and vitrification techniques (Withers and Engelmann, 1998). The protocol and physical mechanism of these techniques are different in a manner that classical techniques involve freeze-induced dehydration while cryopreservation techniques focus on vitrification.

Classical cryopreservation is based on slow cooling resulting in a defined pre-freezing temperature (-40 °C), followed by rapid immersion in LN (-196 °C). Prior to slow cooling (0.5-2.0°C/min), the germplasm is chemically pre-treated and cryoprotected to reduce cellular water thus minimizing freezing injury or preventing ice formation (Engelmann, 2011). Mazur (1984) stated that the decrease in temperature during slow cooling causes the cells and the external medium to be supercool; however, the medium reaches a point where ice is formed. Therefore, the exterior ice is then prohibited from entering the interior of the cells by the protective cell membrane. Further decrease in temperature increases the accumulation of the external ice, resulting in equilibration of cells by losing water to the external ice (Engelmann, 2004; 2011). The removal of most or all freezable water in tissues reduces or prevents lethal intracellular ice from occurring during cooling in LN (Sakai, 1995). There is a profound relationship between WC and the required cooling rate to avoid ice nucleation. However there are some disadvantages that are incurred when cells are intensely dehydrated prior to cryoexposure. These are cell shrinkage (severe changes in cell volume and cell membrane) and damages related to high concentration of intracellular salts (Meryman *et al.*, 1977;

Walters *et al.*, 2008). For the success of classical techniques, it is imperative to limit both desiccation damage and freezing of water (Vertucci *et al.*, 1991) thereby minimising irreparable damage that might occur during cryo-exposure. Rewarming must be faster to avoid re-crystallisation, which is melting of ice and reforming of ice crystals at a thermodynamically more damaging crystal size (Mazur, 1984; Walters *et al.*, 2008). Classical techniques are complex because they require the use of expensive controlled freezers. In some instances, normal domestic or laboratory freezers can be used to perform the slow freezing step to a defined temperature (Kartha and Engelmann, 1994).

Vitrification based techniques - The techniques are based on vitrification, which is characterised as a solidification of liquid by an extreme elevation in viscosity during freezing (Fahy *et al.*, 1984). The aim of the vitrification techniques is to further minimise cryo damage and simplify the standard cryopreservation protocols (Engelmann, 1991). These techniques involve subsequent steps such as cell dehydration prior to cooling by treating samples with concentrated cryoprotective media and/or air desiccation (Engelmann, 2004; 2011). Once the cell water potential diminutions is accomplished, rapid cooling follows avoiding ice nucleation (Sakai *et al.*, 1990). Vitrification based techniques outweighs classical techniques (slow cooling based) because of numerous advantages such as being appropriate for complex organs (shoot apices and embryos), less complex, broad applicability, require less modifications for different cell types (Engelmann, 1997a). The most critical step common to all vitrification techniques is the dehydration step prior to immersion in LN because without proper optimisation there will be no or very low survival. While in classical techniques achieving survival after the freezing step is more important (Engelmann, 2011). Vitrification techniques are divided into seven vitrification based procedures which are encapsulation-dehydration, vitrification, encapsulation-vitrification, dehydration, pre-growth, pre-growth dehydration and droplet vitrification (Engelmann, 2011).

Encapsulation-dehydration protocols are based on developing artificial seeds (Gonzalez-Benito *et al.*, 2004; Engelmann, 2011). The method was initially developed by a French research team using potato shoot apices (Fabre and Dereuddre, 1990), and grape (Plessis *et al.*, 1991a), pear (Scottez *et al.*, 1991) and somatic embryos of carrot (Dereuddre *et al.*, 1991). This technique involves pre-treating the tissues in a liquid medium with enriched sucrose for 1 to 7 d, encapsulation with calcium beads followed by either air drying in the laminar flow or by exposure to silica gel (Engelmann, 1991; Scocchi *et al.*, 2004; Shibli *et al.*, 2006). The alginate beads protect the embedded tissue making it resistant or tolerant to lethal cryopreservation treatments (Engelmann, 1991). Thereafter the beads are frozen and thawed either slowly or rapidly (Engelmann, 1991).

Vitrification is the most promising and widely used cryopreservation method (Sarkar and Naik, 1998a; Golmirzaie and Panta, 2000; Zhao *et al.*, 2005a; Kryszczuk *et al.*, 2006) in which tissues are dehydrated by high osmoticum concentrated solutions to prevent ice nucleation during cryopreservation and re-warming (Moges *et al.*, 2003; Sakai *et al.*, 1990). To avoid the formation of ice, tissues are frozen ultra-rapidly in order for the cell solutes or contents to vitrify (an amorphous glassy structure) thereby avoiding the formation of intracellular ice. Rapid and timed pre-treatment in very high concentration of cryoprotectants is required for attaining vitrification (Engelmann, 1991). Vitrification consists of three critical phases: the loading phase, dehydration with PVS2 (plant vitrification solution) and the unloading solution (Sakai *et al.*, 1990; Ashmore, 1997). This method is simple as it uses highly concentrated vitrification solutions prepared with enriched sucrose and glycerol and does not require expensive cooling apparatus and can be applied to a variety of plant material (Matsumoto *et al.*, 1994; Benson *et al.*, 1996).

Encapsulation-vitrification is the combination of encapsulation-dehydration and vitrification techniques whereby explants are embedded in calcium alginate beads

(Kaczmarzyk, 2008; Engelmann, 2011). Explants are primarily encapsulated and subjected to freezing by vitrification (Ashmore, 1997; Gonzalez-Benito *et al.*, 2004), without the need for physical dehydration (Gonzalez-Benito *et al.*, 2004). Alginate beads are pre-cultured in liquid medium with elevated sucrose levels, loaded with sucrose-glycerol solution, dehydrated with PVS2 and cooled by direct immersion in LN. Thereafter samples are thawed at 40 °C water bath and unloaded in enriched sucrose solution (Sakai *et al.*, 1990; Sakai and Engelmann, 2007). Encapsulation-vitrification is easy to handle, saves time required for desiccation and survival is earlier than that of encapsulation-dehydration (Hirai *et al.*, 1998).

2.5 Advantages of cryopreservation

The major advantage of cryopreservation is that a variety of cells and tissues can be stored such as protoplasts, single cells and organised tissues (for example, meristems and somatic embryos; Bajaj, 1983). It is believed that during cryopreservation, cell division, metabolic and biochemical processes are arrested and thus the biological material should be able to be stored without deterioration or modification for extended periods of time (Kantha, 1981; Withers, 1983; Shibli *et al.*, 2006; Kaczmarczyk, *et al.*, 2012). Once in storage, there is no risk of new contamination by fungus or bacteria, and cryogenically stored material has been reported to retain genetic stability (Harding, 2004; Shibli *et al.*, 2006). Moreover, the space requirements are minimal and maintenance is relatively easy (Pence, 2011).

2.6 Factors affecting cryopreservation

The preservation of biological material cryogenically involves various steps such as pre-culture in media with osmotically active compounds, treatment with cryoprotectants, cooling, thawing, post-thaw treatment and regeneration of plantlets (Shibli *et al.*, 2006; Goveia, 2007). The ability of the explants to survive these cryopreservation procedures is affected by the following factors:

i) Physiological condition of plant material

The ability of explants or plant material to survive post-cryostorage is dependent on factors such as genotype, physiological status, cold sensitivity and pre- and post-freezing manipulation. While the type and nature of explants also has an effect on the post-cryo survival regrowth of the plantlet (Engelmann, 1991; Ashmore, 1997). Cells of vigorously growing (immature) cultures are said to have a dense cytoplasm and minimal vacuolar or unvacuolated volumes which promotes adversely the ability of the explants to withstand storage in the LN, thus maintaining the genetic integrity (Kaczmarczyk *et al.*, 2012).

ii) Size of explant

Tissue size affects thermal mass and surface area to volume ratios, in turn affecting rates of cryoprotectant penetration, dehydration and cooling (Berjak *et al.*, 1998, Walters *et al.*, 2008). The smaller the explants and the lower the WC, therefore lesser time for dehydration (Wesley-Smith *et al.*, 2004a; Kaczmarczyk, *et al.*, 2008). Smaller explants also allow control over the range of attainable cooling rates, therefore the efficacy of rapid cooling in preventing ice formation can be explored (Wesley-Smith *et al.*, 2004a). Most recalcitrant seeds are relatively large and are highly susceptible to the dehydration and freezing which are necessary for successful cryopreservation. In such cases excised zygotic axes are the most suitable explants (Berjak, 2006; Berjak and Pammenter, 2008). However, in many cases the embryonic axes are themselves large and detrimentally affected by excision, dehydration and LN immersion (Goveia, 2007; Varghese *et al.*, 2009). Other explants appropriately small for cryopreservation can be obtained from various sources alternative to the excised axes: these can be buds from *in vitro* shoots and shoot apices. Shoot apices are the most favoured or most ideal explants for cryopreservation because they contain relatively undifferentiated cells that are suggested to remain genetically stable during the regeneration process and direct shoot

development after re-warming is generally obtained (Mroginski *et al.*, 1991; Walters *et al.*, 2008; Kaczmarczyk, *et al.*, 2012).

iii) Water content

Water concentration is the measure of the ratio of water within an entity relative to other constituents. Recalcitrant seeds retain high WC when shed, but the WC at shedding varies with species (Ballesteros *et al.*, 2014), and often within single species from one harvest to the next (Berjak and Pammenter, 2004). High WC in tissue promotes formation of lethal ice crystals upon explant exposure to sub-zero temperatures, thus affecting the success of cryopreservation (Roberts, 1973; Wesley-Smith *et al.*, 1991; Quain *et al.*, 2012). Desiccation-sensitive axes cannot be dehydrated to WC less than 0.3 g H₂O g⁻¹ DW without detrimental damage (Wesley-Smith *et al.*, 1991). This means that if biological material is desiccation tolerant, cryopreservation will be relatively simply achieved (Fu *et al.*, 1994), but not otherwise. Lowered WC of explants can be induced by exposure to abscisic acid, sugars, mannitol and sorbitol (Panis *et al.*, 2002; Walters *et al.*, 2002), exposure to cryoprotectants and vitrification (using chemical agents such as sucrose, glycerol, DMSO, ethylene glycol, proline etc.; Engelmann *et al.*, 1994). Desiccation of tissue prior to cryostorage in activated silica gel (Hatanaka *et al.*, 1994) and by flash drying (Berjak and Pammenter, 1999; Pammenter *et al.*, 1999; 2002) reduces WC thus enhancing cryo-tolerance (Quain *et al.*, 2012).

iv) Drying rate

Dehydration prior to cooling is inevitable for the success of cryopreservation of non-orthodox plant material. Rapid physical dehydration of tissues is often achieved via flash drying (Berjak *et al.*, 1989; Pammenter *et al.*, 2002), a technique used to induce rapid dehydration of

tissues by running an air stream over activated silica gel, which is aimed at decreasing the WC to an amount suitable for freezing (Pammenter *et al.*, 2002; von Fintel, 2006; Ballesteros *et al.*, 2014). Drying rate has been found to have an effect on WC to which recalcitrant seeds can be dried before loss of viability (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999; Varghese *et al.*, 2011). Slow drying of recalcitrant seeds or excised axes leads to damage of tissue at high WCs, which is the consequence of unbalanced metabolism while water is lost, and is termed metabolism-linked desiccation damage (Pammenter *et al.*, 1998; Walters *et al.*, 2001; Varghese *et al.*, 2011). According to Vertucci (1990) and Pammenter *et al.* (1991) water exists in five levels of hydration in desiccation tolerant seed tissues, and at levels below 0.26-0.24 g g⁻¹ DW the water is non-freezable which is understood to be bound to structures, i.e. structure-associated water. Pammenter *et al.* (1991) further stated that in orthodox seeds much of the freezable water can be removed with no detrimental effect on viability, thus such seeds can be successfully cryopreserved. In contrast, recalcitrant seeds or embryonic axes excised from such seeds can only withstand partial removal of bulk water or for that matter little or no structure-associated non-freezable water, which is associated with lethal effects when removed (Pammenter *et al.*, 1991; 1993) due to their desiccation-sensitivity. Those authors describe this as desiccation-damage *sensu stricto*. Flash drying does not render recalcitrant material desiccation tolerant to be stored at ambient or sub-ambient conditions. Rather, it minimises the time during which metabolism-associated damage can occur (Pammenter *et al.*, 1998; Walters *et al.*, 2001) paving way for the possibility of its storage under LN provided the stress incurred due to desiccation and/or cooling is not enormous. Though flash dried embryonic axes do not survive for extended periods at ambient or even refrigerator temperatures, but provide ideal explants for cryopreservation. Thus the material should be ideally cooled in a cryogen immediately after drying.

v) Pre-cooling treatment (cryoprotection)

Pre-cooling treatment (cryoprotection using penetrating and non-penetrating cryoprotectants) can entail cultivating biological material to be preserved in the presence of cryoprotective agents such as sucrose, sorbitol, mannitol, dimethyl sulphoxide (DMSO), amino acids, sugars or polyethylglycol (PEG) etc., which may induce some drying and are also said to protect membranes, proteins and enzymatic binding sites from the freezing stress (Gonzalez-Benito *et al.*, 2004; Humbeck *et al.*, 2008). If tissues are cooled slowly, water is drawn out of cells to nucleate as ice extracellularly, which is considered an effective method of dehydrating living cells (Gonzalez-Benito *et al.*, 2004). Partial dehydration of tissues is essential before cooling, and can be at least partly achieved by exposure to an osmoticum or cryoprotectants (Hirai and Sakai, 1999). If the biological material is treated with osmotic agents, survival after cryostorage may be higher than that of non-treated material (Shibli *et al.*, 2006). The concentration and duration of exposure of plant material to the osmotic agents of course depends on the plant species and type of the explant (Ashmore, 2007). Pre-treatment additives have been shown to facilitate survival of shoot apices of *T. emetica* (Varghese *et al.*, 2009) and explants of *Ekebergia capensis* (Hajari *et al.*, 2011) after cryostorage. In addition, cooling tolerance of biological material can be enhanced by cold hardening (Hirai *et al.*, 1998) and cryoprotection or pre-growth culture of plant material.

A single cryoprotectant (e.g. DMSO) can be effective, but a mixture of cryoprotectants (e.g. DMSO and glycerol) is usually more effective (Withers, 1991). Cryoprotection, however, is seldom adequate to dehydrate material sufficiently for cryopreservation, but is complementary to subsequent physical drying (von Fintel, 2006). The mode of action of cryoprotectants is that they increase the concentration of solutes in the cytoplasm, thus helping to promote intracellular vitrification during dehydration and therefore minimising intracellular ice formation (Rao, 2004; Walters, 2008). There are some disadvantages of

using cryoprotectants such as cytotoxicity, dehydration injury and mechanical damage during the freezing and thawing steps (Wesley-Smith *et al.*, 1995), making the technique variably successful, depending on explant-type and species.

vi) Cooling rates

Cooling rate of explants is critical during cryopreservation as it determines the survival after exposure to LN (Wesley-Smith *et al.*, 1991; 2004a; 2014; 2015). Slow cooling rates have been found to allow the process of protective dehydration to take place in explants from recalcitrant seeds (Withers, 1991; Wesley-Smith *et al.*, 2004a; 2014). However, slow cooling to very low temperatures before transferring the plant material to LN may be injurious, due to excessive cellular dehydration by extracellular ice crystallisation. The injury caused by cooling is related to the rate employed and the WC of the tissue: rapid freezing, successfully used for explants at higher WCs, has been shown to prevent deleterious intracellular ice formation (Wesley-Smith *et al.*, 1991; von Fintel, 2006; Wesley-Smith *et al.*, 2004a). However, generalisations cannot yet be made, as employment of a particular cooling rate promoting cryo-survival (in relation to WC) has been shown to be species and explant related (Wesley-Smith, 2002; Varghese *et al.*, 2009).

vii) Thawing

Prior to regeneration, frozen tissues must be thawed without permitting re-crystallisation during warming (Ashmore, 1997). This can be accomplished only by rapid warming, where frozen tissue is immersed in medium placed in temperature-controlled water bath at 37 - 40°C (Berjak, 1999). Rehydration in distilled water (which has been extensively utilised) is likely to have deleterious effects on dehydrated biological material. The use of a 1:1 CaMg solution, containing 0.5 μ M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Berjak and Mycock,

2004) has been found to promote normal ongoing development of explants after cryostorage and/or dehydration, notably promoting re-assembly of functional cytoskeletons (Berjak *et al.*, 1999; Berjak and Mycock, 2004).

2.7 Oxidative stress accompanying procedural steps for, and after, cryopreservation

Free radicals are highly reactive atoms or molecules that are capable of independent existence, consisting of one or more unpaired electrons (Halliwell, 2006). Reactive oxygen species being versatile molecular species and radicals regulate a complicated network of signalling pathways in plants (Bhattacharjee, 2012). Their role is to regulate cell physiology and responses to the environment. Reactive oxygen species include superoxide ($\cdot\text{O}_2^-$), perhydroxy radical (HO_2^\cdot), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot\text{OH}$), alkoxy radical ($\text{RO}\cdot$), peroxy radical ($\text{ROO}\cdot$), singlet oxygen ($^1\text{O}_2$), organic hydroperoxide (ROOH), molecular O_2 , activated oxygen (O_3) and so forth (Pukacka and Ratajczak, 2006; Berjak and Pammenter, 2008; Bhattacharjee, 2012). These ROS are implicated in normal metabolism, plant growth and development (Gapper and Dolan, 2006), radicle protrusion during germination of seeds (Schopfer *et al.*, 2001; Müller *et al.*, 2009) and oxidative metabolic processes in all aerobic organisms, for example those occurring in photosynthesis, respiration and signalling in chloroplasts, mitochondria and peroxisomes (Forman and Torres, 2002; Apel and Hirt, 2004; Benson and Bremner, 2004). The ROS exhibiting a role in development are produced by NADPH oxidases (NOXs) that are responsible for the generation of $\cdot\text{O}_2^-$. It has been found that the NOX proteins are required in root elongation of *Arabidopsis* (*Arabidopsis thaliana*) (Forman *et al.*, 2003) and also in expansion in maize (*Zea mays*) roots (Liszkay *et al.*, 2004). NOX derived ROS is not only responsible for root development but also growth of other organs e.g. leaf elongation reported by Rodriguez *et al.*, 2002.

Plants have developed signalling and sensing mechanisms protecting them from the various stresses posed by the environment (Bhattacharjee, 2012). Reactive oxygen species are one of the most common responses from abiotic and biotic environmental stresses in plants. These environmental stresses include extreme temperatures, salinity, smog, drought, heavy metals, herbicides and pathogens etc. (Mittler and Blumwald, 2010). The cytotoxicity of ROS (Hendry, 1993; Chaitanya and Naithani, 1994; Varghese and Naithani, 2002) affects plant metabolism and productivity thus resulting in a decrease in crop yield (Bhattacharjee, 2012). Although ROS is associated with cell signalling and redox sensing mechanisms, when produced in uncontrolled levels also results in destructive events such as membrane damage resulting in ion leakage, impaired photosynthesis and respiration, production of toxic compounds, disrupted redox homeostasis of cells (Kranter *et al.*, 2006) eventually leading to cell death (Halliwell and Gutteridge, 1984; Einset *et al.*, 2007; Bhattacharjee, 2012). Excessive production of ROS has also been associated with oxidative stress in tissues resulting in oxidative damage of proteins, lipids and nucleic acids (Chaitanya and Naithani, 1994; Halliwell and Gutteridge, 2007).

A balance between ROS and quenching activity of house-keeping or stress-induced antioxidants is imperative to prevent the damages exerted by various environmental stresses. Although ROS is implicated in plant development, when it released into an extent that overwhelms endogenous antioxidant capacity of small explants such as embryos and shoot apices, it causes oxidative damage (Berjak *et al.*, 2011). In cases where ROS overwhelms endogenous total antioxidant activity, exogenous antioxidants, transition metal chelating agents, various antioxidant enzymes (*viz.*, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), peptides and metabolites (phenolic and nitrogen compounds) can be applied to counteract stress induced by unbalanced ROS activity

(Ali and Alqurainy, 2006). However, the role of antioxidants as a defence system is very important in plant development.

It has been shown that each step of cryopreservation described above from excision to retrieval from cryogenic storage – in cryopreservation protocols for zygotic embryonic axes is accompanied by stress-related surges of ROS production (Roach *et al.*, 2008; Whitaker *et al.*, 2010; Berjak *et al.*, 2011; Pammenter *et al.*, 2011). As there is no reason to believe that the same will not be true when shoot apices are used as alternative explants, determinations of ROS and of aqueous antioxidants was assessed during and after the steps of cryopreservation.

2.8 Cathodic protection

Cathodic protection is a method used to quench unregulated or unbalanced ROS activity to minimise oxidative damage (Pammenter *et al.*, 1974; Berjak *et al.*, 2011). Cathodic protection has been extensively investigated in the metal industry e.g. against corrosion in bridges and pipelines (Baeckmann, 1997; Huck, 2005). Cathodic protection in plant tissues was first investigated by Pammenter *et al.* (1974) who placed *Zea mays* seeds on the cathode (aluminium foil sheet) in a static electric field with a potential difference between cathode and anode of 300 volts. This treatment extended their viability when maintained under accelerated ageing conditions. Thereafter, Berjak *et al.* (2011) extended the principle of cathodic protection by using the cathodic fraction of the electrolysed salt solution (cathodic water) as a solvent in the procedural steps of cryopreservation. Cathodic water is generated by electrolysing a CaMg solution, with the cathode and anode in separate chambers. Cathodic water is distinguished from anodic water by being milky, having a high pH (10.0- 11:50), high dissolved hydrogen content and low oxidative-reduction potential (-800 to -900 mV) (Huang *et al.*, 2008). Cathodic water has been reported to have the potential to quench or scavenge ROS, protect DNA from oxidative damage (Hanaoka, 2001; Shirahata *et al.*, 1997)

and act as a reductant. Based on the beneficial effects of CW stated by Berjak *et al.* (2011) which is its ability to enable seedling development from cryopreserved embryonic axes of *Strychnos gerrardii*, it was used in this study throughout the stages of cryopreservation.

2.9 *In vitro* regeneration

2.9.1 Culture conditions

Aseptic culture conditions are a prerequisite for successful development of seedlings (from axes) or plantlets (from alternative explants) *in vitro*. Sterile conditions prevent the invasion by, and subsequent proliferation of microorganisms which not only compete for nutrients, but also degrade the explant tissues, especially at the fragile regeneration phase (Kioko, 2003). Therefore it is crucial to decontaminate/sterilize the working environment, equipment, culture medium and the plant material. Common decontaminants used include dilute solutions of sodium or calcium hypochlorite or mercuric chloride, 70% ethanol (Hartmann *et al.*, 2002) and NaDCC (sodium dichloroisocyanurate or sodium troclosene).

2.10 Recalcitrant-seeded species studied

The two recalcitrant-seeded species indigenous to South Africa used in this study were *Trichilia dregeana* Sond. and *Trichilia emetica* Vahl. of the family Meliaceae.

2.10.1 *Trichilia emetica* Vahl.

Trichilia emetica is a tropical tree species producing short-lived recalcitrant seeds, which grows in forests and in open riverine-alluvial lowland rainforests (Kioko *et al.*, 2006). It is commonly also known as the Natal mahogany, and also shares the same Zulu name (*umKhuhlu*). Even though both the *Trichilia* spp. shares some similarities there are some distinct differences, one of them being the height of the trees. *T. emetica* grows to a height of 5-10 m whereas *T. dregeana* trees are much taller at 10-35 m (Pooley, 1993). Unlike *T.*

dregeana, *T. emetica* flowers (which are creamy to silvery-green and velvety) between September and November and produces fruits (that are creamy green and turning pale brown when ripe) in January to April (Goveia, 2007). *Trichilia emetica* fruits possess a neck and are both fruits and seeds are smaller than those of *T. dregeana*. Though the actual size of the seeds is different between the two species, they are morphologically similar (Pooley, 1993).

The extensive use of this species in traditional medicine has encouraged scientists to do research on the species (Komane *et al.*, 2011). Its exasperating use has also contributed to its high exploitation and extinction rates (Palmer and Pitman, 1972). Extensive research has been done on *T. emetica* in our laboratory to store and cryopreserve the excised embryonic axes and/or the shoot apices of this species. Koiko *et al.* (2006) reported that *T. emetica* seeds are shed at an average axis water concentration of 2.8 g g⁻¹ and lose viability after 20 days when stored at 6 °C and upon dehydration. However, there is some insight about the responses of *T. emetica* embryonic axis to cryopreservation stresses and ultrastructural responses of seeds in storage (Kioko *et al.*, 2003; 2006). A study was also done by Varghese *et al.* (2009) on the cryopreservation of the shoot tips of *T. emetica*, whereby 71% shoot production were achieved after cryopreservation. The current study was undertaken to improve shoot production and obtain proper plantlet after cryopreservation using shoot apices of *T. emetica*. Extensive work has also been done on its medicinal properties by a number of researchers (for e.g., Komane *et al.*, 2011; Gunatilaka *et al.*, 1998; Diallo *et al.*, 2003; Germano *et al.*, 2005).

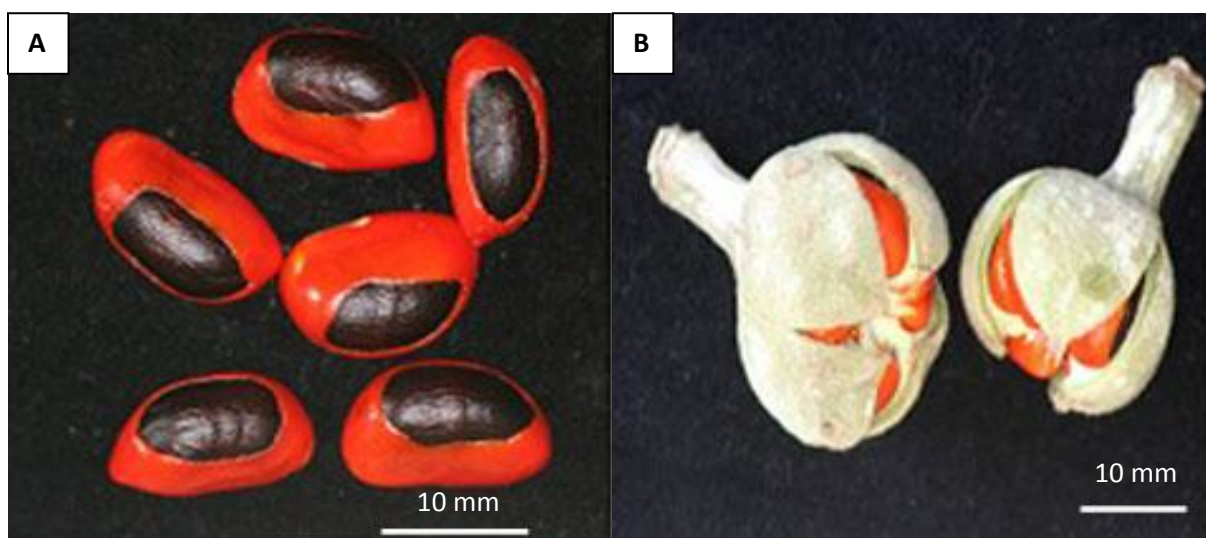


Figure 3: Freshly harvested seeds (A) and fruits (B) of *T. emetica*.

2.10.2 *Trichilia dregeana* Sond.

The common names given to *T. dregeana* are Forest/Natal-Mahogany or *umKhuhlu* (Zulu). The Mahogany trees (noting that this common name is also used for, and should not be confused with, quite different species in Indian/Malaysian areas) are fairly widespread, occurring in the tropics and subtropics of the northern and southern hemispheres. They are indigenous to southern and eastern Africa, being more prevalent in the areas of high rainfall in coastal and montane evergreen forests. *T. dregeana* is a large tree, with impressive heights between 10-35 m and a diameter of up to 1.8 m. The growth form is strikingly uniform, very dense, and the tree has deep green, glossy foliage and rounded canopy with few internal branches visible (Kioko *et al.*, 2006). New leaves are shiny, red brown and turn lime green before darkening (Schmidt *et al.*, 2002; Whitaker *et al.*, 2010). In South Africa *T. dregeana* trees produce creamy-white flowers, between October and December and fruiting occurs mainly between May and July. When ripe the fruit capsule bursts open on the tree to show six

seeds, each of which has a black testa which is almost completely enclosed by a scarlet aril (Goveia, 2007).

The tree provides excellent shade and has a non-aggressive root system, making it ideal for gardens. *Trichilia dregeana* can be grown easily from fresh seeds which have not been allowed to dehydrate, or from cuttings, growing quickly when watered: it can be planted in either shade or sunlight. The tree commercially produces wood suitable for carving, furniture, fishing floats, musical instruments and household implements. The seed arils are cooked as a vegetable or crushed to yield a milky juice taken as a drink or with side dishes. The seeds are rich in fats, which are used in soaps, as body ointment and hair oil as well as for cooking (Schmidt *et al.*, 2002). The oil has properties of hastening healing, particularly of fractures. The species is also an important medicinal plant with the seed, oil, leaves, roots and bark being used for many purposes depending on different cultures (Hutchings *et al.*, 1996). For example, bark preparations are used as remedy for backache, stomach problems, and kidney ailments and also has application as a fish poison.

The fruits are consumed by a variety of animals such as baboons, monkeys, and antelope, various species of birds and humans use them for preparation of a milky soup. The bark is used in herbal medicine, while the roots, leaves, as well as oil from the seeds are used for remedies against stomach, intestine and kidney ailments, indigestion, fever, parasites and eczema. The powdered bark provides an emetic (hence the species name, *emetica*) (Pooley, 1993). In addition to the medicinal properties of the bark, *T. emetica* also produces wood of good quality (Kioko *et al.*, 2006; Goveia, 2007).

Over the years, this species has been widely studied with some trying to understand the characteristics and behaviour of this species which was reported to produce recalcitrant seeds by Choinski (1990). Han *et al.* (1997) reported on the presence of dehydration-related

polypeptides dehydrins during seed maturation and in response to ABA or water deficit related stresses in the axes of these seeds. Berjak *et al.* (2004) investigated strategies for field collection of *T. dregeana*. . Whitaker *et al.* (2010) reported on production of ROS in the steps of cryopreservation whereas Varghese *et al.* (2011) studied the effect of drying rates on survival and ROS in *T. dregeana* embryonic axes. Attempts have been made to grow excised embryonic axes of *T. dregeana* after various steps of cryopreservation by numerous researchers (Kioko *et al.*, 1998; Kioko, 2003; Berjak and Mycock, 2004; Goveia, 2007; Naidoo, 2012) but with little or no success. Hence the present work was conceived with the idea of establishing a cryopreservation protocol for *T. dregeana* using shoot apices.



Figure 4: Three-lobed mature fruits and seeds of *T. dregeana*.

2.11 AIM AND OBJECTIVES

2.11.1 Aim

The aim of this investigation is to produce and cryopreserve explants alternative to embryonic axes, for *T. emetica* and *T. dregeana*, where difficulties with the latter have precluded their successful cryopreservation. The research was also focusing on assessing the possible physiological and biochemical factors associated with cryopreservation of alternate explants of these species and evaluating reasons for poor performance after cryo survival.

2.11.2 Objectives

- Collection of *T. emetica* and *T. dregeana* seeds, storage and *in vitro* germination.
- Optimising seedling, shoot apex (explant) excision, and medium and culture conditions for the *in vitro* regeneration of complete plantlets from these explants of *T. dregeana* and *T. emetica*.
- Establishing a cryopreservation protocol using meristems (shoot apices) from *in vitro* regenerated seedlings of *T. emetica* and *T. dregeana* were investigated.
- Investigate possible ways of improving post-cryo vigour in plantlets regenerated from cryopreserved meristems (shoot apices), if vigour is compromised.
- Testing the effect of highly reducing antioxidant cathodic water reported to ameliorate extensive oxidative stress accumulated during various procedures of cryopreservation on the survival of meristems (shoot apices) post-cryo and their growth and vigour after regeneration.
- During the different stages of cryopreservation protocol, biochemical (to study ROS mediated oxidative stress) and microscopical (to study ultrastructure) studies were considered in the context of cryo-survival and normal plantlet formation.

3. MATERIALS AND METHODS

3.1 Seed collection and storage

Mature capsules of *T. emetica* were hand-harvested from the trees in St. Lucia (28° 22' 59, 88" S, 32° 25' 0, 12; google earth), South Africa (in January-February 2012 and 2013) and *T. dregeana* seeds were harvested from trees in the Glenwood area (29° 22' 0, 12" S, 30° 58' 59, 88", google earth), Durban, South Africa or from trees at the University of Kwa-Zulu Natal (Westville campus) (29° 49' 4, 43" S, 3° 56' 37,58" E; google earth), Durban, South Africa from April-July 2012 and 2013. Once in the laboratory, the seeds were removed from the fully matured and opened fruits and those seeds damaged by insects were discarded. The aril covering the seed and seed coat were removed and the seeds were subjected to further processing and hydrated storage.

3.2 Seed processing

Cleaned seeds were surface-decontaminated by immersing in 1% (v/v) sodium hypochlorite (NaOCl) containing a few drops of Tween 20[®] (wetting agent) for 20 min. Seeds were then soaked in an antifungal 'cocktail' containing 0.5 ml L⁻¹ Early Impact (active ingredient, triazole and benzimidazole; Zeneca Agrochemicals, South Africa) and 2.5 ml L⁻¹ Previcur N (active ingredient, propamocarb-HC, AgrEvo, S. Africa) for 60 min and 240 min for *T. emetica* and *T. dregeana* respectively. These fungicides have shown to curtail fungal contamination in storage (Calistru *et al.*, 2000; Goveia, 2004; Myeza, 2005). Seeds were then rinsed with sterile distilled water, placed on dry paper towel on a laboratory bench and dried overnight back to the original batch fresh weight and either used immediately or prepared for hydrated storage.

3.3 Hydrated storage

The decontaminated seeds were dusted with Benomyl 500 W fungicidal powder (active ingredient, benzimidazole; Villa protection, South Africa) and were stored in a monolayer on plastic mesh grids suspended about 100 mm over few sheets of paper towel moistened with 1% (v/v) NaOCl, within sealed, sterile plastic buckets and placed at 16 °C. The lid of the bucket was lined with dry paper towel to prevent condensate dripping onto the stored seeds. The seeds were stored in hydrated storage at 16°C for a maximum period of three months until further use.

3.4 Cryopreservation of shoot apices

The methodology developed by Varghese *et al.* (2009) was followed to cryopreserve the shoot apices of *T. emetica*, the details of which are given below.

3.4.1 Production and excision of shoot apices

Seeds of *T. emetica* and *T. dregeana* were germinated *in vitro* on half-strength MS medium in magenta jars (Murashige and Skoog, 1962). The shoots obtained were multiplied on MS medium with 0.5 mg L⁻¹ BAP (SIGMA, Germany) using single nodal segments, to establish sterile stock material. The stock cultures were maintained in a growth room under cool fluorescent light (Philips TL-D 90 Deluxe Pro 58W/965, Amsterdam, Holland; 52 µmol m⁻² s⁻¹ photosynthetically active radiation) and 16 h photoperiod at 25 ± 2°C. The shoot apices (0.5 – 0.8 mm) in length, consisting of the meristematic dome and 1 or 2 pairs of leaf primordia were aseptically isolated from axillary buds of the shoots using a dissecting microscope, in a laminar flow hood (J10 Tech Lab. Companion, BC-01E; Korea). These shoot apices were then used for various cryopreservation procedures as described below.

3.4.2 Pre-culture and cryoprotection (PVS2) of shoot apices

The shoot apices immediately after excision were pre-cultured on MS medium containing 0.3 M sucrose and 0.5 M glycerol for 3 d (Matsumoto *et al.*, 1998; Varghese *et al.*, 2009), in the dark at 25 ± 2 °C. The pre-cultured shoot apices were initially osmo protected by treating with a loading solution (2.0 M glycerol and 0.4 M sucrose) for 15 min followed by an exposure to chilled PVS2 (30% [w/v] glycerol, 15% [w/v] ethylene glycol and 15% [w/v] DMSO in liquid MS medium containing 0.4 M sucrose [pH 5.6- 5.8]) on ice. Due to the potential toxicity of PVS2 to the shoot apices of the *Trichilia* species used in this study, exposure to the vitrification solution was done sequentially (Benson *et al.*, 2007) by treating with 50% PVS2 solution for 5 min followed by 100% PVS2 for a further 15 min (Varghese *et al.*, 2009). All the cryoprotectant solutions were made with laboratory grade chemicals and autoclaved or filter sterilised before use.

3.4.3 Water content determination

Water content of the shoot apices was determined immediately after excision, after pre-culture and immediately after pre-culture + cryoprotection (shoot apices were blotted with filter paper before WC determination). The WC was determined gravimetrically by weighing before and after oven-drying at 80°C for 48 h and was expressed on a dry mass basis (g H₂O per g dry weight). Five replicates of three shoot apices per replicate (total of 15 shoot apices) were weighed using a six-place balance (Mettler MT5, Germany) before drying in an oven.

3.4.4 Survival and shoot production prior to cooling

Survival of the shoot apices were assessed after excision, pre-culture and pre-culture + cryoprotection. Greening and opening of the leaf primordia was recorded as survival. Ten shoot apices from each treatment were cultured on MS medium with 0.05 mg L⁻¹ BAP and 0.1 mg L⁻¹ GA₃ (Varghese *et al.*, 2009) solidified with 8% agar (Bacteriological Agar; Merck,

South Africa) to assess the survival at each of the stages. The number of shoot apices showing visible leaf expansion and organised re-growth after eight to ten weeks of culture on the same medium was recorded as shoot production and was expressed as percentage.

3.4.5 Cooling

Two rates of cooling viz., slow cooling or two-step cooling and relatively rapid cooling of shoot apices within cryotubes plunged into LN, were used for the cryopreservation of the shoot apices of *Trichilia emetica*.

i. Slow or two-step cooling

Cryoprotected shoot apices (n=30) were placed in sterile 2 ml cryotubes (Greiner bio-one, Germany; ten shoot apices per cryotube) with 1 ml of fresh PVS2 and were cooled at a slow rate of $1^{\circ}\text{C min}^{-1}$ using Mr Frosty[®] (Nalgene[™], USA) to an intermediate temperature of -40°C in a -80°C deep freezer. Thereafter, the cryotubes were immediately plunged into LN and stored in a LN Dewar for a minimum of 1 h.

ii. Rapid cooling

The cryoprotected shoot apices (n=30) were placed in cryotubes (ten shoot apices per cryotube) with 1 ml of fresh PVS2, mounted on cryocanes and cooled by plunging into LN for 1 h.

3.4.6 Rewarming (Thawing)

Irrespective of the mode of cooling, the cryotubes containing the shoot apices were rapidly warmed in a water bath at 40°C for 2 min. The PVS2 was drained off and immediately replaced with an unloading solution (1.2 M sucrose) in which shoot apices were held for 15 min at 25°C .

3.4.7 Recovery

The shoot apices (n=30) were then transferred to sterile filter paper which was placed on WPM (woody plant medium; Lloyd and Mc Cown, 1980) containing 0.05 mg L⁻¹ BAP, 0.1 mg L⁻¹ IBA and 0.1 mg L⁻¹ GA₃ (Varghese *et al.*, 2009) or (WPM with 1 mg L⁻¹ BAP and 0.1 mg L⁻¹ IBA) in Petri dishes. After a day in culture (in the dark), the filter paper with the shoot apices was placed on fresh WPM. The cultures were incubated in dark for a week and then exposed to partial light for 7d followed by exposure to full light at culture room conditions (as described before). The survival of shoot apices and subsequent shoot development were assessed as mentioned in section 3.4.4.

3.5 Use of cathodic water in the cryopreservation of shoot apices

Highly reducing cathodic water (CW), reported to possess strong antioxidant activity (Berjak *et al.*, 2011) was used during the procedural steps of cryopreservation of the shoot apices to ascertain its effects on ameliorating oxidative stress, if any.

3.5.1 Generation of cathodic water

Cathodic water was generated using CaMg solution (1:1 solution of 0.5 µM CaCl₂·2H₂O and 0.5 mM MgCl₂·6H₂O; Berjak and Mycock, 2004). The solution was electrolysed using a Bio-Rad™ Powerpac (BioRad, Hercules, Carlifonia, USA) equipped with two platinum electrodes (anode and cathode). The two electrodes were immersed in two 250 ml glass beakers each containing 200 ml of the CaMg solution. The circuit was completed by an agar-based salt bridge (3 g KCl and 0.3 g bacteriological agar dissolved in 10 mL of distilled water) after which the solution was electrolysed at 60 V potential differences for 60 min. The CaMg solution in the beaker in which the cathode was immersed was used as the cathodic water within an hour of preparation. A detailed set up of the equipment for the generation of this CW is given below.

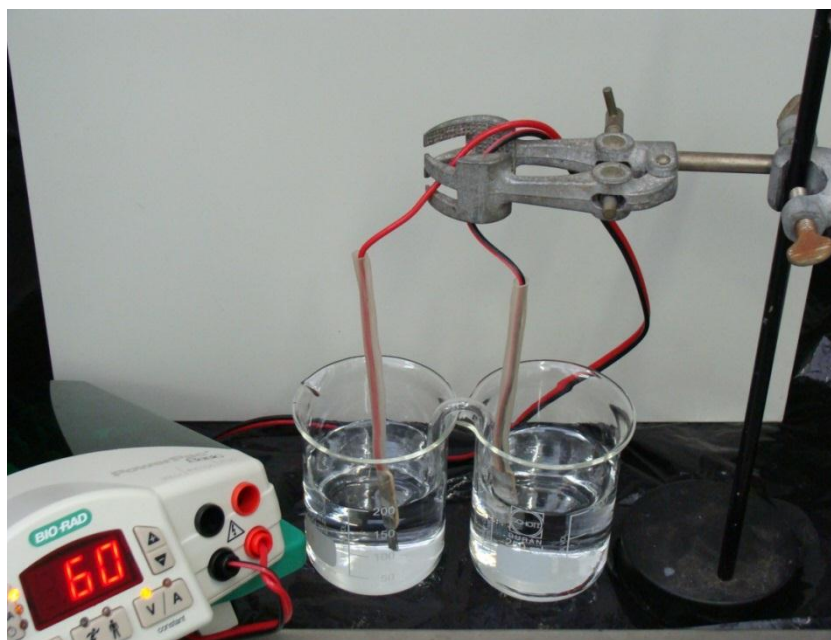


Figure 5: Electrolysis apparatus used to generate cathodic water.

3.5.2 Treatment of shoot apices with cathodic water (CW)

The shoot apices were treated with CW at four different stages of cryopreservation. Immediately after excision, the shoot apices were immersed in CW for 15 min. For the pre-culture step, the shoot apices were cultured on filter paper soaked in CW placed over the pre-culture medium. During cryoprotection, the shoot apices treated with loading solution and PVS2 were made up in CW. In the retrieval step, shoot apices were treated with unloading solution made in CW. Water content of the shoot apices (five replicates of three shoot apices each) immediately after CW treatment was assessed after excision, pre-culture and pre-culture + cryoprotection. Survival of the CW treated shoot apices (n=30) was recorded during the procedural steps leading to cryopreservation and following retrieval from cryostorage while shoot production (n=30) was assessed just before and after retrieval from cryostorage.

3.6 Biochemical estimations

In order to assess the oxidative stress, if any, to which the shoot apices are subjected to during the procedural steps of cryopreservation, estimation of extracellular superoxide as a representative of ROS and total aqueous antioxidant activity were carried out.

3.6.1 Estimation of extracellular superoxide production

Extracellular production of superoxide ($\cdot\text{O}_2^-$) was estimated immediately after excision (n=20), pre-culture, pre-culture + cryoprotection and 1 h after retrieval from cryostorage for CW treated and untreated shoot apices. Extracellular production of $\cdot\text{O}_2^-$ was measured by the oxidation of epinephrine (SIGMA, Germany) to adrenochrome using a spectrophotometer (Cary 50 Conc UV Vis spectrophotometer, Varian, Palo Alto, CA) at 490 nm (Misra and Fridovich, 1972), at room temperature in the dark. Four replicates of five shoot apices each were shaken in 2.0 ml Eppendorfs® with 0.5 ml of 1 mM epinephrine (pH 7.0) and 1.5 ml distilled water for 15 min in the dark at 25° C (Roach *et al.*, 2008). The blank consisted of 0.5 ml epinephrine and 1.5 ml distilled water without the shoot apices. After $\cdot\text{O}_2^-$ was measured, the shoot apices from each replicate were placed in an oven at 80° C for 48 h to obtain DW. The extinction coefficient of adrenochrome at 490 nm, 4.47 mM⁻¹ cm⁻¹, was used to calculate $\cdot\text{O}_2^-$ production and was expressed as $\mu\text{mol min}^{-1} \text{g}^{-1}$ dry weight (DW).

Validation test for superoxide assay

As epinephrine can be oxidized non-specifically and possibly by some enzymes (e.g. tyrosinases; Baker and Orlandi 1995), the validity of the epinephrine assay for the detection of extracellular $\cdot\text{O}_2^-$ production (Misra and Fridovich 1972) was assessed. SOD (from horseradish) was added to 2.0 ml of 1 mM epinephrine (pH 7.0) to give a final concentration of 0.1 $\mu\text{g ml}^{-1}$ before apices were incubated in the assay mixture. The addition of SOD inhibited the oxidation of epinephrine by more than 50% after 15 min of incubation (data not

shown), validating the use of the assay for the detection of extracellular $\bullet\text{O}_2^-$ production in this study. The assay was performed using the UV-Vis spectrophotometer used in the $\bullet\text{O}_2^-$ assay described above.

3.6.2 Estimation of total aqueous antioxidant activity

The total aqueous antioxidants in the shoot apices were assayed immediately after excision, pre-culture, pre-culture + cryoprotection and 1 h after retrieval from LN. This assay was conducted following the method described by Johnstone *et al.* (2006). In the estimation of total antioxidant, the depletion of azino-3-ethylbenzothiazoline-6 sulfonic acid radical is measured using a spectrophotometer (Cary 50 Conc UV Vis spectrophotometer, Varian, Palo Alto, CA). Thereafter antioxidant activity was expressed as trolox equivalents, trolox being a water soluble vitamin E analogue. Prior to assessing the TAA in shoot apices a number of solutions were prepared, such as phosphate buffer saline (PBS; 5 mM Na_2HPO_4 and 37.5 mM mM NaCl, pH 7.4), extraction buffer (50 mM KH_2PO_4 buffer; pH 7.0, containing 1 mM CaCl_2 , 1mM KCl and 1 mM EDTA) and ABTS radical solution (7 mM ABTS and 2.45 mM $\text{K}_2\text{S}_2\text{O}_8$ in 1 ml distilled water and incubate in the dark for 12-16 h before use). The buffers were prepared prior to the extraction and were kept in a constant temperature at 4°C in a cold room; the ABTS solution however was kept in the dark at room temperature.

The fresh weight of three replicates of 10 shoot apices (n=30) each were weighed, prior to extracting the antioxidants. Liquid nitrogen was used to cool the mortar and pestle before homogenising the tissue with 50 mg polyvinylpyrrolidone (PVP) to a fine powder and thereafter the powder was extracted sequentially with 1 ml extraction buffer (0.5 ml at a time). The homogenates were transferred to pre-chilled 2 ml Eppendorf[®] tubes, then immersed in LN to snap-freeze the contents and stored in a -80°C freezer until further processing. When samples were removed from the -80°C freezer, the spectrophotometric

analyses of TAA were done on the same day. The samples were thawed, vortexed for 15 min (5 min intervals) and kept on ice before centrifuging (using a Hermle Labortechnik centrifuge, Germany) at 15000 rpm for 30 min at 4°C and the supernatants were pipetted out into Eppendorf® tubes and placed on ice for TAA estimation. The working reagent containing (50 ml PBS and about 800µl ABTS radical solution) was prepared, and then the spectrophotometer was zeroed with 1 ml PBS. Optimisation of the working reagent to an absorbance of 0.70 to 0.72 at a wavelength of 734 nm was the done and used as the initial absorbance for the assay. Thereafter 1 ml of this working solution was pipetted into a cuvette and the absorbance was read at zero min before the reaction. Immediately after, 100 µl of extract from each sample was added. The reaction was facilitated by placing Parafilm® on the mouth of the cuvette and inverting it three times. The absorbance was read at 30 sec intervals for 2 min.

Standard curve for ABTS: A standard curve for ABTS was prepared for each set of experiments for the calculation of TAA. The extraction buffer was used as a solvent to prepare a 5 mM Trolox® (6- hydroxyl-2.5.7.8-tetramethylchromeane-2-carboxylic acid; SIGMA) solution. From the stock solution, 1 ml of the following standard mM concentrations was prepared: 0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12 and 0.14 mM, using the extraction buffer for dilutions. The assay for Trolox was done in a similar way with that of the ABTS assay, except that instead of 100 µl of the extract in the ABTS working solution, 100 µl of each standard solution was added. Absorbance readings were taken from each of the standard solutions three times (as described above). Calculations for TAA activity were expressed on fresh mass basis.

3.7 Methods to improve shoot production and post-cryo vigour of shoots obtained from cryopreserved shoot apices

3.7.1 Media manipulation studies

a. Type of medium

Excised shoot apices were cultured on five different types of media, the details of which are given below:

- (i) WPM with 0.05 mg L⁻¹ BAP, 0.1 mg L⁻¹ GA₃ and 0.1 mg L⁻¹ IBA (**M1**)
- (ii) MS medium with 1 mg L⁻¹ BAP and 0.1 mg L⁻¹ IBA (**M2**)
- (iii) WPM with 1 mg L⁻¹ BAP and 0.1 mg L⁻¹ IBA (**M3**)
- (iv) MS medium with 1.0 mg L⁻¹ trans-zeatin and 0.1 mg L⁻¹ IAA (**M4**)
- (v) WPM with 1.0 mg L⁻¹ trans-zeatin and 0.1 mg L⁻¹ IAA (**M5**)

The shoot apices (n=30) which were treated with CW or not were cultured on each of the above media with weekly transfers into respective fresh medium. Observations on survival, shoot production, shoot length; number of shoots produced per shoot apex and number of expanded leaves per shoot were recorded at monthly interval up to three months. The best medium was selected based on the survival and shoot production and used as the recovery medium following cryopreservation.

b. Method of cathodic water treatment

The effectiveness of CW was assessed using different methods of treatments such as treating shoot apices (n=30) with CW immediately upon excision, excising shoot apices in CW and treating the shoot apices with 1% ascorbic acid (AsA) made in CW. Immediately after

treatment with CW, the shoot apices were cultured on WPM with 1 mg L⁻¹ BAP and 0.1 mg L⁻¹ IBA which was found to be the best medium based on the previous studies described in 3.7.1a. Similar observations as mentioned in 3.7.1a were taken at monthly interval up to three months.

c. Solid and liquid medium

The shoot apices (n=30) were cultured on solid and liquid MS and WPM medium with the best growth regulator combination as obtained from the experiments as described in section 2.8.1a. When liquid medium was used, the shoot apices were placed over a filter paper bridge inserted into the liquid medium in culture tubes. Observations on survival, shoot production, shoot length, number of shoots per shoot apex and number of expanded leaves per shoot was recorded.

3.7.2 Cryopreservation of shoot apices using PVS3 as the cryoprotectant

Plant vitrification solution 2 (PVS2) is a very concentrated solution, consisting of different components such as DMSO, ethylene glycol, glycerol and sucrose. PVS2 constituents are considered to be toxic, especially DMSO (Benson, 2008) in some cases; therefore PVS3 [sucrose (40% w/v) and glycerol (40% w/v) (Yap *et al.*, 1999; Quain *et al.*, 2009)] was used as an alternative cryoprotectant solution. The shoot apices, following excision, were pre-cultured for 3 d and then were treated with PVS3 in a similar way as described for PVS2 treatment in section 3.5.3. The only difference was that the shoot tips (n=30) were directly treated with 100% PVS3 after the loading step unlike with PVS2 where a sequential treatment was followed. The shoot apices were observed weekly and the number of shoot apices showing visible leaf expansion and organised re-growth after eight to ten weeks of

culture on WPM with 1 mg L⁻¹ BAP and 0.1 mg L⁻¹ IBA was recorded as the shoot production and was expressed as percentage.

3.7.3 Cryopreservation of shoot apices using encapsulation-dehydration technique

The shoot apices were excised and cultured overnight on MS medium with 0.3 M sucrose and 0.5 glycerol (Matsumoto *et al.*, 1995; Varghese *et al.*, 2009). They were then encapsulated in 2 % sodium alginate (Matsumoto *et al.*, 1995) and the encapsulated beads were cultured on MS medium with 0.75 M sucrose overnight (Sakai, 2000; Engelmann *et al.*, 2008). Thereafter, the beads were desiccated under a sterile air flow in a laminar air flow hood at room temperature for various periods (1 h, 2 h, 3 h, 4 h, 5 h and 6 h). They were then transferred to sterile cryotubes and put directly into LN for an hour. The beads were then rewarmed in a 40 °C water bath for 2 min and cultured on WPM with 1 mg L⁻¹ BAP and 0.1 mg L⁻¹ IBA in the dark for 24 h. After 24 h, the beads were transferred onto fresh medium and exposed to light. Survival (n=30) and WC (n=10) of the beads were recorded at each drying time. Following retrieval from cryostorage, survival and shoot production from the shoot apices within the beads (n=30) were recorded after four to eight weeks. Survival was defined as all forms of visible viability (evidence of green structures or callus) while shoot production denoted the formation of fully formed shoots. The removal of the alginate bead after retrieval and before culturing was also done to improve the survival and re-growth after exposure to cryogenic temperatures (Plessis *et al.*, 1991b; Niino *et al.*, 1992; Engelmann *et al.*, 2008).

3.8 Ultra-structural studies

Shoot apices of *T. emetica* (n=30) which were not treated with CW were examined using transmission electron microscopy at different steps of cryopreservation prior to cooling (excision, pre-culture and cryoprotection) and after exposure to the cryogen. Apices were immersed in a solution of 2.5% glutaraldehyde in 0.1 M Sorensen phosphate buffer, pH 7.2, at 4°C for 2 to 24 h. After three washes of five min each with phosphate buffer (0.1 M, pH 7.2), shoot apices were post-fixed in 0.5% osmium tetroxide for 1 h. Then the shoot apices were washed with the same buffer three times for five min and subsequently dehydrated in a graded acetone series (30%, 50%, 75%, 100%), after which they were infiltrated with low viscosity epoxy resin (1:1 mixture of resin: 100% acetone; Spurr, 1969) for 4 h. Polymerisation was done in fresh resin at 70° C for 8 h. Ultrathin sections (100 nm) of shoot apices were cut with an Ultracut EM UC7 microtome (Leica, Austria), using glass knives and picked up with uncoated cooper grids (200 mesh). The grids were then stained with aqueous uranyl acetate and lead citrate (Reynolds, 1963), thereafter viewed with a Hitachi H7000 Jeol JEM 1010 (JOEL, Japan) transmission electron microscope at an accelerating voltage of 95 KV. Images of shoot apices ultrastructure were taken using the iTEM Soft Imaging System.

3.9 Statistical Analysis

Where applicable, data were analysed using the statistical software programme SPSS (SPSS Inc., Chicago, Illinois, USA) Version 21 for Windows. All data where statistical analyses were performed were initially tested for normality and equality of variance using a 1- sample Kolmogorov Smirnov test and a Lavene's test of equality of error variances respectively. Normally distributed data (WC, O_2^- and TAA) were analysed using paired t- test (to compare between CW treated and untreated shoot tips) at 5% significance level and univariate ANOVA (to compare between the stages of cryopreservation for both CW treated and untreated shoot apices. Percentage data for survival and shoot production were arcsine

transformed to make sure that the data conformed to parametric test assumptions and then subjected to ANOVA to test for inter-treatment differences. Multiple comparisons between the means across the different treatments were done using Tukey's post-hoc test at 5% level of significance.

4. RESULTS AND DISCUSSION

4.1 *In vitro* germination of seeds of *Trichilia emetica* and *T. dregeana*

Seeds of *T. emetica* and *T. dregeana* were decontaminated and germinated on half-strength MS medium. Compared with seeds of *T. emetica*, those of *T. dregeana* required more time to germinate. By 14 d after sowing, 50% of *T. emetica* seeds germinated, whereas with *T. dregeana* it took more than thrice the time for the germination of 50% seeds (Table 3). Slow germination of seeds of *T. dregeana* has been reported earlier as well (Eggers, 2007). Even when WPM and water agar were tried as germination medium for *T. dregeana* seeds, germination was slow and sufficient shoot apices could not be obtained for cryopreservation experiments.

Table 3: Comparison of germination rate of *T. emetica* and *T. dregeana* seeds. *T. emetica* seeds were germinated only on half-strength MS medium.

Plant species	Time taken for germination of 50% of seeds (d)		
	MS medium	WPM	WA
<i>Trichilia emetica</i>	14	-	-
<i>Trichilia dregeana</i>	40-60	30-40	30

4.2 Explants for cryopreservation

Size of explants is one of the crucial factors affecting survival after cryopreservation (Benson, 1995). Shoot apices consisting of the meristematic dome and one or two pairs of leaf primordia often serve as ideal explants for cryopreservation (Shibli *et al.*, 1999). Presence of leaf primordia surrounding the meristematic dome was found to be essential for cryo-survival in this study. The ultrastructure of the shoot apices were observed on the tunica layer (cells in the outer most layer) which exhibited well organized organelles, seen in Figs

6A and 6B. Shoot meristems comprise a small number of relatively undifferentiated cells that are suggested to remain genetically stable during the regeneration process (Mroginski *et al.*, 1991), although this latter contention remains to be rigorously tested (Harding, 2004). These explants are composed of a relatively homogeneous population of small cells with the potential for active division; these cells contain few vacuoles and have a high nucleocytoplasmic ratio (Engelmann, 2000) predisposing them to be more tolerant to desiccation than more differentiated cells. For successful cryopreservation of *T. emetica* in a previous study, the ideal size for shoot apices was between 0.5 – 0.8 mm (Varghese *et al.*, 2009). Hence in this study, shoot apices of 0.5 – 0.8 mm were aseptically isolated from the axillary buds of *in vitro* grown shoots and used as explants.

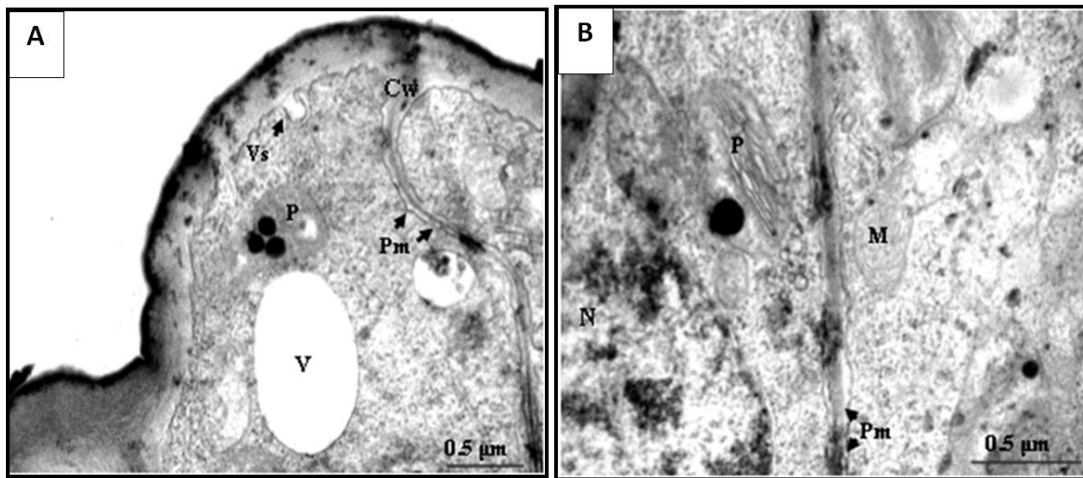


Figure 6 (A and B): Typical meristem cells of a shoot apex showing well organized cells. *P*, plastid; *V*, vacuole; *Cw*, cell wall; *Vs*, vesicle; *Pm*, plasma membrane; *N*, nucleus and *M*, mitochondria.

Unlike *T. emetica*, the sub-cultured shoots of *T. dregeana* produced less prominent axillary buds which made it extremely difficult to obtain shoot apices of the desired size to carry out cryopreservation experiments. Culture of single node or two-node shoot segments have been

reported to activate the axillary buds (Marcotrigiano and McGlew, 1991). Nodal segments of *T. dregeana* were also cultured on WPM with 0.5 mg L⁻¹ BAP to obtain more prominent axillary buds; however, this attempt was also not successful. Due to the difficulties encountered in obtaining sufficient number of shoot apices and problems in excising viable shoot apices from the axillary buds, *T. dregeana* was not further investigated. Experiments described henceforth in this dissertation are limited to *T. emetica*, the results of which are discussed below.

4.3 Water content, survival and shoot production

Water content is one of the most critical factors affecting the success of cryopreservation. Cryopreservation is more often successful if the formation of intracellular ice crystals is avoided, thus preventing the physical damage of the cell membranes (Helliott *et al.*, 2003) or even if formed are not damaging to cell membranes and organelles (Wesley-Smith *et al.*, 2014). Figure 7A illustrates the WC of *T. emetica* shoot apices with cathodic water (hereafter referred to as + CW) and without cathodic water (hereafter referred to as – CW) treatment during the various procedural steps involved in cryopreservation prior to cryogen exposure. The medium and procedure for cryopreservation of shoot apices as described in Varghese *et al.* (2009) was followed; the survival and shoot production using this method are given in Figs 7A and 7B.

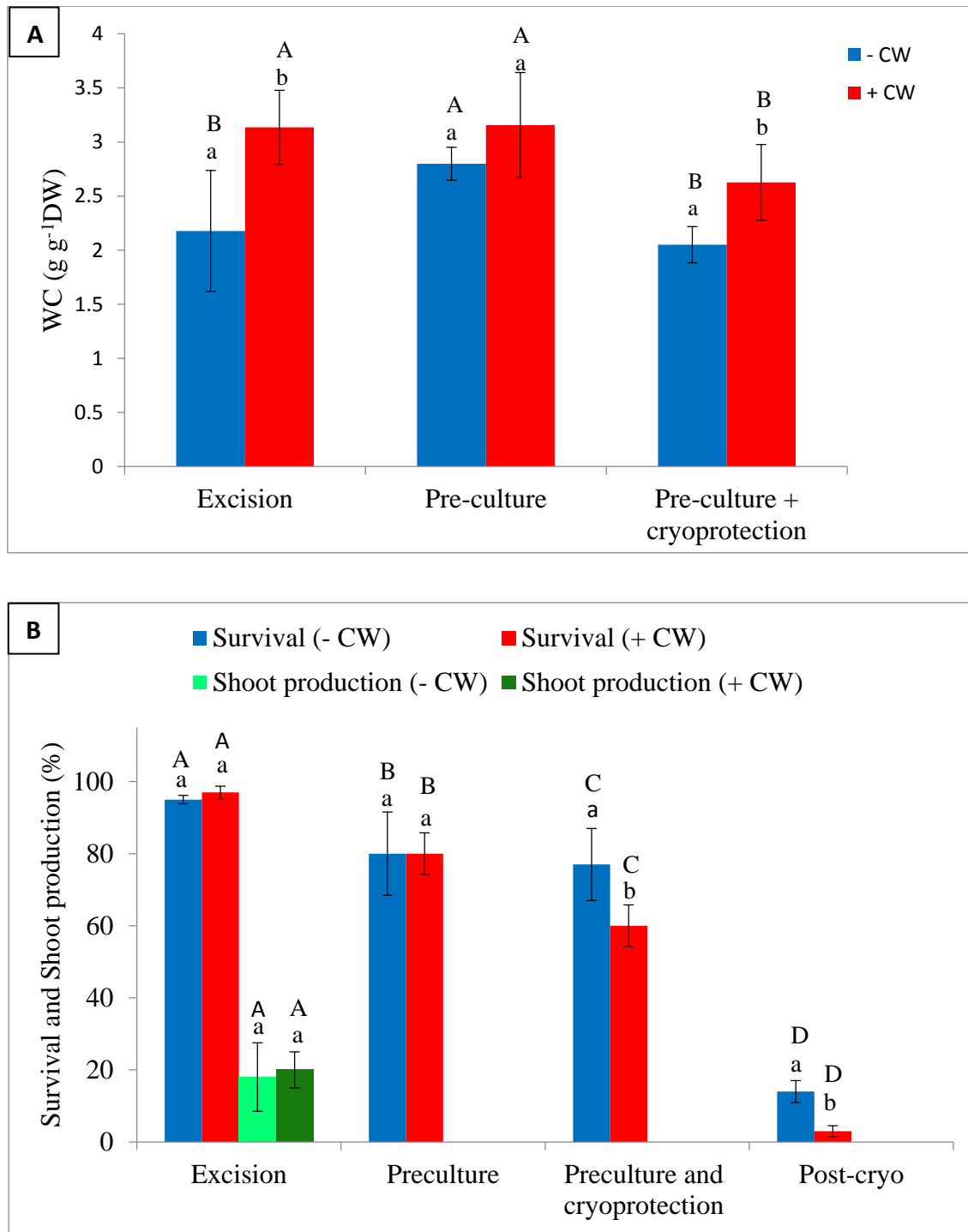


Figure 7: Water content (7A), survival and shoot production (7B) from *T. emetica* shoot apices when treated with and without cathodic water during the different steps of cryopreservation using medium (WPM + 0.05 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA + 0.1 mg L⁻¹ GA₃) and procedure described in Varghese *et al.* (2009). Vertical bars in figures represent mean SD. Columns labelled with different letters are significantly different; $p < 0.05$ [uppercase letters for differences in WC, survival and shoot production across different steps

(ANOVA) and lowercase between WC, survival and shoot production at each step (*t* test) with and without CW].

The initial WC of *T. emetica* shoot apices excised from shoots grown *in vitro* for a month upon excision (– CW treatment) was *ca.* 2.2 g g⁻¹ DW. The initial WC of the shoot apices in this study was relatively higher when compared with that reported by Varghese *et al.* (2009) where the initial WC was only *ca.* 1.53 g g⁻¹ DW. The lower WC of apices in the study reported by those authors might have been caused by the source plantlets being maintained on MS medium devoid of growth regulators for at least three months without sub-culturing before excising the shoot apices (Varghese *et al.*, 2009). In the present study, the shoot apices were excised from the shoots within a month of *in vitro* seed germination. Excising apices from donor plants that were in culture for a longer duration is believed to reduce the WC of the apices, rendering the cytosol more concentrated and amenable to vitrification on cooling (Sant *et al.*, 2006), and is also reported to enhance post-LN recovery (Makowska *et al.*, 1999; Thinh *et al.*, 1999).

Treatment with CW significantly increased the WC of the shoot apices within treatments (Fig. 7A), at excision, pre-culture and cryoprotection stages (excision, $p < 0.001$; pre-culture and cryoprotection, $p < 0.05$). The excised shoot apices when soaked in CW would have imbibed the water causing an increase in the WC unlike the shoot apices that were cultured immediately upon excision without CW treatment. In the shoot apices without CW treatment, there was a significant increase ($p < 0.05$) in the WC following the pre-culture step while the WC of the CW treated apices slightly decreased after the pre-culture stage (Fig. 7A). Regardless of the effect of the pre-culture medium (MS with 0.3 M sucrose and 0.5 M glycerol) on the shoot apices, WC of the treated shoot apices was still high after the pre-culture stage. Sucrose is considered to be non-penetrating and lowers WC by osmotic dehydration (Engelmann *et al.*, 1994; Santos and Stushnoff, 2003; Quain *et al.*, 2009)

whereas glycerol is permeable, penetrative and has dehydrating effects (Gao *et al.*, 1995; Benson, 2008). Even though both cryoprotectants in general are believed to have a dehydrating effect, in this study, it actually increased the WC of shoot apices. This might have been due to the higher concentration of glycerol in the medium which first shrinks the cells for the reason of osmotic efflux of intracellular water, then increasing the volume as glycerol permeates and water re-enters the cells (Meryman and Williams, 1985; Du *et al.*, 1994; Benson, 2008). This particular pre-culture medium was chosen as culturing on this medium has been reported to produce high shoot recovery from Wasabi (*Wasabia japonica*) (Matsumoto *et al.*, 1998) and *T. emetica* (Varghese *et al.*, 2009) shoot meristems on retrieval from cryostorage. Similar pre-culturing medium has been reportedly beneficial for embryonic axes too. For instance, Thammasiri (1999) reported high survivals following cryopreservation of embryonic axes of jackfruit (*Artocarpus heterophyllus*) after the explants were pre-cultured in this medium.

However, the WC of the shoot meristems after cryoprotection and before cooling is most important as it directly affects survival and shoot production following retrieval from cryostorage. The WC of the shoot apices after cryoprotection with PVS2 and before cooling was slightly lower than at excision (the WC decreased to *ca.* 2.05 g g⁻¹ DW without CW treatment and *ca.* 2.63 g g⁻¹ DW with CW treatment). During cryoprotection with PVS2, the shoot apices were exposed to an extremely concentrated solution of 7 to 8 M (Sakai and Engelmann, 2007) which would have partially dehydrated the tissues. However, in this study, exposure to PVS2 did not induce sufficient dehydration as was expected (Fig 7A) and is reported in some other studies.

The survival and shoot production from the apices without (– CW) and with (+ CW) cathodic water treatment is given in Fig. 7B. Although 95-97% of the shoot apices survived following excision without and with CW treatment respectively, only 18-20% shoot formation was

observed (Fig. 7B). Moreover, none of the survived shoot apices produced any shoots after pre-culture, cryoprotection and post-cryo recovery. The preliminary studies on the cryopreservation of the shoot apices were based on the protocol established by Varghese *et al.* (2009). It can be seen that when a similar protocol was followed in this study, although shoot apices survived cryopreservation (Fig. 7B) when cooled slowly by the method described by those authors, they did not produce any shoots when cultured on the recovery medium (WPM + 0.05 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA + 0.1 mg L⁻¹ GA₃) they used. Even when apices were cultured immediately after excision, only 18% shoots production was observed (Fig. 7B). Moreover, the shoots showed poor growth. Hence, various experiments were undertaken with an objective to improve shoot production from the shoot apices. These were performed with the expectation that an appropriate culture medium would increase shoot production following retrieval from LN.

The effects posed by CW treatment, which has antioxidant properties and was used to counteract the over production of ROS as a consequence of excision and dehydration (Berjak *et al.*, 2011) might have also quenched the ROS essential for plantlet development (Gapper and Dolan, 2006). The cell division and cell expansion are two major factors contributing to the growth of a plant. Cell growth is a necessary biological process which is the differentiation of plant cells into mature organs, resulting in plant development (Sugimoto-Shirasu and Roberts, 2003). One role of ROS is to regulate cell growth which subsequently controls plant development (Gapper and Dolan, 2006).

4.4 Methods to improve shoot production from shoot apices

The various methods to improve shoot production from the apices included media manipulations, method of treatment with CW and the use of solid or liquid media the details of which are discussed below.

4.4.1 Type of medium

Different media combinations were considered to obtain the best combination giving the highest survival and shoot production from the shoot apices in the presence and absence of CW. Survival and shoot production from the shoot apices were assessed when cultured on two types of media (MS and WPM) with different types of growth regulators (i.e. BAP, trans-zeatin, IBA, IAA and GA₃). The effects of media on survival and shoot production from shoot apices of *T. emetica* are given in Tables 4 and 5.

Table 4: The effects of media on survival and shoot production from shoot apices of *T. emetica* without CW treatment.

Observation (s)	Treatments *														
	M1			M2			M3			M4			M5		
	Culture period (d)														
	30	60	90	30	60	90	30	60	90	30	60	90	30	60	90
Survival (%)	97	97	95	97	97	97	100	100	100	100	100	97	100	80	53
Shoot formation (%)	58	58	18	52	52	52	70	70	70	27	27	27	53	50	50
Shoot length (mm)	0	13	17	0	16	12	0	9	10	0	6	8	0	8	10
No. of shoots per shoot apex	0	0	0	0	25	32	2	5	14	4	5	5	0	4	4
No. of expanded leaves per shoot	2	5	9	3	14	17	3	18	30	1	3	4	2	5	6

* **M1** - WPM + 0.05 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA + 0.1 mg L⁻¹ GA₃

M2 - MS + 1 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA

M3 -WPM + 1 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA

M4 - MS + 1 mg L⁻¹ trans-zeatin + 0.1 mg L⁻¹ IAA

M5 - WPM + 1 mg L⁻¹ trans-zeatin + 0.1 mg L⁻¹ IAA.

Survival and shoot production were significant among the different types of media ($p < 0.05$), there were no significant difference between treatments (– CW)

Table 5: The effects of media on survival and shoot production from shoot apices of *T. emetica* treated with CW before culturing.

Observation (s)	Treatment (s) *														
	M1			M2			M3			M4			M5		
	Culture period (d)														
	30	60	90	30	60	90	30	60	90	30	60	90	30	60	90
Survival (%)	100	97	97	100	93	93	100	100	100	100	83	83	100	65	61
Shoot formation (%)	25	30	20	13	23	23	100	67	67	0	0	0	10	22	17
Shoot length (mm)	0	3	3	0	11	11	0	8	10	0	0	0	0	8	10
No. of shoots per shoot apex	1	2	1	1	5	6	2	6	6	1	1	1	4	1	4
No. of expanded leaves per shoot	2	2	2	5	23	24	4	22	27	0	0	0	4	9	10

* **M1** - WPM + 0.05 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA + 0.1 mg L⁻¹ GA₃

M2 - MS + 1 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA

M3 -WPM + 1 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA

M4 - MS + 1 mg L⁻¹ trans-zeatin + 0.1 mg L⁻¹ IAA

M5 - WPM + 1 mg L⁻¹ trans-zeatin + 0.1 mg L⁻¹ IAA.

There was difference in survival and shoot production between the types of media ($p < 0.05$), but there were no significant difference between treatments.

The results in Tables 4 and 5 showed significant differences ($p < 0.001$) in the survival of shoot apices after 90 d *in vitro*, across the type of media and survival ranged from 50 – 100% for untreated and 60 – 100% for CW treated shoot apices. However, shoot production is considered to be more important as obtaining a complete plantlet is the ultimate aim of any regeneration or cryopreservation study. Shoot production showed significant differences across the type of media especially after 90 d. Comparison of the different media combinations showed that WPM + 1 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA was the best for shoot

production (Tables 4 and 5. Shoot production in that medium was 67% for untreated (Table 4) and 70% for cathodic water treated shoot apices (see Table 5); the differences however were not significant. While shoot length (10 mm) was the same after 90 d for both treatments (– CW and + CW), number of shoots per shoot apex decreased after CW treatment from 14 (– CW) to 6 (+ CW) and the number of expanded leaves slightly decreased from 30 (– CW) to 27 (+ CW).

Compared with the original medium for culturing and recovery of the shoot apices of *T. emetica* (WPM + 0.05 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA + 0.1 mg L⁻¹ GA₃; Varghese *et al.*, 2009), doubling the concentration of BAP in the medium was found to be better for shoot production. Cytokinins have been reported to decrease the phase of apical dominance and induce axillary and adventitious shoot formation (Madhulatha *et al.*, 2004). Borthakur *et al.* (2011) got the best results (68.33% shoot formation with an average of three to seven shoots per explant and shoot length of 4.47 ± 0.49 cm) when apical buds of *Albizia lebbeck* were cultured on MS basal medium supplemented with 1 mg L⁻¹ BAP. Bhosale *et al.* (2011) also reported an increase in shoot production from the shoot apices of banana, with an enhanced BAP concentration. Considering the best survival, shoot production and associated factors, WPM with 1 mg L⁻¹ BAP and 0.1 mg L⁻¹ IBA was used for culturing and recovery of shoot apices following cryopreservation.

When MS medium was used with the same combination of growth regulators as WPM, excessive amount of callus was produced from the base of the explants. Further, on MS medium, although 97% of the untreated shoot apices survived, the shoot production was only 52%, which was significantly lower than that obtained on WPM with similar growth regulators (Table 4). When shoot apices were treated with CW, there was no significant difference in survival, but there was a significant decrease in shoot production ($p < 0.001$) regardless of the type of medium used.

Table 6: Summative results of survival and shoot production from shoot apices grown in different types of media

Media	Untreated shoot meristems (– CW)			Treated shoot meristems (+ CW)		
	L*	WPM	MS	L*	WPM	MS
Survival (%)	95	100	97	97	100	93
Shoot formation (%)	18	70	52	20	67	23

* L- Medium composition from Varghese *et al.* (2009); WPM + 0.05 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA + 0.1 mg L⁻¹ GA₃.

4.4.2 Treatment with cathodic water

Experiments were also carried out to compare the effects of methods of CW treatment on survival and shoot production from excised shoot apices of *T. emetica*. Significant differences ($p < 0.001$) in survival and shoot production were observed among the shoot apices when treated with CW, excised in CW or treated with AsA made in CW (Table 7).

Table 7: Comparative study of cathodic water treatment methods on survival and shoot production from shoot apices.

Observation (s)	Treatments								
	Shoot apices treated with CW immediately after excision			Shoot apices excised in CW			Shoot apices treated with a solution of CW + 1% AsA immediately after excision		
	Culture period (d)								
	30	60	90	30	60	90	30	60	90
Survival (%)	100	100	100	87	87	80	87	87	67
Shoot formation (%)	100	67	67	27	43	50	0	30	30
Shoot length (mm)	0	0	0	0	0	8	0	6	7
No. of shoots per shoot apex	2	6	6	0	0	2	1	3	3
No. of expanded leaves per shoot	4	22	27	3	3	5	0	11	11

There were significant differences between the treatments ($p < 0.05$)

Treatment of shoot tips with CW immediately after excision gave the highest shoot-production (67%), which was significantly higher ($p < 0.001$) than excising in CW (50%) or treating with AsA made in CW (30%). Although treatment with ascorbic acid made in CW has been reported to promote survival and shoot production from zygotic axes of *T. emetica* (Naidoo, 2012), it did not improve survival or shoot formation from the shoot apices in this study. Excision of the shoot apices in CW would have possibly over quenched the ROS affecting normal metabolism (Berjak *et al.*, 2011; Varghese *et al.*, 2011), signalling, germination and plantlet establishment because the plants were treated prior to the oxidative burst that is associated with excision (Kranter *et al.*, 2010). It is important to maintain a fine balance between the ROS production and its scavenging. In the present study, treating the excised shoot apices in CW immediately up on excision was considered to be the best option for shoot production.

4.4.3 Solid and liquid medium

The shoot tips were cultured on solid and liquid medium (WPM and MS) with 1 mg L⁻¹ BAP and 0.1 mg L⁻¹ IBA to compare its effects on shoot formation and growth (Tables 8 and 9).

Table 8: Growth of excised shoot apices on solid and liquid WPM medium

Days in culture (d)	Survival (%)		Shoot formation (%)		Shoot length (mm)		No. of shoots per shoot apex		No. of expanded leaves per shoot	
	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid
30	100	79.33	70	16.66	0	0	2	0	3	1
60	100	53.33	70	6.66	9.3	2.66	5	-	18	1
90	100*	53.33*	70*	6.66*	9.9	3	14	-	30	2

Table 9: Growth of excised shoot apices on the solid and liquid MS medium

Days in culture (d)	Survival (%)		Shoot formation (%)		Shoot length (mm)		No. of shoots per shoot apex		No. of expanded leaves per shoot	
	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid
30	97	79	52	6.66	0	0	0	0	3	1
60	97	63.33	52	0	16	0	25	0	14	0
90	97*	60*	52*	0*	12	0	32	0	17	0

*There were significant differences between the survival and shoot production ($p < 0.05$) in both WPM and MS.

There were significant differences in the survival and shoot production from the shoot apices cultured on solid and liquid medium ($p < 0.05$). For both survival and shoot formation, solid medium was found to be better than liquid medium. The highest shoot production was observed on solid WPM which was significantly higher than that obtained on solid MS medium (Tables 8 and 9). There was also less or no callus formation on the shoot apices when cultured on solid WPM (data not shown). MS medium has a higher salt concentration (Fadel *et al.*, 2010) compared with WPM which might have overwhelmed the tiny shoot apices leading to a decline in survival and shoot production from the shoot apices of *T. emetica*.

Based on the results of the studies described in section 4.4.1, a medium similar to that described in Varghese *et al.* (2009), but with higher BAP concentration and without GA₃ (WPM + 1 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA) was used as the recovery medium for the rest of the cryopreservation experiments. On this modified medium which 100% of the shoot apices showed survival when cultured immediately after excision (both – CW and + CW) with 70% and 67% shoot production (– CW and + CW respectively).

4.5 Pre-conditioning of explants and cryoprotection

Successful cryopreservation by vitrification lies in the ability of the explants to acquire dehydration tolerance to PVS2, which is the most commonly, used vitrification solution (Sakai and Engelmann, 2007). Vitrification involves the treatment of tissues in a mixture of highly concentrated cryoprotectants (penetrating and non-penetrating), followed by cooling in LN (Sakai and Engelmann, 2007). At low temperatures, these cryoprotective solutions solidify and become a “glassy state” without intracellular crystallisation (Sakai and Engelmann, 2007, Benson, 2008). When tissues are treated with PVS2, it replaces cellular water and changes the behaviour of freezable water in the cells. It increases cellular viscosity and hinders coming together of water molecules (Volk and Walter, 2006) which results in ice

formation. The glassy state is a pre-requisite for the successful cryopreservation of explants. Determination of the WC, time and temperature for glass transition of the explants can improve the chances of successful cryopreservation (Zamecnik *et al.*, 2011). Hence, when cells vitrify, intracellular ice crystallisation is avoided during ultra-rapid freezing by transition of the aqueous solution into an amorphous glassy state of the cells (Zamecnik *et al.*, 2012). Therefore, protection from lethal ice damage and viability post-cryo is assured.

However, treatment with PVS2 has been found to be toxic to many explants, as DMSO which is one of its constituents is reported to be cytotoxic (Matsumoto *et al.*, 1994; Engelmann, 1997b; Benson, 2008; Berjak and Pammenter, 2014). Hence, research has been carried out to minimise its toxicity while using this solution to an advantage. One of the ways to reduce toxicity of PVS2 is to use it sequentially by treating with 50% PVS2 solution followed by 100% PVS2 (Sarkar and Naik, 1998b; Benson *et al.*, 2007) or using it at half concentration for each component (Quain *et al.*, 2012). The ultrastructure of the shoot apices treated with 100% PVS2 (Figs 8C and 8D) was indicative of extensive cellular deterioration while some amount of cellular integrity was retained when PVS2 treatment was done sequentially (Figs 8A and 8B). Details on ultrastructure of the shoot apices during the procedural steps of cryopreservation are given in section 4.9.

The tissue must be sufficiently dehydrated by PVS2, without causing any injury in order to be able to vitrify upon cooling. Tolerance to dehydration induced by the highly concentrated PVS2 solution can be achieved by proper pre-conditioning and loading treatments. Pre-culture on sucrose-enriched medium has been reported to produce high levels of shoot recovery after cryopreservation (Dumet *et al.*, 1993; Engelmann, 1991; Yap *et al.*, 2011). In this study, the shoot apices were pre-cultured on MS medium with 0.3 M sucrose and 0.5 M glycerol. Additionally, the shoot apices were treated with a loading solution consisting of 2M glycerol and 0.4M sucrose. Loading step has been reported to be very effective in inducing

dehydration tolerance of shoot apices to PVS2 (Matsumoto *et al.*, 1994; Sakai *et al.*, 2000) though its mode of action is not very well understood (Sakai, 2000). Although the presence of sucrose and glycerol in the pre-culture and loading steps prior to PVS2 treatment has been reported to be a significant factor in achieving high post-cryo recovery (Turner *et al.*, 2001), in this study, the post-cryo shoot production was low. It is probable that these solutions may be effective under different experimental conditions such as prolonged exposure periods; these aspects were not investigated in this study.

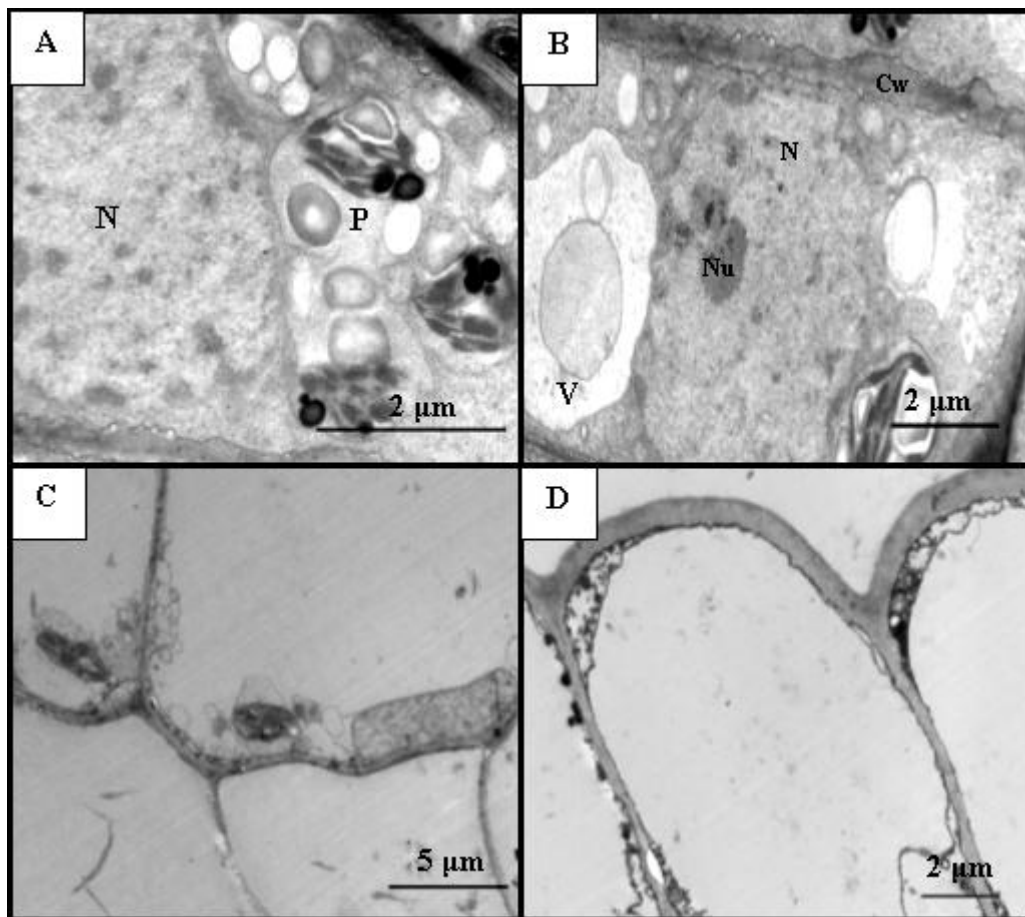


Figure 8: Micrographs showing the effects of sequential cryoprotection with PVS2 (50% followed by 100%; A and B) and 100% PVS2 (C and D) on the shoot apical meristem cells. *P*, plastid; *V*, vacuole; *Cw*, cell wall and *Nu*, nucleolus.

4.6 Cooling rates

For successful cryopreservation, an appropriate balance between tissue WC and cooling rates is crucial (Wesley-Smith *et al.*, 2004a; Wesley-Smith *et al.*, 2014; Wesley-Smith *et al.*, 2015). The rate of cooling affects the location, size and number of ice crystals formed within a biological tissue when exposed to low cryogenic temperatures (Wesley-Smith *et al.*, 2004a; 2014). The pre-cultured and cryoprotected shoot apices of *T. emetica* were subjected to slow cooling (two-step cooling) and a relatively rapid cooling by plunging cryovials with the shoot apices into LN. The slow cooling method cooled the shoot apices at the rate of 1 °C min⁻¹ to a temperature of -40 °C. Thereafter the cryovials were plunged into LN at -196 °C. Slow cooling resulted in 80% of the shoot apices surviving without CW treatment and 67% survived when shoot apices were treated with CW; data not shown) while the relatively rapid cooling had a lower survival of 18% (– CW) and 3% (+ CW; data not shown). The better survival with slow cooling could be because an initial slow rate of cooling might have resulted in extracellular ice crystals that would have caused a flow of water from the cells due to the osmotic imbalance, effectively concentrating the cytoplasm (Wesley-Smith *et al.*, 2004b; Kaczmarczyk *et al.*, 2012) thus enabling vitrification upon cooling. In this study, slow cooling was used in association with PVS2. Dimethyl sulphoxide (DMSO) and glycerol present in PVS2 can penetrate the cell wall and plasma membrane to minimise cellular WC to avoid ice nucleation (Benson, 2008). Extracellular ice formation during freeze-induced dehydration removes free water molecules through osmotic gradient from cytoplasm to intracellular spaces where it crystallizes (Benson, 2004). Due to cell dehydration and increase in solute concentration, cytoplasmic viscosity is enhanced (Leprince *et al.*, 1999) thus becoming too viscous for ice to nucleate during cooling. Slow cooling might also have had better result because of the effective balance between WC and cooling rate (Normah and Makeen, 2008). Rapid cooling at high WC as in this study, would have resulted in the

formation of lethal ice crystals (Dumet and Benson, 2000) thus affecting the survival of the shoot apices following retrieval from LN. Successful cryopreservation of shoot apices using slow cooling has been reported by many others as well (Reinhoud *et al.*, 2000; Varghese *et al.*, 2009). Based on the better survival obtained with slow cooling, only this cooling rate was henceforth used for further studies.

Shoot production from the apices immediately upon excision and following cryopreservation by slow cooling is given in Fig. 9. Comparison of CW water treated and untreated shoot apices showed that the shoot apices without CW treatment showed 68% survival following retrieval from cryostorage and 40% of them produced shoots when cultured on the revised medium (WPM + 1 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA; Fig. 9) whereas only 39% of the CW treated shoot apices survived and only 20% of those produced shoots (Fig. 9). It is important to note that the shoots produced from cryopreserved shoot apices that were CW treated turned brown and died in culture (Fig. 12).

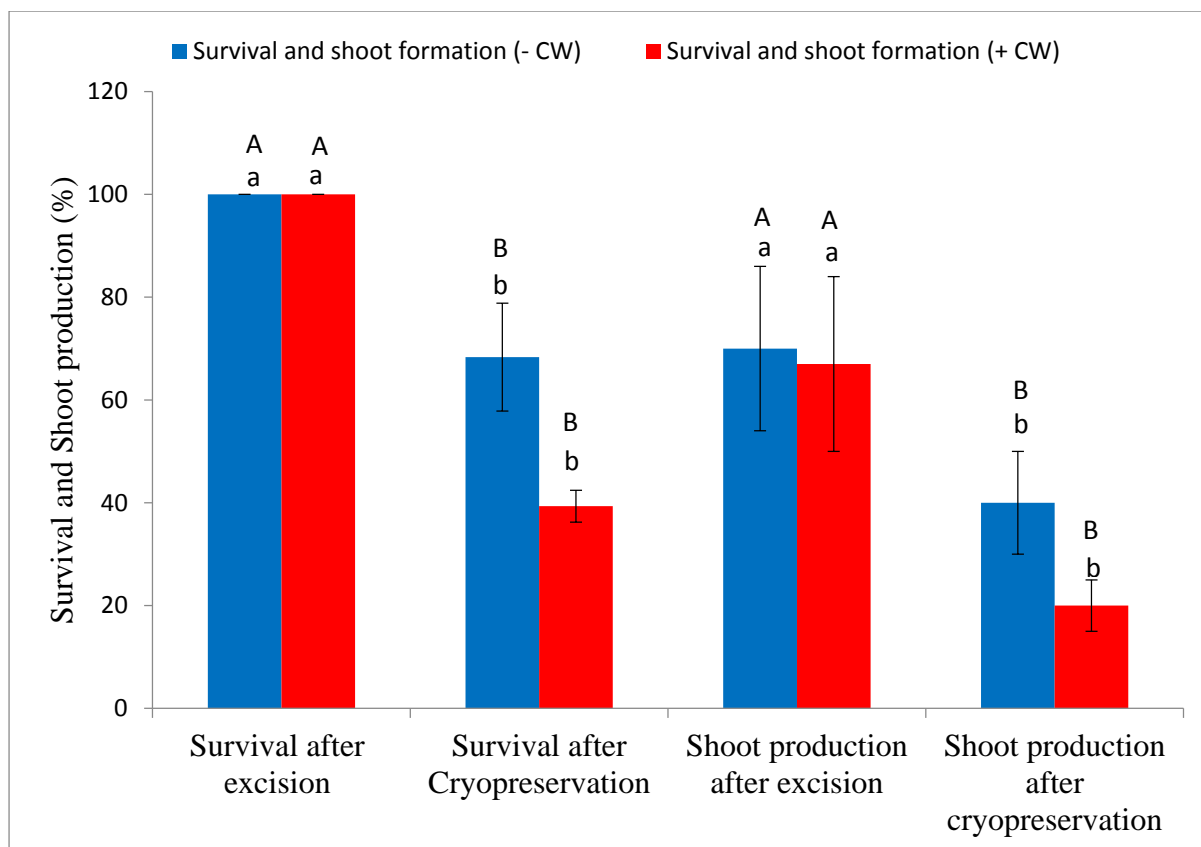


Figure 9: Survival and shoot production from excised shoot apices of *T. emetica* following excision and after cryopreservation. Apices were excised, pre-cultured, cryoprotected with PVS 2; cooled slowly and recovered on WPM with 1 mg L⁻¹ BAP and 0.1 mg L⁻¹ IBA. Letters above the graph represent significant differences in survival and shoot production $p < 0.05$ before and after cryogen exposure.

Cathodic water treatment did not significantly influence either survival or shoot production from the shoot apices before cryopreservation (Fig. 9). It was surprising to note that in spite of the shoot apices treated with CW having quite a high WC after the cryoprotection step, there was only 53% survival prior to LN exposure (Fig. 10). The survival further reduced to 39% after post-cryo recovery (Fig 10). The survival of the untreated apices however remained reasonably high (68%; Fig. 10). The poor survival of the CW treated apices could be a direct consequence of the CW treatment.

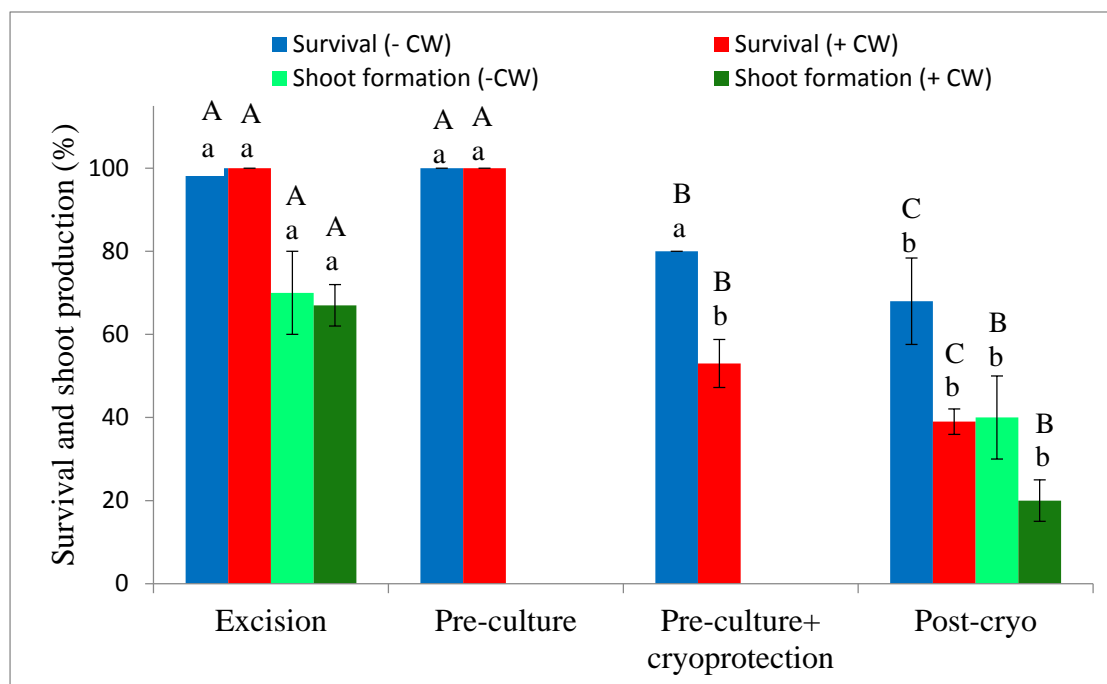


Figure 10: Survival and shoot production of *T. emetica* shoot apices during the steps of cryopreservation. Apices were excised, pre-cultured, cryoprotected with PVS 2; cooled slowly and recovered on WPM with 1 mg L⁻¹ BAP and 0.1 mg L⁻¹ IBA. Letters above the graph represent significant differences in survival and shoot production $p < 0.05$ before and after cryogen exposure.

However, CW treated apices significantly reduced survival ($p < 0.05$) and shoot production (only 20%, $p < 0.05$) after cryopreservation. Irrespective of whether the shoot apices were treated with CW or not, cryopreservation in general, significantly reduced the survival and shoot production (Fig. 9). The poor survival and shoot production observed may be a consequence of the stresses posed by the various procedural steps in cryopreservation as well as the storage in LN (Roach *et al.*, 2008; Berjak *et al.*, 2011). In the current study, 40% of the shoot apices cryopreserved at a WC of *ca.* 2.05 g g⁻¹ DW (without CW treatment) formed shoots while many studies have shown lower WCs of 0.40 - 0.25 g g⁻¹ DW to be appropriate for successful cryostorage e.g. Berjak *et al.* (2011) and Wesley-Smith *et al.* (2001) when

using embryonic axes. Although 68% of the apices survived cryogen exposure, with 40% of those producing shoots (Fig. 9), the development *in vitro* was extremely slow, and root production had not occurred even after 6 months. In a previous investigation, 71% shoot production was obtained when the shoot apices of the same species was cryopreserved (Varghese *et al.*, 2009), but they too failed to establish vigorous plants. In the present study, although similar explants were used, the shoot apices were excised from mother material which was cultured only for one month. This could have resulted in insufficiently developed axillary buds with high WC.

Cathodic water was expected to improve the survival and shoot production following retrieval from cryostorage as it has been reported to have antioxidant properties which minimise the damaging effects of ROS during the steps leading to, and during cryopreservation (Berjak *et al.*, 2011). It has also been reported to promote shoot production of *Strychnos gerrardii* (Berjak *et al.*, 2011). However, the results from this study showed that treatment with CW did not improve survival and shoot production after cryopreservation, regardless of the type of medium used and/or the cooling rate. One of the possible reasons for the poor response of CW treated shoot apices of *T. emetica* to cryopreservation might be the high WC of the explant (*ca.* 2.6 g g⁻¹ DW) prior to cooling which would have resulted in irreversible injury caused by the formation of intracellular ice crystals during cooling, consequently affecting the survival and shoot production. Water content being one of the most influential factors affecting survival and re-growth after cryogen exposure, it must be controlled to avoid ice nucleation (Benson, 2008). Ice formation results in physical rupture of the structure affects osmotic and colligative integrity of cells and causes mechanical injury (Benson, 2008) thus leading to loss of viability and/or cell death.

The type of explant used in this study (shoot apex, a vegetative tissue, compared with zygotic axis) could also have possibly affected the results. It could also be due to the over quenching of ROS, which has been reported to be essential for plantlet development (Gapper and Dolan, 2006). Reactive oxygen species has also been implicated as signal transducers responsible for redox signalling (Halliwell, 2006) and homeostasis needed by the cell for normal metabolism (Chen and Li, 2002; Forman and Torres, 2002; Apel and Hirt, 2004). Additionally, it might be the effect of a high pH of cathodic water (pH between 10 -11) or prolonged time exposure to CW (15 min), which could be too long for a very small explant such as a shoot apex. Though speculative at this juncture, this area needs further investigation as use of cathodic water for plant tissue is a relatively new concept.

4.7 Vitrification using PVS3 as the cryoprotectant

The most commonly used vitrification solution for cryopreservation is PVS2 (Sakai and Engelmann, 2007). PVS2, consisting of DMSO as one of the major components, is regarded as a highly toxic cryoprotectant for various plant species (Engelmann, 1997; Benson, 2008). Unlike PVS2, PVS 3 contains only sucrose (40% w/v) and glycerol (40% w/v) and hence has a less toxic effect on tissues (Yap *et al.*, 1999; Quain *et al.*, 2009). The comparative results on the response of shoot apices to cryopreservation when PVS2 and PVS3 were used as cryoprotectants are given in Table 10.

Table 10: Survival and shoot production following cryopreservation of shoot apices cryoprotected with PVS 2 and PVS 3

Observations	PVS2		PVS3	
	Without CW	With CW	Without CW	With CW
Survival (%)	68	39	90	63
Shoot formation (%)	40	20	0	0

Preliminary results on the comparison of PVS2 and PVS3 showed that survival of the shoot apices cryoprotected with PVS3 following slow cooling was higher. While only 68% (– CW) and 39% (+ CW) of the shoot apices cryoprotected with PVS2 survived cryopreservation, 90% (– CW) and 63% (+ CW) survived cryopreservation when cryoprotected with PVS3. The highest survival was also obtained when the shoot apices were not treated with CW. However, there was no shoot production when PVS3 was used as the cryoprotectants unlike PVS2. The beneficial effect of PVS2 when compared with PVS3 has been reported elsewhere (Halmagyi *et al.*, 2004).

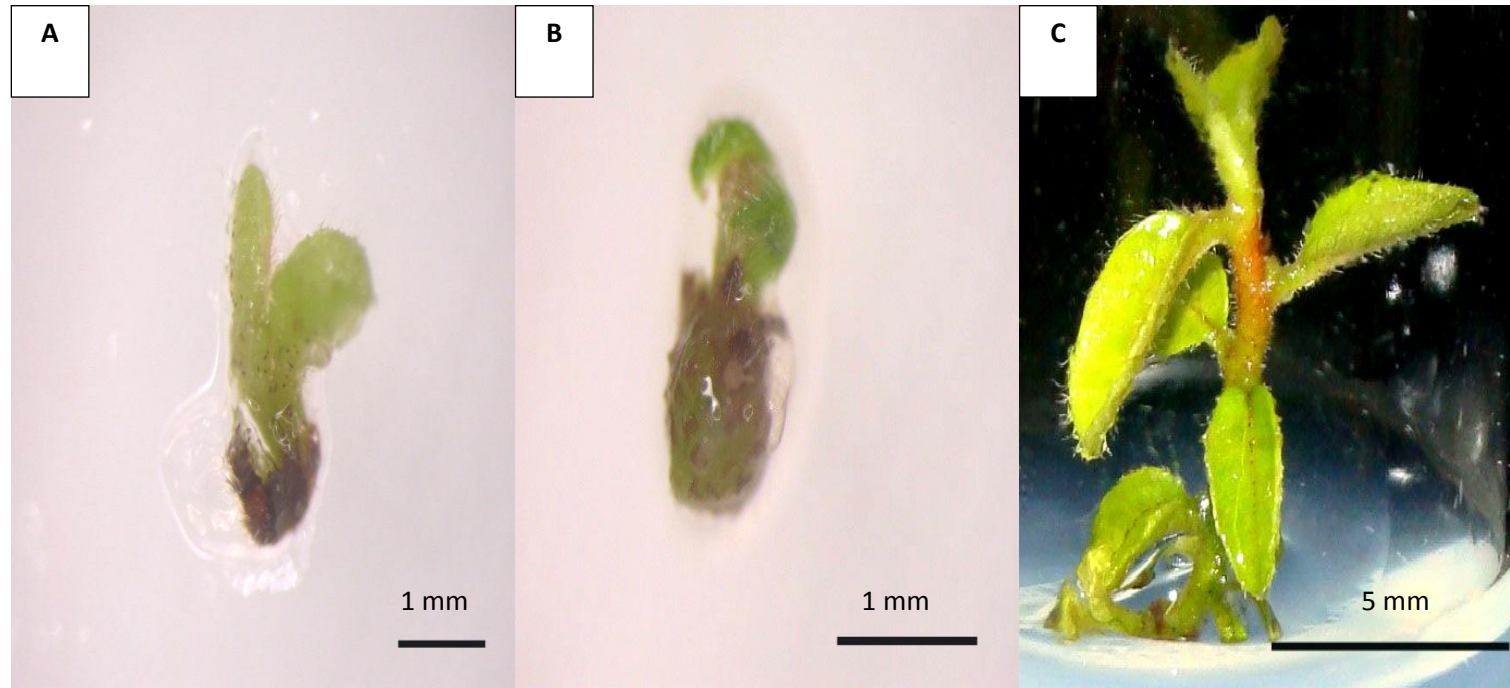


Figure 11: Shoot formation from cryopreserved shoot apices of *T. emetica* (– CW) using slow cooling method, 3 months (A), 4 months (B) and 5 months (C) into recovery.

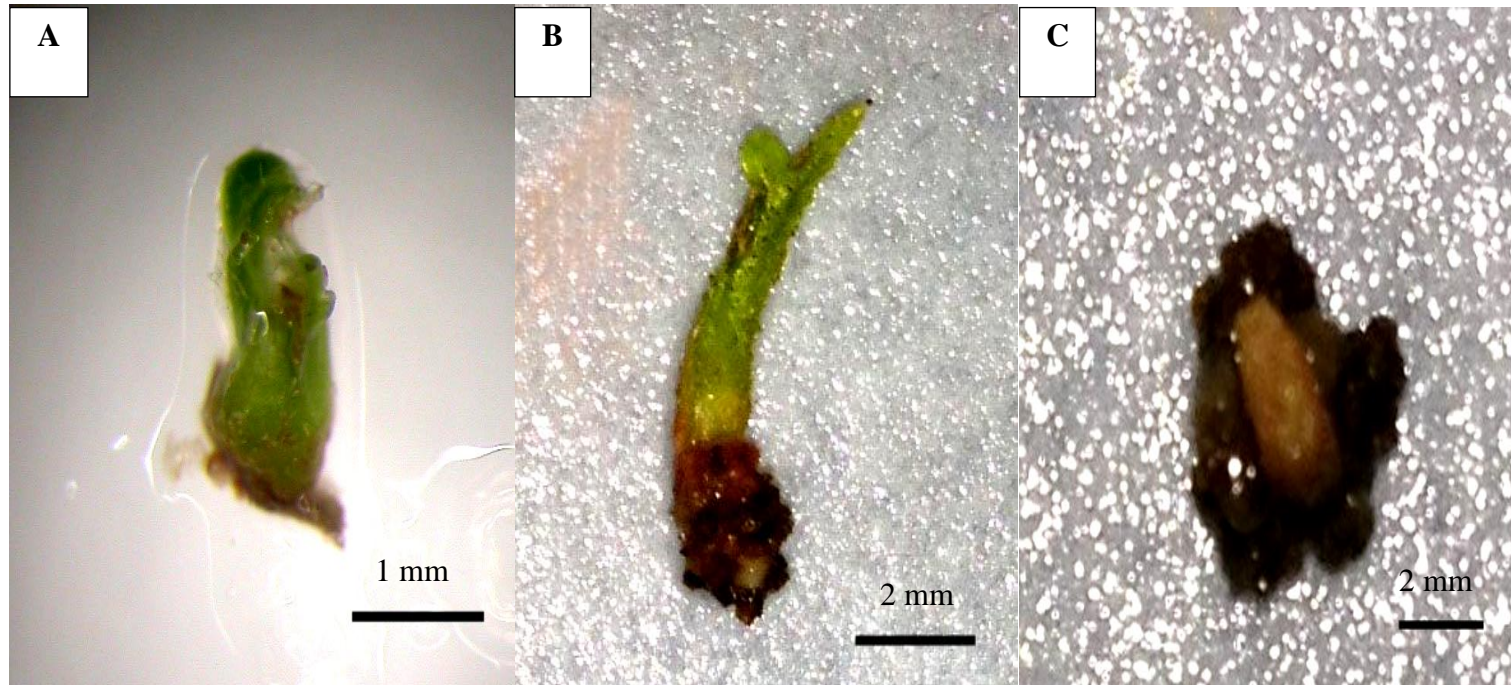


Figure 12: Shoot formation from cathodic water treated and cryopreserved shoot apices of *T. emetica* using slow cooling method, 3 months (A), 4 months (B) and 5 months (C) into recovery.

4.8 Oxidative stress during the procedural steps of cryopreservation

During the procedural steps leading to cryopreservation, and cooling and thawing, plant tissues are susceptible to various stresses, including oxidative stress due to the uncontrolled production of ROS, which can affect post-cryogenic survival (Benson and Bremner, 2004). An effective quenching of the ROS at source or an enhancement of the endogenous antioxidant machinery of the tissue could be probable solutions to this problem (Berjak *et al.*, 2011). In this study, the production of extracellular superoxide and the total aqueous antioxidant activity were assessed to determine the oxidative stress to which the shoot apices were subjected to during the steps leading to cryopreservation.

4.8.1 Production of extracellular superoxide

Figure 13 shows the production of extracellular superoxide during the various steps of cryopreservation. There were significant differences in the production of extracellular $\cdot\text{O}_2^-$ during the various steps of cryopreservation +CW ($p < 0.001$) and – CW ($p < 0.05$) treatments. Excision of the explants and the procedural steps that lead to, and follow cryogenic exposure often result in an over production of ROS (Roach *et al.*, 2008; Whitaker *et al.*, 2010; Berjak *et al.*, 2011 and Sershen *et al.*, 2012b). The data in Fig. 13 showed an enhanced generation of extracellular $\cdot\text{O}_2^-$ during the recovery stage ($p < 0.05$) following cryopreservation irrespective of whether the shoot apices were treated with CW or not. Such an over production of ROS, if not quenched adequately, may have resulted in the poor survival and shoot production from the shoot apices of *T. emetica*. Excessive ROS production has been reported during rapid changes in temperature when the samples are first cryostored and then rewarmed (Benson and Bremner, 2004; Roach *et al.*, 2008 and Sershen *et al.*, 2012b). Many authors have described the consequences of subjecting biological material to low temperatures, resulting in an increase in ROS production (Einset *et al.*, 2007, Berjak *et al.*, 2011; Whitaker *et al.*, 2010 and Sershen *et al.*, 2012b); as seen in Fig. 13.

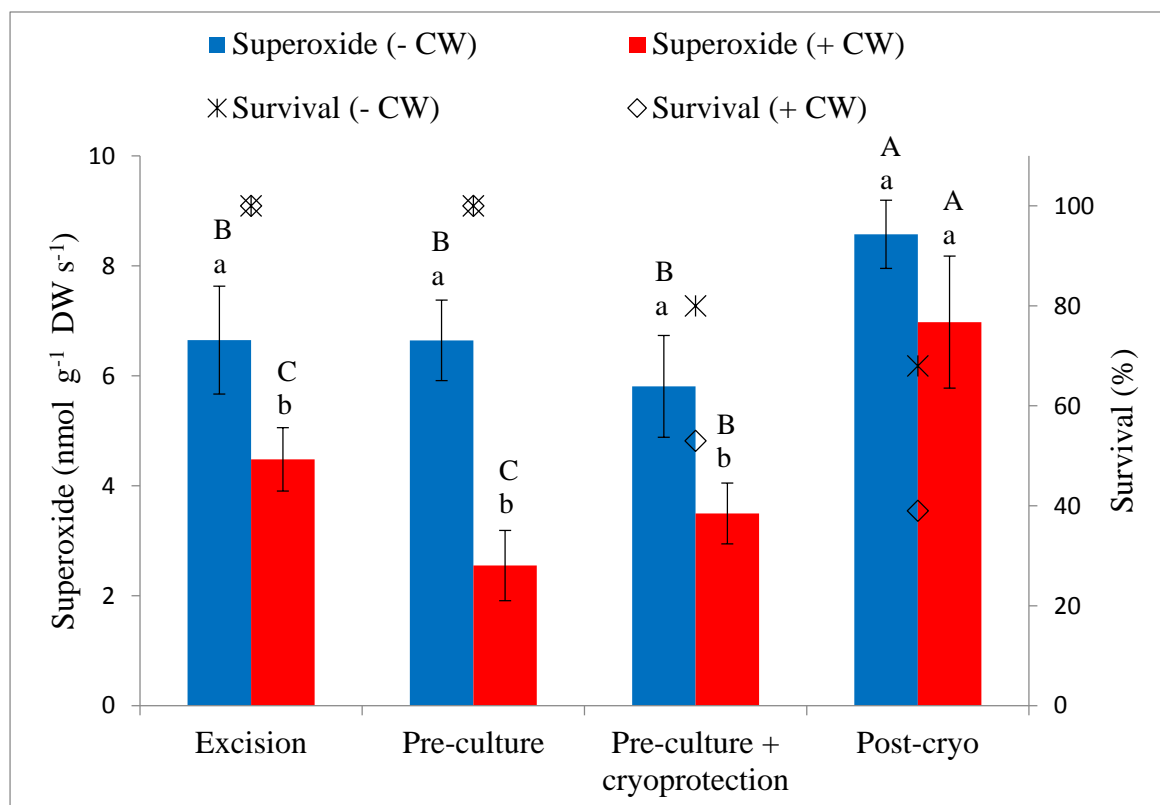


Figure 13: Production of extracellular superoxide in shoot apices of *T. emetica* during the steps of cryopreservation. Bars represent mean \pm SD, columns labelled with different letters are significantly different; $p < 0.05$ (uppercase letters for differences in ' O_2^- ' across different steps and lowercase between ' O_2^- ' at each step with and without CW).

In plants, ROS are produced as by-products of different metabolic pathways in both stressed and unstressed cells in various compartments such as the chloroplasts, mitochondria, plasma membranes, peroxisomes, apoplast, endoplasmic reticulum and cell wall (Apel and Hirt, 2004). The imbalance between the production of ROS and its quenching by antioxidants results in oxidative damage and phytotoxic reactions such as lipid peroxidation, protein degradation and DNA mutation (Ali and Alqurainy, 2006). Wojtaszek (1997) stated that when cellular structures produce superoxide, other types of ROS (hydrogen peroxide and hydroxyl radical) are also formed (Benson and Bremner, 2004), especially when cells

undergo mechanical stress. Besides normal generation of superoxide via cellular metabolic activity, a number of abiotic stress factors also induce its production, such as high light intensity, drought, chilling, high temperature, mechanical stress and injury or wounding of tissue (Apel and Hirt, 2004; Shao *et al.*, 2008; Varghese *et al.*, 2011 and Sharma *et al.*, 2012).

It is essential to strictly control unregulated ROS production as it is said to cause extensive oxidative damage. As ROS accumulates, it overwhelms the endogenous antioxidant capacity of the tissue leading to excessive cellular damage (Berjak *et al.*, 2011). Treatment with CW has been reported to quench the excessive ROS produced (Berjak *et al.*, 2011) which was observed in this study as well. However, the quenching of extracellular $\cdot\text{O}_2^-$ did not improve the survival of the CW treated shoot apices during the cryopreservation stages, especially after cryoprotection. This could be because of the over-quenching of the 'good' ROS that might have been required for survival and shoot formation (Gapper and Dolan, 2006). Although uncontrolled production of ROS has been reported to be harmful, in regulated amounts, ROS have been implicated in cell signalling (Apel and Hirt, 2004; Halliwell, 2006) which is vital for plantlet development. Reactive oxygen species responsible for plant development are said to be produced by NADPH oxidases (NOXs) that generate superoxide radical ($\cdot\text{O}_2^-$) (Gapper and Dolan, 2006). Liskay *et al.* (2004) suggested that NOX derived ROS regulates cell elongation in maize (*Zea mays*) roots while Rodrigues *et al.* (2002) and Sagi *et al.* (2004) state that NOX-derived ROS is not only responsible for root elongation but also for growth of other organs. Rodrigues *et al.* (2002) also reported that a certain concentration of ROS is necessary for leaf elongation. Therefore it is crucial to attain a balance between ROS and antioxidants for growth and development of plantlets post-cryo.

4.8.2 Total aqueous antioxidant activity

Figure 14 shows the total aqueous antioxidant activity in the shoot apices of *T. emetica* at the various stages of cryopreservation.

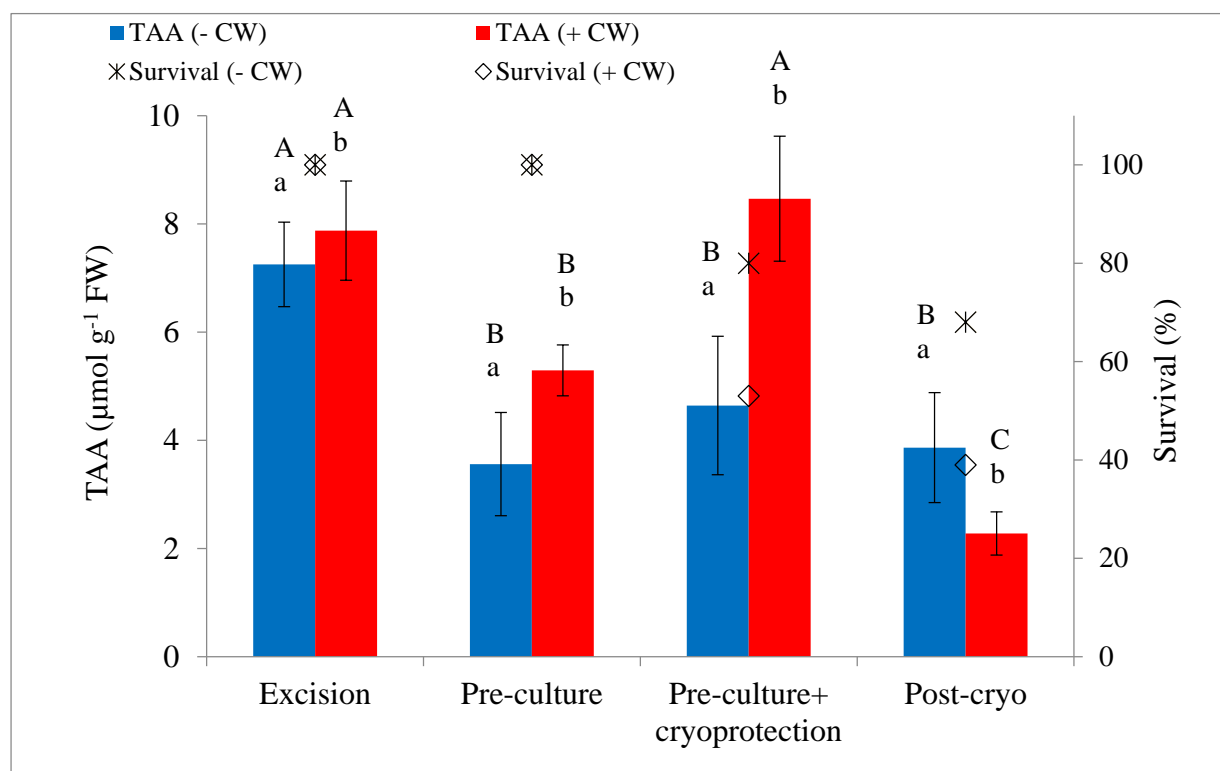


Figure 14: Total aqueous antioxidant activity in shoot apices of *T. emetica* during the stages of cryopreservation. The numbers above columns represent survival after each step of cryopreservation. Vertical bars represent mean \pm SD, columns labelled with different letters are significantly different; $p < 0.05$ (uppercase letters for differences in TAA across different steps and lowercase between TAA at each step with and without CW).

Significant differences ($p < 0.001$) were observed in the TAA activity between the various steps in cryopreservation, the lowest activity was recorded at the recovery stage where apices treated with CW showed reduced TAA activity (Fig. 14). Cathodic water treatment of the shoot apices significantly enhanced ($p < 0.001$) the TAA during the cryoprotection step while

the activity was significantly low ($p < 0.05$) during recovery (Fig. 14). Despite the increase in TAA, the survival of the treated shoot apices following cryoprotection was lower (53%) than those shoot tips which were not treated with CW (80%; Fig. 10). According to Ali and Alqurainy (2006), an increase in the endogenous antioxidant capacity does not ascertain a higher degree of protection during elevated stress conditions.

Production of ROS is triggered by different types of stresses that a plant undergoes; it is prevented or minimized by an antioxidant system of low molecular weight antioxidants, antioxidant enzymes and ROS-interacting enzymes (Ali and Alqurainy, 2006). Antioxidants are one of the most important defence mechanisms a plant poses, which is induced during late embryogenesis. This kind of protection is stimulated when biological tissues are stressed and it is generally produced from molecular antioxidants, carbohydrates and proteins (Walters *et al.*, 2008). Excessive production of ROS can devastate the defence mechanism of a plant which necessitates the use of alternative methods to enhance the endogenous TAA such as applying exogenous antioxidants (Berjak *et al.*, 2011). In this study, CW served as an exogenous antioxidant. Shoot apices treated with the highly reducing CW resulted in an increase in the endogenous TAA compared with the untreated shoot apices during the steps prior to cryopreservation (Fig. 14). However, the antioxidant activity showed a rapid decrease during recovery especially in the CW treated shoot apices which might have led to the poor survival and shoot production from these shoot apices. This impairment in TAA during cooling and thawing maybe critical in the ability of explants to overcome the oxidative stress, thereby probably affecting survival and shoot production after retrieval from LN.

When the antioxidant capacity of the plant tissues is inadequate to quench the excessive ROS produced, there is consequent oxidative stress (Scandalios, 1993). A comparison of the production of extracellular O_2^- and TAA in the shoot apices of *T. emetica* showed an increase

in the extracellular O_2^- production during the recovery stage, whilst TAA significantly decreased (Figs 13 and 14), more so in the shoot apices treated with CW. The consequent oxidative stress due to the inability of the antioxidant machinery to quench the excessive extracellular O_2^- would have resulted in the poor survival and shoot production from these shoot apices (39% survival and 20% shoot production from the CW treated shoot apices).

4.9 The effects of excision, cryoprotection (PVS2) and subsequent cryopreservation on the ultrastructure of *T. emetica* shoot apices

Shoot apices not treated with cathodic water (see section, 4.6) exhibited 68% survival (assessed as greening) when cryopreserved at a WC of *ca.* 2.05 g g^{-1} ; however, just 40% of these subsequently produced shoots (Fig. 9). Though some of the shoot apices remained green for several months, they either eventually formed callus or turned necrotic. Visible plantlets were obtained after 8 weeks *in vitro* but their development was extremely slow, with root production taking longer than 6 months to occur.

Work on zygotic explants from recalcitrant seeds has suggested that cooling can have severe metabolic and physiological consequences on subsequent growth (Berjak *et al.*, 1999). Mycock (1999) also suggested that biochemical imbalances and perturbation of the sub-cellular matrix (which includes the cytoskeleton) during pre-freezing manipulations, such as partial dehydration, may be responsible for the abnormal post-cryo growth often observed in zygotic germplasm of recalcitrant seeds (e.g. Pence, 1992; Steinmacher *et al.*, 2007). Ultrastructural and other microscopical studies have been particularly valuable in providing evidence for the explanation of poor/ abnormal growth of plant material retrieved from cryostorage (e.g. Sershen *et al.*, 2012a). In order to better understand the possible basis of the poor/ abnormal growth of shoot apices following cryopreservation in this study Transmission

Electron Microscopy was used to investigate the ultrastructure of meristematic cells from *T. emetica* shoot tips after excision (untreated), pre-culture, cryoprotection (with PVS2) and after retrieval from LN.

Following excision, freshly isolated shoot apices displayed 100% survival and exhibited ultrastructural features characteristic of normal actively dividing meristematic cells (as described by Kaczmarczyk *et al.*, 2008 and Yap *et al.*, 2011): well-defined undamaged cell walls, intact plasma membranes, a dense cytoplasm filled with many cell organelles such as the nucleus, mitochondria, plastids, vacuoles and vesicles (Fig. 15 A-C). The large nuclei seen in many of these untreated cells (Fig. 15A) is recognized as a feature of species with a small genome size (Nagl and Fussenig, 1979; Helliot *et al.*, 2003), and in the case of *T. emetica* was mainly euchromatic with heterochromatic regions limited to the nuclear periphery (Fig. 15A).

After 3 days of pre-culture on MS medium (supplemented with 0.3 M sucrose and 0.5 M glycerol) and cryoprotection in PVS2 (50% PVS2 for 5 min and 100% PVS2 for 15 min) for 20 min, survival of shoot tips decreased to 80%. This decline in survival relative to fresh samples was accompanied by a number of ultrastructural abnormalities (Fig. 16 A-C): a number of meristematic cells showed the development of numerous small vesicles along the cell membrane, abnormalities in the structure of the nuclear envelope and derangement of plastids. Similarly, Helliot *et al.* (2003) reported a number of ultrastructural changes in banana shoot meristems following pre-culture for 2 weeks on MS medium supplemented with 0.4 M sucrose. Changes induced in banana shoot meristems included, fragmentation of large vacuoles into smaller ones, differentiation of proplastids into amyloplasts containing starch, abnormalities in the structure of the nuclear envelope, partial plasmolysis and the swelling of other organelles such as the endoplasmic reticulum. Kaczmarczyk *et al.* (2008) also reported the appearance of small vesicles in the cytoplasm, swelling of mitochondria and chloroplasts

and a change in shape of the large central vacuole when potato shoot tips were cryoprotected with 10% DMSO in MS with hormones (MSH). The derangement of plastid ultrastructure following pre-culture and cryoprotection (Fig. 16B) was a particularly important observation since plastids are one of the most important organelles in plant cells (Pyke, 1999). This importance is based largely on their role in synthesising chlorophyll and storing starch but a number of metabolic pathways also take place in plastids, such as lipid biosynthesis and amino acid metabolism (Galili, 1995; Ohlrogge and Browse, 1995). Dividing meristematic cells consist of undifferentiated plastids called proplastids which differentiate as the cell differentiates, producing other types of plastids based on the type of cell they inhabit.

Ultrastructural damage inflicted during preconditioning steps such as cryoprotection is generally exacerbated during subsequent cooling and rewarming (Sershen *et al.*, 2012a). Given the ultrastructural abnormalities induced during pre-culture and cryoprotection in *T. emetica* shoot apex meristematic cells observed here (Fig. 15 A-C), it was therefore not surprising that only 40% of the 68% of shoot apices that survived cryopreservation produced shoots. An investigation of the ultrastructure of meristematic cells in shoot tips recovered from LN revealed small areas of surviving cells (Fig. 16A) within the shoot meristems or in the leaf primordial tissues surrounded by dead cells that exhibited little to no ultrastructural integrity (Fig. 16B). This mixture of surviving, dead and dying cells in material recovered from cryostorage is a common observation and may explain the poor/ abnormal growth that is often observed during *in vitro* recovery of explants of various types (Kaczmarczyk *et al.*, 2008; Sershen *et al.*, 2012a). Importantly, the plastids in a number of the surviving *T. emetica* cells appeared to be even more deranged (Fig. 16C) than in material that was preconditioned, but not exposed to LN (Fig. 15 B). This suggests that damage incurred during the preconditioning steps used in this study may have predisposed *T. emetica* shoot tips to even more damage and associated viability loss during cooling and subsequent rewarming.

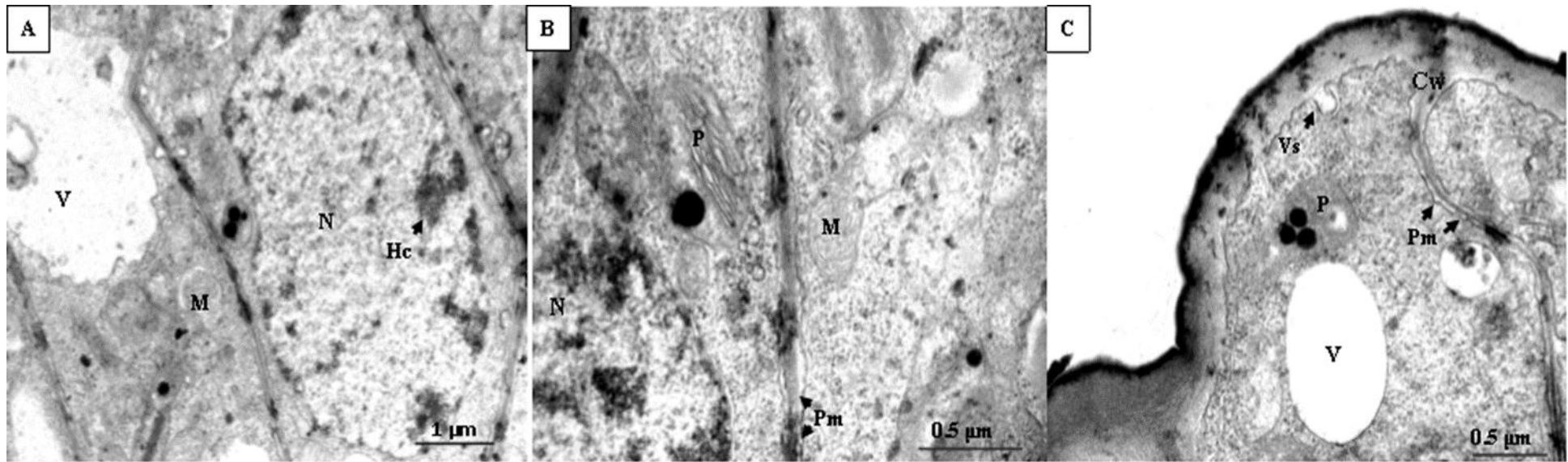


Figure 15 A-C: TEM micrographs showing ultrastructure of freshly (control) excised shoot meristematic cells of *T. emetica*. A-C Control meristematic cells: A over view of the whole cell, B and C show different organelles such as mitochondria, plastids and nucleus. *P*, plastid; *V*, vacuole; *Cw*, cell wall; *Pm*, plasma membrane; *NE*, nuclear envelop; *N*, nucleus; *M*, mitochondria; *Vs*, vesicles; *Nu*, nucleolus; *Hc* Heterochromatin.

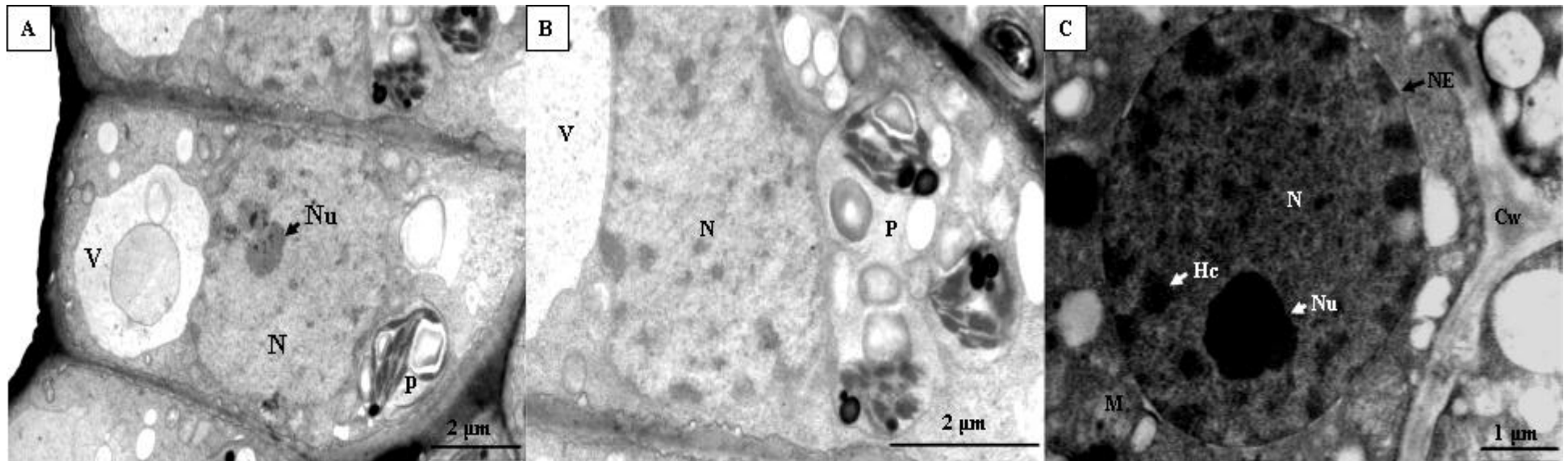


Figure 16 A-C: Micrographs of pre-cultured (0.3 M sucrose and 0.5 M glycerol) and cryoprotected (PVS2) shoot apices meristematic cells of *T. emetica*. An entire meristematic cell of a tunica layer, B plastid derangement following PVS2 treatment, C alteration of nuclear envelop. *P*, plastid; *V*, vacuole; *Cw*, cell wall; *Pm*, plasma membrane; *NE*, nuclear envelop; *N*, nucleus; *M*, mitochondria; *Vs*, vesicles; *Nu*, nucleolus; *Hc*, Heterochromatin.

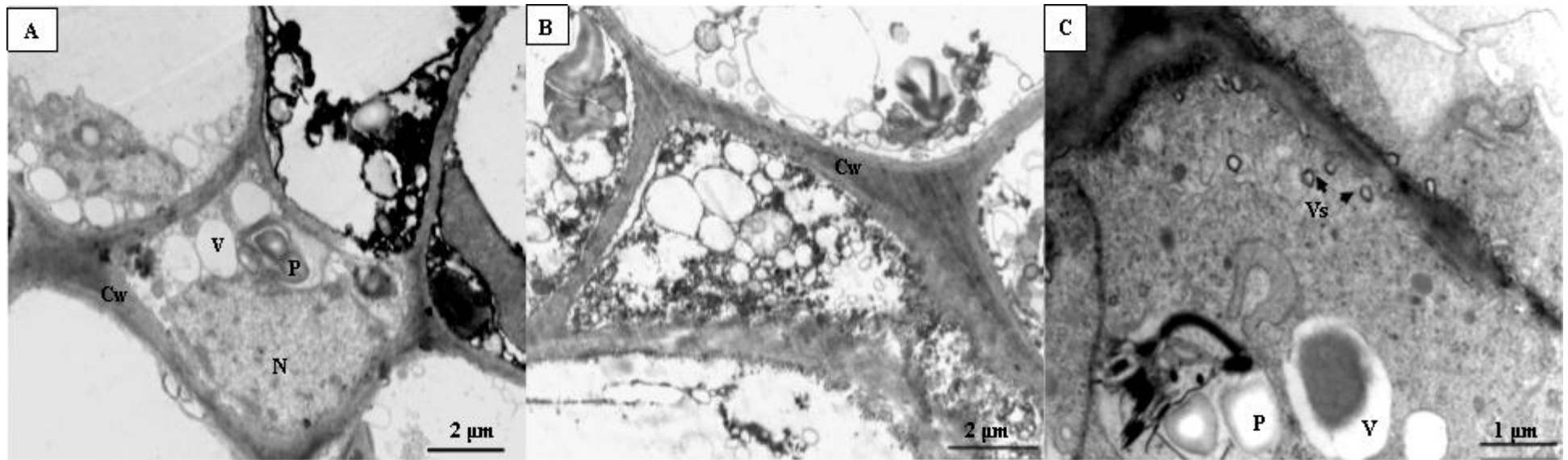


Figure 17 A-C: Ultrastructure of meristematic cells of *T. emetica* shoot apices after cryo-exposure. A showing small areas of surviving nearby cells after cryoprotection and also cryopreservation, B Total destruction of apical meristem cells after cryo-exposure. C further derangement of plastids and appearance of many small vesicles. *P*, plastid; *V*, vacuole; *Cw*, cell wall; *Pm*, plasma membrane; *NE*, nuclear envelop; *Vs*, vesicles; *Nu*, nucleolus; *Hc*, Heterochromatin.

4.10 Encapsulation-dehydration as a method of cryopreservation

The shoot apices were encapsulated in an alginate bead and subjected to slow drying in a laminar air flow chamber. Encapsulation dehydration is one of the improved cryopreservation techniques used to obtain better survival and re-growth after cryogen exposure (Engelmann, 1991; Rao, 2004). Encapsulation protects the plant material from extreme treatments prior to cryopreservation, which might be lethal to non-encapsulated samples (Engelmann, 1991; Cho *et al.*, 2002; Engelmann *et al.*, 2008).

4.10.1 Dehydration of the encapsulated shoot apices

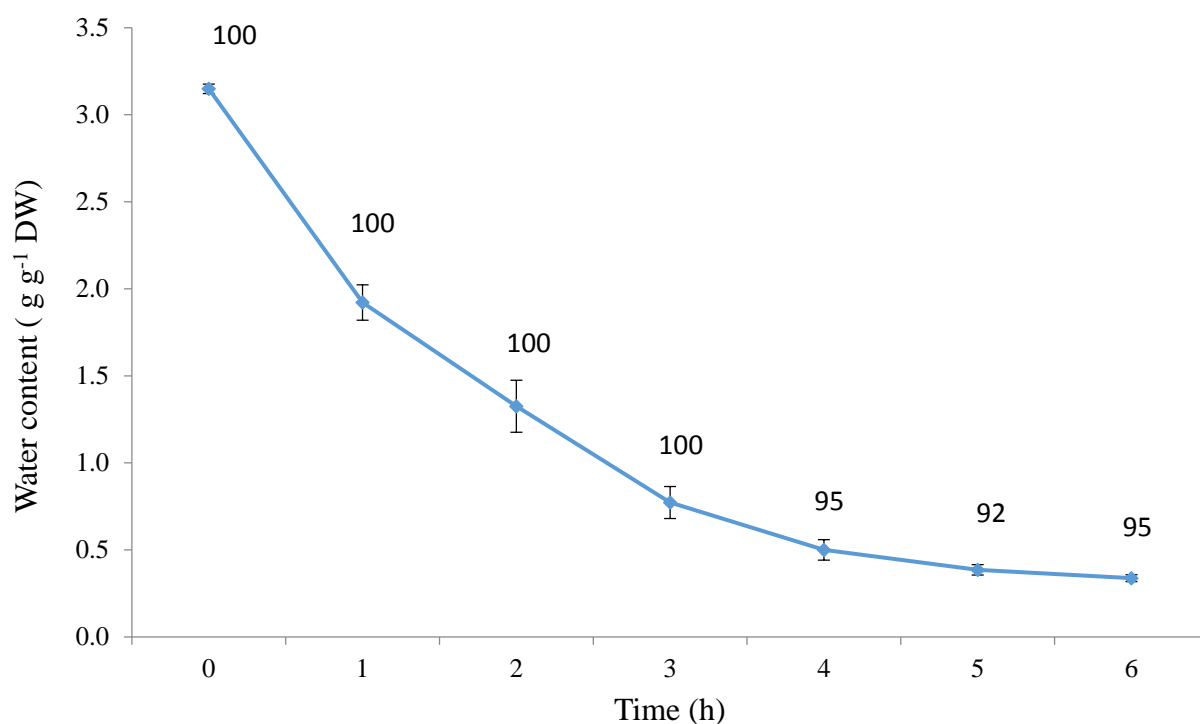


Figure 18: Change in water content of encapsulated shoot apices of *T. emetica* up on slow drying in a laminar air flow chamber. The numbers above the points represents survival percentage after dehydration in the laminar flow at different time intervals. Bars represent mean WC \pm SD.

The initial WC of encapsulated shoot apices of *T. emetica* was 3.1 g g⁻¹ DW (Fig. 18). These encapsulated shoot apices were dried slowly and the changes in WC over a 6 h period is given in Figure 18. The beads could be dried down to 0.3 g g⁻¹ DW after a period of 6 h. After 5 h of dehydration, the WC of the beads was 0.4 g g⁻¹ DW which was considered to be in the range of the required WC for cryopreservation of many recalcitrant seeded species (Sershen *et al.*, 2007). At that WC, 92% of the shoot apices showed survival (Table 11).

4.10.2 Cryopreservation

Data in Table 11 shows the survival and shoot production from the encapsulated shoot apices of *T. emetica*. The encapsulated shoot apices were dehydrated and plunged into LN within cryotubes.

Table 11: Survival of encapsulated shoot apices of *T. emetica* after cryopreservation

Treatment	Time (h)	Survival before cryopreservation (%)	Survival after cryopreservation (%)
ED	4 h	95	90
ED	5 h	92	90
ED	6 h	95	90

ED = encapsulation-dehydration

Cryopreservation following encapsulation-dehydration of the shoot apices resulted in 90% survival (Table 11) at WCs ranging from 0.3 – 0.5 g g⁻¹ DW. However, the shoot apices failed to produce any shoots although it has been reported that this technique results in high survival and direct re-growth in many species (Engelmann, 2000). In order to improve survival and re-growth, the beads were given a slight cut to expose the shoot apices before being cultured (Engelmann, 2008), but they turned brown after 3 days on the recovery medium. In some cases it was also found necessary to extract the explants from the beads

before placing them on recovery medium to improve recovery, as in the case of apices of grape (Plessis *et al.*, 1991b) and mulberry (Niino *et al.*, 1992). However, in this study, that method also failed to produce any shoots.

5. CONCLUSION

This study explored various factors leading to successful cryopreservation of shoot apical meristems of *T. emetica*, a recalcitrant seeded tree species. Although 95% of the shoot apices survived before cryopreservation, only 18% of these produced shoots when recovery medium (WPM + 0.05 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA + 0.1 mg L⁻¹ GA₃) from a previous investigation by Varghese *et al.* (2009) was used. After cryopreservation, 90% of the shoot apices survived on the same medium, but the shoot apices failed to produce shoots even after 6 months in culture. Hence, based on extensive studies on the type of medium to be used, a similar medium but with higher BAP concentration and without GA₃ (WPM + 1 mg L⁻¹ BAP + 0.1 mg L⁻¹ IBA) was used for recovery of the cryopreserved shoot apices. On this recovery medium, the shoot apices showed 68% survival following retrieval from cryostorage and 40% of these produced shoots (Fig. 9).

Although the cryopreserved shoot apices produced shoots upon recovery, shoot production was poor. Moreover, the development *in vitro* was extremely slow, and root production had not occurred after 6 months. High WC of the explants, presumed to be the consequence of insufficiently developed axillary buds, was the most likely cause of poor shoot production after cryopreservation. The WC of the freshly excised shoot apices from the younger shoots presently used was considerably higher than described by Varghese *et al.*, 2009 (2.2 g g⁻¹ as opposed to 1.53 g g⁻¹), where the donor shoots were in culture for a longer duration. The use of sufficiently developed shoot apices before excision has been shown to be important in yielding better plantlet recovery after cryopreservation. This is due to the fact that sufficiently matured explants ensure desiccation and cooling tolerance (Normah and Makeen, 2008). The treatment with CW further increased the WC leading to poor survival of the CW treated shoot apices upon cryo-recovery. Furthermore, exposure to PVS2 did not bring about the expected dehydration, thus compromising the chances of vitrification of the cell contents upon cooling.

Water content is not the only factor that might have contributed to poor development or shoot production after cryogen exposure. Excessive production of ROS which are produced by plant tissues during stressful conditions have been shown to have negative effect i.e. resulting in oxidative damage. Fig. 13 showed that there was an increase in $\cdot\text{O}_2^-$ generation after retrieval, thus contributing to poor re-growth and shoot production after cryopreservation. Varghese *et al.* (2011) and Serksen *et al.* (2012b) have also shown that oxidative stress throughout the steps of cryopreservation contributed to the loss of viability of recalcitrant zygotic embryos/ axes.

The use of PVS2 in the procedural steps of cryopreservation, i.e. cryoprotection, had the most damaging effect on shoot production because of its reported toxic effect (Matsumoto *et al.*, 1994; Engelmann, 1997; Benson, 2008; Berjak and Pammenter, 2014). The most striking effect of PVS2 at the ultrastructural level in shoot meristem cells was derangement of plastids both before (Fig. 16B) and after cryogen exposure (Fig. 17A and C). Following cryo-recovery, shoot apical meristem cells were extensively deteriorated – indicating non-survival (Fig. 17B), although there were small regions of surviving cells (Fig. 17A). The ultrastructural effects of PVS2 (originally developed for explants of temperate species) provide a clue to repeated failure to cryopreserve embryonic axes of many tropical recalcitrant-seeded species after treatment with plant vitrification solutions. Although other cryopreservation methods were attempted, i.e. encapsulation-dehydration, encapsulation-vitrification and the use of PVS3 instead of PVS2, there was no success in attaining proper plantlets after cryopreservation.

Shoot apices excised from bulked-up material derived from seedlings ensure the same genetic diversity as do the seeds from which they develop. Even though shoot apices shows the ideal characteristics (compact cells with a large nuclear: cytoplasmic ratio) of alternative explants

for cryopreservation (Engelmann, 2011) their use should be explored further. This study suggests that the shoot apices used were not sufficiently developed, and with the commensurately high WC, proved to be unsuitable explants for germplasm conservation of *T. emetica*. Maintaining mother material in culture for longer durations before explant excision and optimising the exposure duration to loading solution and concentration of sucrose in the loading solution might however, provide sufficient dehydration tolerance to PVS2 leading to successful vitrification upon cooling. Ultimately, in pursuit to establish a successful cryopreservation protocol for germplasm conservation of recalcitrant-seeded species, other techniques such as *in vitro* slow growth and *ex situ* conservation including establishment of field gene banks to secure the germplasm should also be practiced.

6. REFERENCES

- Abbas, H. and Qaiser, M.** 2011. *Ruellia aribacteolata*: conservation assessment and strategies to avoid extirpation. *Pakistan Journal of Botany*, 43: 2351-2357
- Ali, A. and Alqurainy, F.** 2006. Activities of antioxidants in plants under environmental stress. In: Matohashi, N. (ed) *The Lutein Prevention and Treatment for Diseases*. Transworld Research Network. India, pp. 187-256.
- Apel, K. and Hirt, H.** 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology*, 55: 373-399.
- Ashmore, S.E.** 1997. Status report on the development and application of *in vitro* techniques for the conservation of plant genetic resources, International Plant Genetic Resources Institute, Rome, Italy.
- Baeckmann, W.V.** 1997. Handbook of Cathodic Corrosion Protection. (3rd ed) Gulf Publications, Texas, USA.
- Ballesteros, D., Sershen, Varghese, B., Berjak, P. and Pammenter, N.W.** 2014. Uneven drying of zygotic embryos and embryonic axes of recalcitrant seeds: challenges and considerations for cryopreservation. *Cryobiology*, 69: 100-109.
- Bajaj, Y.P.S.** 1983. Cryopreservation and international exchange of germplasm. In: Sen, S.K. and Giles, K.L. (eds) *Plant Cell Culture in Crop Improvement*. Plenum Press, New York, USA, pp. 19-41.
- Baker, C.J. and Orlandi, E.W.** 1995. Active oxygen in plant pathogenesis. *Annual Review of Phytopathology*, 33: 299–321.
- Benson, E.E.** 1995. Cryopreservation of shoot-tips and meristems. In: McClenna, M. and Day, J.D. (eds) *Cryopreservation and Freeze Drying Protocols*. Humana press, 38: 121-132.

- Benson, E.E.** 2004. Cryopreservation of algal and plant diversity: Historical perspectives and future challenges. In: Fuller, B., Lane, N. and Benson E.E. (eds) *Life in the Frozen State*. CRC Press, London, pp. 299-328.
- Benson, E.E.** 2008. Cryopreservation theory. In: Reed, B.M. (ed) *Plant cryopreservation: A practical guide*. Springer, Netherlands, 2: 15-31.
- Benson, E.E. and Bremner, D.** 2004. Oxidative stress in the frozen plant: a free radical point of view. In: Fuller, B.J., Lane, N. and Benson, E.E. (eds) *Life in the Frozen State*. Boca Raton, CRC Press, pp. 205-241.
- Benson, E.E., Harding, K. and Johnston J.W.** 2007. Cryopreservation of shoot tips and meristems. In: Stacey, G.N. and Day, J.G. (eds) *Methods in Molecular Biology, Cryopreservation and Freeze-Drying Protocols*. Humana Press Inc., Totowa, NJ. 368: 163-183.
- Benson, E.E., Wilkinson, M., Todd, A., Ekuere, M. and Lyon, J.** 1996. Developmental competence and ploidy stability in plants regenerated from cryopreserved potato shoot apices. *CryoLetters*, 17: 119-128.
- Berjak, P.** 2005. Protector of the seeds: Seminal reflections from Southern Africa. *Science*, 307: 47-49.
- Berjak, P.** 2006. The challenge of recalcitrant germplasm cryopreservation. *Journal of Horticultural Science and Biotechnology*, 81: 781-782.
- Berjak, P., Farrant, J.M., Mycock, D.J. and Pammenter, N.W.** 1990. Recalcitrant (homoiohydrous) seeds: the enigma of their desiccation sensitivity. *Seed Science and Technology*, 18: 297-310.
- Berjak, P., Farrant, J.M. and Pammenter, N.W.** 1989. The basis of recalcitrant seed behaviour. In: Taylorson, P.B. (ed) *Recent Advances in the Development and Germination of Seeds*. Plenum Press, New York, pp. 89-108.

- Berjak, P., Kioko, J.I., Makhathini, A. and Watt, M.P.** 2004. Strategies for field collection of recalcitrant seed and zygotic embryonic axes of the tropical tree, *Trichilia dregeana* Sond. *Seed Science and Technology*, 32: 825-836.
- Berjak, P., Kioko, J., Walker, M., Mycock, D.J., Wesley-Smith, Watt, P. and Pammenter, N.W.** 1998. Cryopreservation an elusive goal. *Proceedings of the IUFRO Conference on Recalcitrant Seeds*. Kuala Lumpur, Malaysia. Marzarlina, M., Khoo, K.C., Jayanthi, N., Tsan, F.Y. and Krishnapillay, B. (eds) Forest Research Institute of Malaysia (FRIM), 1: 96-106.
- Berjak, P. and Mycock, D.** 2004. Calcium, with magnesium, is essential for normal seedling development from partially dehydrated recalcitrant axes: a study on *Trichilia dregeana* Sond. *Seed Science Research*, 14: 217-231.
- Berjak, P., Mycock, D.J., Walker, M., Kioko, J.I., Pammenter, N.W. and Wesley-Smith, J.** 1999. Conservation of genetic resources naturally occurring as recalcitrant seeds. In Bradford, M.B., Bradford, K.J. and Vázquez-Ramos (eds) *Seed Biology Advances in Applications*, pp. 223-228.
- Berjak, P., Mycock, D.J., Wesley-Smith, J., Dumet, D. and Watt, M.P.** 1996. Strategies for *in vitro* conservation of hydrated germplasm, In: Normah, M.N., Narimah, M.K., Clyde, M.M. (eds), *In vitro Conservation of Plant Genetic Resources*. Percetakan, Kuala Lumpur, pp. 19-52.
- Berjak, P. and Pammenter, N.W.** 1999. Importance considerations pertaining to desiccation sensitivity of seeds (recalcitrant seed behaviour) In: Edwards, D.G.W. and Naithani, S.C. (eds) *Seed and Nursery Technology of Forest Trees*. New Age International Limited Publishers, New Delhi, India.
- Berjak, P. and Pammenter, N.W.** 2000. What ultrastructure has told us about recalcitrant seeds. *Revista Brasileira de Fisiologia Vegetal*, 12: 22-55.

- Berjak, P. and Pammenter, N.W.** 2004. Recalcitrant seeds. In: Benech-Arnold, R.L. and Sánchez, R.A. (eds) *Handbook of Seed Physiology*. Food Products Press, the Haworth Reference Press, New York. pp. 305-345
- Berjak, P. and Pammenter, N.W.** 2008. From *Avicennia* to *Zizania*: Seed recalcitrance in perspective: a review. *Annals of Botany*, 101: 213-228.
- Berjak, P. and Pammenter, N.W.** 2014. Cryostorage of germplasm of tropical recalcitrant-seeded species: approaches and problems. *International Journal of Plant Science*, 175: 29-39.
- Berjak, P., Sershen, Varghese, B. and Pammenter, N.W.** 2011. Cathodic amelioration of the adverse effects of oxidative stress accompanying procedures necessary for cryopreservation of embryonic axes of recalcitrant-seeded species. *Seed Science Research*, 21:187-203.
- Berjak, P., Walker, M., Watt, M.P. and Mycock, D.J.** 1999. Experimental parameters underlying failure or success in plant germplasm cryopreservation: a case study on zygotic axes of *Quercus robur* L. *CryoLetters*, 20: 251-262.
- Bhattacharjee, J.** 2012. The language of reactive oxygen species signalling in plants. A review, *Journal of Botany*.
- Bhosale, U.P., Dubhashi, S.V., Mali, N.S. and Rathod, H.P.** 2011. *In vitro* shoot multiplication in different species of banana. *Journal of Plant Science and Research*, 1: 23-27.
- Blackesley, D., Pask, N., Henshaw, G.G. and Fay, M.F.** 1996. Biotechnology and the conservation of forest genetic resources; in vitro strategies and cryopreservation. *Plant Growth Regulation*, 20: 11-16.

- Borthakur, A., Das, S.C., Kalita, M.C. and Sen, P.** 2011. A protocol for *in vitro* shoot regeneration from apical buds of *Albizzia lebbeck* (L.) Benth. *Annals of Biological Research*, 2: 275- 282.
- Botanic Gardens Convention International (BGCI)**, 2010. BG-Journal, 1: 4-6. Available: <http://www.bgci.org/>. Last accessed: 05 November 2014.
- Brush, S. B.** 1991. A farmer based approach to conserving crop germplasm. *Springer*, 45: 153-165.
- Calistru, C., McLean, M., Pammenter, N.W. and Berjak, P.** 2000. The effects of mycofloral infection on the viability and ultrastructure of wet-stored recalcitrant seeds of *Avicennia marina* (Forssk.) Vierh. *Seed Science Research*, 10: 341-353.
- Chaitanya, k.S.K. and Naithani, S.C.** 1994. Role of superoxide, lipid peroxidation and superoxide dismutase in membrane perturbation during loss of viability in seeds of *Shorea robusta* Gaertn. F. *New Phytologist*, 126: 623–627
- Cheeseman, J.** 2007. Hydrogen peroxide and plant stress: a challenging relationship. *Plant Stress*, 1: 4–15.
- Chen, W.P. and Li, P.H.** 2002. Attenuation of reactive oxygen production during chilling in ABA-treated maize cultured in cells. In: *Plant Cold Hardening- Gene Regulation and Genetic Engineering*. Kluwer Academies/ Plenum Publishers, New York, pp. 223-233.
- Cho, E.G., Hor, Y.L., Kim, H.H., Rao, R.V. and Engelmann, F.** 2002. Cryopreservation of *Citrus madurensis* zygotic embryonic axes by vitrification: Importance of pregrowth and preculture conditions. *CryoLetters*, 23: 325-332.

- Choinski, J.S.** 1990. Aspects of viability and post-germinative growth in seeds of tropical tree, *Trichilia dregeana* Sonder. *Annals of Botany*, 437-442.
- Day, J.G., Harding, K.C., Nadarajan, J. and Benson, E.E.** 2008. Cryopreservation, conservation of bioresources at ultra-low temperatures. In: Walker, J.M. and Rapley, R. (eds.) *Molecular Biomechanics Handbook*. Humana Press, ISBN 978-1-60327-374-9, Totowa, NJ. pp. 917-947
- Dereuddre, J., Hassen, M., Blandin, S. and Kaminski, M.** 1991. Resistance of alginate-coated somatic embryos of carrot (*Daucus carota* L.) to desiccation and freezing in liquid nitrogen: Thermal analysis. *CryoLetters*, 12:135-148.
- Dhillon, B.S. and Saxena, S.** 2003. Conservation and access to plant genetic resources. In: Mandel, B.B., Chaudhury, R., Engelmann, F., Bhag, M., Tao, K.L. and Dhillon, B.S. (eds) *Conservation Biotechnology of Plant Germplasm*. NBPGR, New Delhi, India/IPGRI, Rome, Italy/FOA, Rome, Italy, pp. 3-18.
- Diallo, D., Paulsen, B.S., Liljebe, T.H.A. and Michaelsen, T.E.** 2003. The malian medicinal plant *Trichilia emetica*; studies on polysaccharides with complement fixing ability. *Journal of Ethnopharmacology*, 84: 279-287.
- Du, J., Kleinhans, F. W., Mazur, P. and Critser, J.K.** 1994. Human spermatozoa glycerol permeability and activation energy determined by electron paramagnetic resonance. *Biochimica et biophysica acta*. 1194:1-11.
- Dumet, D. and Benson, E. E.** 2000. The use of physical and biochemical studies to elucidate and reduced cryopreservation-induced damage in hydrated/desiccated plant germplasm. In: Engelmann, F. and Takagi, H. (eds) *Cryopreservation of Tropical Plant Germplasm*, IPGRI, Italy.

- Dumet, D., Engelmann, F., Chabrillange, N., Duval, Y. and Dereuddre, J.** 1993. Importance of sucrose for the acquisition of tolerance to desiccation and cryopreservation of oil palm somatic embryos. *CryoLetters* 14: 243-250.
- Eggers, S.K.** 2007. Sub-imbibed storage of recalcitrant seeds of four species. M.Sc. Dissertation, University of KwaZulu-Natal, Durban, South Africa.
- Einset, J., Winge, P. and Bones, A.** 2007. ROS signaling pathways in chilling stress. *Plant Physiology*, 5: 365-367.
- Ellis, R.H. and Roberts, E.H.** 1980. Improved equations for the prediction of seed longevity. *Annals of Botany*, 45: 13-30.
- Engelmann, F.** 1991. In vitro conservation of tropical plant germplasm: a review. *Euphytica*, 57: 227-243.
- Engelmann, F.** 1997a. *In vitro* conservation methods. In: Biotechnology and Plant Genetic Resources (Callow, J.A.; Ford-Lloyd, B.V.D.; Newbury, H.J., Eds.). CABI, Oxon, UK, pp. 119-161.
- Engelmann, F.** 1997b. Importance of desiccation for the cryopreservation of recalcitrant seeds and vegetatively propagated species. *Plant Genetic Resources Newsletter*, **112**: 9-18.
- Engelmann, F.** 2000. Importance of cryopreservation for the conservation of plant genetic resources. In: Engelmann, F. and Takagi, H. (eds) Cryopreservation of tropical plant germplasm-current research progress and applications. JIRCAS, Tsukuba, pp. 8-20.
- Engelmann, F.** 2004. Plant cryopreservation: progress and prospects. *In vitro Cellular Development Biology Plant*, 40: 427-433.
- Engelmann, F.** 2011. Use of biotechnologies for the conservation of plant biodiversity. *In vitro Cell Development Biology Plant*, 47: 5-16.

- Engelmann, F., Dambier, D. and Ollitraut, P.** 1994. Cryopreservation of cell suspension and embryogenic callus of citrus using a simple freezing process. *CryoLetters*, 15: 53-58.
- Engelmann, F., Gonzalez-Arno, M.T., Wu, W.J., Escobar, R.E.** 2008. Development of encapsulation-dehydration. In: Reed, B.M. (ed) *Plant Cryopreservation: A Practical Guide*. Springer, Berlin, pp. 59-76.
- Fabre, J. and Dereuddre, J.** 1990. Encapsulation-dehydration: a new approach to cryopreservation of *Solanum* shoot tips. *CryoLetters*, 11: 413-426.
- Fadel, D., Kintzios, S., Economou, A.S., Moschopoulou, G. and Constantnidou, H.A.** 2010. Effect of different strength of medium on organogenesis phenolic accumulation and antioxidant activity of spearmint (*Mentha spicata* L.). *The Open Horticulture Journal*, 3: 31-35.
- Fahy, G.M., Macfarlane, D.R., Angell, C.A. and Meryman, H.T.,** 1984. Vitrification as an approach to cryopreservation. *Cryobiology*, 21: 407-426.
- Farrant, J.M., Pammenter, N.W. and Berjak, P.** 1989. Germination-associated events and the desiccation sensitivity of recalcitrant seeds - a study on three unrelated species. *Planta*, 178: 189-198.
- Food and Agriculture Organisation of the United Nations, FAO.,** 2010. The state of the world's plant genetic resources for food and agriculture. Rome, Italy.
- Forman, H.J. and Torres, M.** 2002. Reactive oxygen species and cell signalling (respiration burst in Macrophage signalling). *American Journal of Respiratory and Critical Care Medicine*, 166: S4-S8.

- Forman, H.J., Demidchik, V., Bothwell, J.H.F., Mylona, P., Mledema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D.G., Davies, J.M. and Dolan, L.** 2003. Reactive oxygen species produced by NADPH oxidases regulate plant cell growth. *Nature*, 422: 442-446.
- Fu, J.R., Jin, J.P., Peng, Y.F. and Xia, Q.H.** 1994. Desiccation tolerance in two species with recalcitrant seeds: *Clausena lansium* (Lour.) and *Litchi chinensis* (Sonn.). *Seed Science Research*, 4: 257-261.
- Galili, G.** 1995. Regulation of lysine and threonine biosynthesis. *American Society of Plant Physiologists*, 7: 899–906.
- Gapper, C. and Dolan, L.** 2006. Control of plant development by reactive oxygen species. *Plant Physiology*, 141: 341-345.
- Gao, D. Y., Liu, J., Lui, C., McGann, L.E., Watson, P. F., Kleinhans, F.W., Mazur, P., Critser, E.S. and Critser, J.K.** 1995. Prevention of somatic injury to human spermatozoa during addition and removal of glycerol. *Human Reproduction*, 10: 1109-1122.
- Geldenhuys, C.J.** 2000. Commercial products from the wild: sustainable utilization, commercialization and domestication of products from indigenous forest and woodland ecosystems: a progress report. In: Seydack, A.H.W., Vermeulen, W.J. and Vermeulen, C. (eds) *Towards Sustainable Management Based on Scientific Understanding of Natural Forests and Woodlands, Proceedings of the second Natural Forests and Savanna Woodlands Symposium, Knysna*. South African Government Printer, Pretoria, pp. 342-348.

- Germano, M.P., Angelo, V.D., Sanogo, R., Catania, S., Alma, R., De Pasquale, R. and Bisignano, G.** 2005. Hepatoprotective and antibacterial effects of extracts from *Trichilia emetica* Vahl. (Meliaceae). *Journal of Ethnopharmacology*, 96: 227–232
- Golmirzaie, A. and Panta, A.** 2000. Advances in potato cryopreservation at the International Potato Centre, Peru. In: Cryopreservation of tropical plant germplasm - current research progress and applications. JIRCAS, Tsukuba, pp. 250-254.
- Gonzalez-Benito, M.E., Clavero-Ramirez, I. and Lopez-Aranda, J.M.** 2004. The use of cryopreservation for germplasm conservation of vegetatively propagated crops. *Spanish Journal of Agricultural Research*, 2: 341-351.
- Goveia, M.** 2007. The effects of developmental status and excision injury on the success of cryopreservation of germplasm from non-orthodox seeds. MSc. Dissertation, University of Kwa-Zulu Natal, Durban.
- Goveia, M., Kioko, J.I. and Berjak, P.** 2004. Developmental status is a critical factor in the selection of excised recalcitrant axes as explants for cryopreservation: a study on *Trichilia dregeana* Sond. *Seed Science Research*, 14: 241-248.
- Graham, D. and Patterson, B.D.** 1982. Responses of plants to low, non-freezing temperatures: proteins, metabolism and acclimation, *Annual Review of Plant Physiology*, 33: 347-374.
- Grout, B.W.W.** 1990. *In vitro* conservation of germplasm. In: Bhonjwani, S.S. (ed) *Plant Tissue Culture: Applications and Limitations*. Elsevier, Amsterdam, pp.394-411.
- Gunatilaka, A.A.L., Bolzani, V.S., Dagna, E., Hofmann, G.A., Johnson, R.K., McCabe, F.L., Mattern, M.R. and Kingston, D.G.I.** 1998. Limonoids showing selective

- toxicity to DNA repair-deficient yeast and other constituents of *Trichilia emetica*. *Journal of Natural Products*, 61: 179-184.
- Hajari, E., Berjak, P., Pammenter, N.W. and Watt, M.P.** 2011. A novel means for cryopreservation of germplasm of the recalcitrant-seeded species, *Ekebergia capensis*. *CryoLetters*, 32: 308-316.
- Halliwell, B.** 2006. Reactive oxygen species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiology*, 141: 312-322.
- Halliwell, B. and Gutteridge, J.M.C.** 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemistry Journal*, 219: 1-14.
- Halliwell, B. and Gutteridge, J.M.C.** 2007. Free Radicals in Biology and Medicine. (4th ed) Oxford University Press Inc., New York, USA.
- Halmagyi, A., Fischer-Kluver, G., Mix-Wagner, G. and Schumacher, H.M.** 2004. Cryopreservation of *Chrysanthemum morifolium* (*Dendranthema grandiflora* Ramat.) using different approaches. *Plant Cell Reports*, 22: 371-375.
- Hamilton, K.N., Tunner, S.R. and Ashmore, S.E.** 2009. Cryopreservation. In: Oxford, C.A. and Meagher, P.F. (eds) *Plant Germplasm in Australia: Strategies and Guidelines for Developing, Managing and Utilising Ex situ collections*. Australian Network for Plant Conservation Inc., ISBN 978-0-9752191-1-9, Canberra.
- Han, B., Berjak, P., Pammenter, N., Farrant, J. and Kermode, A.R.** 1997. The recalcitrant plant species, *Castanospermum australe* and *Trichilia dregeana*, differ in their ability to produce dehydrin-like polypeptides during seed maturation and in response to ABA or water deficit related stresses. *Journal of Experimental Botany*, 48: 1717-1726.

- Hanaoka, A.** 2001. Antioxidant effects of reduced water produced by electrolysis of sodium chloride solutions. *Journal of Applied Electrochemistry*, 31: 1307-1313.
- Harding, K.** 2004. Genetic integrity of cryopreserved plant cells: a review. *CryoLetters*, 25: 3-22.
- Hartmann, H.T., Kesler, D.E., Davies, F.T. and Geneve, R.L.** 2002. *Plant Propagation Principles and Practices*. (7th ed) Prentice Hall, Upper Saddle River, NJ.
- Hatanaka, T., Yasuda, T., Yamaguchi, T. and Sakai, A.** 1994. Direct regrowth of encapsulated somatic embryos of coffee (*Coffea canephora*) after cooling in liquid nitrogen. *Cryoletters*, 15: 47-52.
- Helliot, B., Swennen, R., Poumay, Y., Frison, E., Lepoivre, P. and Panis, B.** 2003. Ultrastructural changes associated with cryopreservation of banana (*Musa* spp.) highly proliferating meristems. *Plant Cell Reports*, 21: 690-698.
- Hendry, G.A.F.** 1993. Oxygen, free radical processes and seed longevity. *Seed Science Research*, 3: 141-153.
- Hirai, D. and Sakai, A.** 1999. Cryopreservation of *in vitro*-grown axillary shoot-tip meristems of mint (*Mentha spicata* L.) by encapsulation-vitrification. *Plant Cell Reports*, 19: 150- 155.
- Hirai, D., Shirai, K., Shirai, S. and Sakai, A.** 1998. Cryopreservation of *in vitro*-grown meristems of strawberry (*Fragaria x ananassa* Duch.) by encapsulation-vitrification, *Euphytica*, 101: 109-115.
- Hong, T.D. and Ellis, R.H.** 1996. A protocol to determine seed storage behaviour. Rome, IPGRI.

- Huang, Y-R., Hung, Y-C., Hsu, S-Y., Huang, Y-W. and Hwang, D-F.** 2008. Application of electrolyzed water in the food industry. *Food Control*, 19: 329-345.
- Huck, T.** 2005. Designing cathodic protection for power plant applications. Case history, pp. 1-4.
- Humbeck, K., Graner, A. and Hinch, D.** 2008. Physiological, biochemical, histological and ultrastructural aspects of cryopreservation in meristematic tissue of potato shoot apices.
- Hutchings, A., Scott, A.H., Lewis, G. and Cunningham, A.B.** 1996. Zulu medicinal plants-an inventory. Natal University Press, Pietermaritzburg, pp. 157-158.
- Jackson, P. W. and Kennedy, K.** 2009. The global strategy for plant conservation: a challenge and opportunity for the international community. *Trends in Plant Science*, 14: 578-580.
- Johnston, C., Day, J.G., Staines, H. and Benson, E.E.** 2006. An *in vitro* oxidative stress test for determining pollutant tolerance in algae. *Ecological Indicators*, 6: 770-779.
- Kaczmarczyk, A.** 2008. Physiological, biochemical, histological and ultrastructural aspects of cryopreservation in meristematic tissue of potato shoot tips. Dissertation. der Martin-Luther-Universität Halle-Wittenberg.
- Kaczmarczyk, A. Rutten, T., Melzer, M. and Keller, E.R.J.** 2008. Ultrastructural changes associated with cryopreservation of Potato (*Solanum tuberosum* L.) shoot tips. *CryoLetters*, 29: 145-156.
- Kaczmarczyk, A., Funnekotter, B, Menon, A., Ye Phang, P., Al-Hanbali1, A., Bunn, E., and Mancera, L.R.** 2012. Current Issues in Plant Cryopreservation, In: Katkov, I.

(ed) *Current Frontiers in Cryobiology*, ISBN: 978-953-51-0191-8, InTech, DOI: 10.5772/32860. Available from: <http://www.intechopen.com/books/current-frontiers-in-cryobiology/current-issues-in-plant-cryopreservation>.

Kartha, K.K. 1981. Meristem culture and cryopreservation methods and applications. In: Thorpe, T.A., (ed) *Plant Tissue Culture, Methods and Applications in Agriculture*. Academic Press, New York, USA, pp.181-212.

Kartha, K.K. 1985. Cryopreservation of plant cells and organs. CRC Press Inc., Boca Raton, Florida.

Kartha, K. K. and Engelmann, F. 1994. Cryopreservation and germplasm storage. In: Vasil, I.K. and Thorpe, T.A. (eds) *Plant Cell and Tissue Culture*, Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 195-230.

King, M.W. and Robert, E.H. 1980. Maintenance of recalcitrant seeds in storage. In: Chin, H.F. and Roberts, E.H. (eds) *Recalcitrant Crop Seeds*. Tropical Press, Kuala Lumpur, Malaysia, SDN. BHD., pp.53-89.

Kioko, J.I., Berjak, P., Pammenter, N.W., Watt, M.P. and Wesley-Smith, J. 1998. Desiccation and cryopreservation of embryonic axes of *Trichilia dregeana* Sond. *CryoLetters*, 19: 15-26.

Kioko, J.I. 2003. Aspects of post-harvest seed physiology and cryopreservation of the germplasm of three medicinal plants indigenous to Kenya of South Africa. PhD. Thesis, University of Natal, Durban, South Africa.

Kioko, J., Berjak, P. and Pammenter, N.W. 2006. Viability and ultrastructural responses to seeds of embryonic axes of *Trichilia emetica* to different dehydration and storage conditions. *South African Journal of Botany*, 72: 167-176.

- Komane, B.M., Olivier, E.I. and Viljoen, A.M.** 2011. *Trichilia emetica* (Meliaceae) A review of traditional uses, biological activities and phytochemistry. *Phytochemistry Letters*, 4: 1-9.
- Kramer, A.T. and Havens, K.** 2009. Plant genetics in a changing world. *Trends in Plant Science*, 14: 599-607.
- Kranner, I., Birtic, S., Anderson, K.M. and Pritchard, H.W.** 2006. Glutathione half-cell reduction potential: a universal stress marker and modulator for programmed cell death? *Free Radical Biology and Medicine*, 40: 2155-2165.
- Kranner, I., Minibayeva, F., Beckett, R.P. and Seal, C.E.** 2010. What is stress? Concepts, definitions and applications in seed science. *New Phytologist*, 188: 655-673.
- Kryszczuk, A., Keller, J., Grube, M. and Zimnoch-Guzowska, E.** 2006. Cryopreservation of potato (*Solanum tuberosum* L.) shoot tips using vitrification and droplet method. *Food, Agriculture and Environment*, 4:196-200.
- Leprince, O., Buitink, J. and Hoekstra, F.A.** 1999. Axes and cotyledon of recalcitrant seeds of *Castanea sativa* Mill. exhibit contrasting responses of respiration to drying in relation to desiccation sensitivity. *Journal of Experimental Biology*, 50: 1515-1524.
- Li, D. and Pritchard, H.W.** 2009. The science and economics of *ex situ* plant conservation. *Trends in Plant Science*, 14: 614-621.
- Linington, S.H., 2003.** The design of seed banks. In: Smith, R.D., Dickie, J.B., Linington, S.H., Pritchard, H.W. and Probert, R.J. (Eds) *Seed Conservation Turning Science into Practice*. Royal Botanic Gardens, Kew, pp. 591-536.

- Lipavska, H. and Vreugdenhil, D.** 1996. Uptake of mannitol from the media by *in vitro* grown plants. *Plant Cell, Tissue and Organ Culture*, 45: 103-107.
- Liszka, A., van der Zalm, E. and Schopfer, P.** 2004. Production of reactive oxygen intermediates O_2^- , H_2O_2 , and OH by maize roots and their role in wall loosening and elongation growth. *Journal of Plant Physiology*, 136: 3114–3123.
- Lloyd, G. and McCown, B.** 1980. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. *Combined Proceedings of the International Plant Propagation Society*, 30: 421-427.
- Lynch, P.T., Benson, E.E. and Harding, K.** 2007. Climate change: the role of economically important, vegetatively propagated plants. *Journal of Horticultural Science and Biotechnology*, 82: 157-160.
- Lynons, J.M.** 1973. Chilling injury in plants. *Annual Review of Plant Physiology*, 24: 445-466.
- Madhulatha, P., Anbalagan, M., Jayachandran, S. and Sakthivel, N.** 2004. Influence of liquid pulse treatment with growth regulators on *in vitro* propagation of banana (*Musa* spp. AAA). *Plant Cell Tissue Organ Culture*, 76: 189-192.
- Makowska, Z., Keller, J. and Engelmann, F.** 1999. Cryopreservation of apices isolated from garlic (*Allium sativum* L.) bulbils and cloves. *CryoLetters*, 20: 175-182.
- Mandal, B.B. and Dixit-Sharma, S.** 2007. Cryopreservation of *in vitro* shoot apices of *Dioscorea deltoidea* Wall, an endangered medicinal plant: effect of cryogenic procedure and storage duration. *CryoLetters* 28: 461-470.

- Mandal, B.B., Tyagi, R.K., Pandey, R., Sharma, N. and Agrawal, A.** 2000. *In vitro* conservation of germplasm of agri-horticultural crops at NBPGR: an overview. In: Razdan, M.K. and Cocking, E.C. (eds) *Conservation of plant genetic resources in vitro*. Volume 2: *applications and limitations*. Science Publishers Inc, New Hampshire. pp. 279-307.
- Marcotrigiano, M. and McGlew, S.P.** 1991. A two-stage micropropagation system for cranberries. *Journal of the American Society for Horticultural Science*, 116: 911–916.
- Matsumoto, T., Sakai, A. and Nako, Y.** 1998. A novel pre-culturing for enhancing the survival of *in vitro*- grown meristems of wasabi (*Wasabia Japonica*) cooled to -196 °C by vitrification. *CryoLetters*, 19: 27-36.
- Matsumoto, T., Sakai, A. and Yamanda, K.** 1994. Cryopreservation of *in vitro* grown apical meristems of wasabi (*Wasabia japonica*) by vitrification and subsequent high plant regeneration. *Plant Cell Reports*, 13: 442-446.
- Matsumoto, T., Sakai, A. and Yamanda, K.** 1995. Cryopreservation of *in vitro* grown apical meristems of lily by vitrification. *Plant Cell Tissue Organ Culture*, 28: 261-266.
- Mazur, P.** 1984. Freezing of living cells: mechanisms and applications. *American Journal of Physiology*, 247: C125-C142.
- Meryman, H.T. and Williams, R.J.** 1985. Basic principles of freezing injury to plant cells; natural tolerance and approaches to cryopreservation. In: Kartha, K.K., (ed) *Cryopreservation of plant cells and organs*. CRC Press, Boca Raton, FL, pp. 13-47.

- Meryman, H.T., Williams, R.J. and Douglas, M.S.J.** 1977. Freezing injury from solution effects and its prevention by natural or artificial cryoprotection. *Cryobiology*, 14: 287-302.
- Misra, H.R. and Fridovich, I.** 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *Journal of Biological Chemistry*, 247: 3170-3175.
- Mittler, R. and Blumwald, E.** 2010. Genetic engineering for modern agriculture: challenges and perspectives, *Annual Review of Plant Biology*, 61: 443–462.
- Moges, A. D., Karam, N. S. and Shibli, R.A.** 2003. Slow growth *in vitro* preservation of African violet (*Saintpaulia ionantha* Wendl.) shoot tips. *Advances in Horticultural Science*, 17: 1-8.
- Mroginski, L.A., Roca, W.M. and Kartha, K.K.** 1991. Crioconservacion de germoplasma. In: Roca, W.M., Kartha, K.K. and Mroginski, L.A (eds) *Cultivo de Tejidos en la Agricultura: Fundamentos Aplicaciones*, CIAT, Cali, Colombia. pp. 715-730.
- Mycock, D.** 1999. Addition of calcium and magnesium to a glycerol and sucrose cryoprotectant solution improves the quality of plant embryo recovery from cryostorage. *CryoLetters*, 20: 77-82.
- Mycock, D.J. and Berjak, P.** 1990. Fungal contamination associated with several homoiohydrous (recalcitrant) seed species. *Phytophylactica*, 22: 413-418.
- Mycock, D.J., Blakeway, F.C. and Watt, M.P.** 2004. General applicability of *in vitro* storage technology to the conservation and maintenance of plant germplasm. *South African Journal of Botany*, 70: 31-36.

- Müller, K., Linkies, A., Vreeburg, R.A., Fry, S.C., Krieger-Liskay, A. and Leubner-Metzger, G.** 2009. *In vitro* cell wall loosening by hydroxyl radicals during cress seed germination and elongation growth. *Plant Physiology*, 150: 1855-1865.
- Myeza, N.** 2005. Effects of various potentially anti-fungal pre- treatments on hydrated storage life span of recalcitrant seeds. MSc Thesis, University of KwaZulu-Natal, Durban.
- Nagl, W. and Fusenig, H. P.** 1979. Types of chromatin organisation in plant nuclei. *Plant Systematics and Evolution Supplement*, 2: 221-235.
- Naidoo, C.** 2012. Oxidative status and stress associated with cryopreservation of germplasm of recalcitrant-seeded species. M.Sc. Thesis, University of KwaZulu-Natal, Durban, South Africa.
- Niino, T., Sakai, A. and Yakuwa, H.** 1992. Cryopreservation of dried shoot tips of mulberry winter buds and subsequent plant regeneration. *CryoLetters*, 13: 51-58.
- Normah, M.N. and Makeen, A.M.** 2008. Cryopreservation of excised embryos and embryonic axes. In: Reed, B.M. (ed.) *Plant Cryopreservation: A Practical Guide*. Springer, New York, pp. 140-155.
- Ohlrogge, J. and Browse, J.** 1995. Lipid biosynthesis. *American Society of Plant Physiologists*, 7: 957–970.
- Ohmiya, K., Sakka, K., Kimura, T. and Morimoto, K.** 2003. Application of microbial genes to recalcitrant biomass utilization and environmental conservation. *Journal of Bioscience and Bioengineering*, 95: 549-561.
- Palmer, E. and Pitman, N.** 1972. Trees of South Africa. A.A. Balkema, South Africa.

- Pammenter, N.W., Adamson, J.H. and Berjak, P.** 1974. Viability of stored seeds: extension by cathodic protection. *Science*, 186: 1123-1124.
- Pammenter, N.W. and Berjak, P.** 1999. A review of recalcitrant seed physiology in relation to desiccation tolerance mechanisms. *Seed Science Research*, 9: 13-37.
- Pammenter, N.W., Berjak, P., Goveia, M., Sershen., Kioko, J.I., Whitaker, C. and Beckett, R.P.** 2011. Topography determines the impact of reactive oxygen species on shoot apical meristems of recalcitrant embryos of tropical species during processing for cryopreservation. *Acta Horticulturae*, 908: 83-92.
- Pammenter, N.W., Berjak, P. and Walters, C.** 1999. The effect of drying rate, and processes leading to viability loss in recalcitrant seeds. In: Marzalina, M., Khoo, K.C., Jayanthi, N., Tsan, F.Y. and Krishnapillay, B. (eds) *Recalcitrant Seeds*. Forest Research Institute, Kuala Lumpur, pp. 14-24.
- Pammenter, N.W., Berjak, P., Wesley-Smith, J. and Vander Willigen, C.** 2002. Experimental aspects of drying and recovery. In: *Desiccation and Survival in Plants: Drying Without Dying* (eds) Black, M. and Pritchard, H.W. CABI, Wallingford, UK. pp. 93-110
- Pammenter, N.W., Greggains, V., Kioko, J.I., Wesley-Smith, J., Berjak, P. and Finch-Savage, W.E.** 1998. Effects of differential drying rates on viability retention of recalcitrant seeds. *Seed Science Research*, 8: 463-471.
- Pammenter, N.W., Vertucci, C.W. and Berjak, P.** 1991. Homeohydrous (recalcitrant) seeds: Dehydration, the state of water and viability characteristics in *Landolphia kirkii*. *Plant Physiology*, 96: 1093-1397.
- Pammenter, N.W., Vertucci, C.W. and Berjak, P.** 1993. Responses to desiccation in relation to non-freezable water in desiccation-sensitive and-tolerant seeds. In: Come,

- D. and Corbineau, F. (eds) *Basic and Applied Aspects of Seed Biology*. AFSIS, Paris, pp. 867-872.
- Pan, S-M. and Yau, Y-Y.** 1991. The isozymes of superoxide dismutase in rice. *Botanical Bulletin of Academia Sinica*, 32: 253-258.
- Panis, B., Piette, B. and Swennen, R.** 2005. Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all Musaceae. *Plant Science*, 168: 45-55.
- Panis, B., Strosse, H., Van Den Hende, S., and Swennen, R.** 2002. Sucrose preculture to simplify cryopreservation of banana meristem cultures. *Cryoletters*, 23: 375-384.
- Paunescu, A.,** 2009. Biotechnology for endangered plant conservation: a critical overview. *Romanian Biotechnological Letters*, 14: 4095-4103.
- Pence, V.C.** 1991. Cryostorage of immature embryos of *Theobroma cacao*. *Plant Cell Reports*, 10: 144-147.
- Pence, V.C.** 1992. Desiccation and the survival of *Aesculus*, *Castanea* and *Quercus* embryo axes through cryopreservation. *Cryobiology* 29: 391-399.
- Pence, V.C.** 2011. Evaluating costs for the *in vitro* propagation and preservation of endangered plants. *In Vitro Cellular and Developmental Biology*, 47: 176-187.
- Perán, R., Berjak, P., Pammenter, N.W. and Kioko, J.I.** 2006. Cryopreservation, encapsulation and promotion of shoot production of embryonic axes of a recalcitrant species. *Ekebergia capensis* Sparrm. *CryoLetters*, 27: 5-16.
- Plessis, P., Leddet, C. and Dereuddre, J.** 1991a. Resistance to dehydration and to freezing in liquid nitrogen of alginate coated shoot tips of grape vine (*Vitis vinifera* L. cv. Chardonnay). *Comptes Rendus de l'Académie des Sciences Paris*, 313: 373-380.

- Plessis, P., Leddet, C. and Dereuddre, J.** 1991b. Cryopreservation of alginate-coated shoot tips of grape after a progressive increase of sucrose concentration in preculture medium. In: Proceedings of the 28th Annual meeting of the Society for Cryobiology, Leuven, Belgium, 7-12 July, 106.
- Pooley, E.** 1993. *The Complete Field Guide to Trees of Natal, Zululand and Transkei*. Natal Floral Publication Trust, Durban. pp. 204-419
- Pukacka, S. and Ratajczak, E.** 2006. Antioxidative response of ascorbate-glutathione pathway enzymes and metabolites to desiccation of recalcitrant *Acer saccharinum* seeds. *Journal of Plant Physiology*, 163: 1259-1266.
- Pyke, K.A.** 1999. Plastid division and development. *American Society of Plant Physiologists*, 11: 549-556.
- Quain, M.D., Berjak, P., Acheampong, E. and Kioko, J.I.** 2009. Sucrose treatment and explant water content: critical factors to consider in development of successful cryopreservation protocols for shoot tip explants of the tropical species *Dioscorea rotundata* (Yam). *CryoLetters*, 30: 212-223.
- Quain, M.D., Berjak, P., Acheampong, E. and Engnin, M.** 2012. Cryopreserving vegetatively propagated tropical crops- The case of *Dioscorea* species and *Solenostemon rotundifolius*. *Cryobiology*, pp. 485-504. In Tech, Available from: <http://www.intechopen.com/books/current-frontiers-in-cryobiology/cryopreserving-vegetatively-propagated-tropical-crops-the-case-of-dioscorea-species-and-solenostemon>.
- Rao, N.K.** 2004. Plant genetic resources: Advancing conservation and use through biotechnology: A review. *African Journal of Biotechnology*, 3: 136-145.

- Reed, B. M., Sarasan, V., Kane, M., Bunn, E. and Pence, C.E.** 2011. Biodiversity conservation and conservation tools. *In vitro cellular and developmental biology plant*, 47: 1-4.
- Reinhoud, P.J., Van Iren, F. and Kijne, J.W.** 2000. Cryopreservation of undifferentiated plant cells. In: Engelmann, F. and Takagi, H. (eds) *Cryopreservation of Tropical Plant Germplasm*. Current Research Progress and Application. IPGRI, Rome, pp. 91-102.
- Roberts, E.H.** 1973. Predicting the storage life of seeds. *Seed Science and Technology*, 1: 499-514.
- Roach, T., Ivanova, M., Beckett, R.P., Minibayeva, F.V., Green, I., Pritchard, H.W. and Kranner, I.** 2008. An oxidative burst of superoxide in embryonic axes of recalcitrant sweet chestnut seeds as induced by excision and desiccation. *Physiologia Plantarum* 133: 131-139.
- Rodriguez, A.A., Grunberg, K.A. and Taleisnik, E.L.** 2002. Reactive oxygen species in the elongation zone of maize leaves are necessary for leaf extension. *Plant Physiology*, 129: 1627–1632.
- Sagi, M., Davydov, O., Orazova, S., Yesbergenova, Z., Ophir, R., Stratmann, J.W. and Fluhrb, R.** 2004. Plant respiratory burst oxidase homologs impinge on wound responsiveness and development in *Lycopersicon esculentum*. *The Plant Cell*, 16: 616-628.
- Sakai, A.** 1995. Cryopreservation of germplasm of woody plants. In: Bajaj, Y.P.S. (ed) *Biotechnology in Agriculture and Forestry: Cryopreservation of Plant Germplasm*, Springer, Heidelberg, 32: 53-69.

- Sakai, A.** 2000. Development of cryopreservation techniques. In: Engelmann, F. and Takagi, H. (eds) *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*. Japan International Research Centre for Agricultural Sciences, Tsukuba, Japan/International Plant Genetic Resources Institute, Rome, Italy, pp. 1-7.
- Sakai, A. and Engelmann, F.** 2007. Vitrification, encapsulation-vitrification and droplet-vitrification: A review. *CryoLetters*, 28: 151-172.
- Sakai, A., Kobayashi, S. and Oiyama, I.** 1990. Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Reports*, 9: 30-33.
- Sakai, A., Matsumoto, T., Hirai, D. and Niino, T.** 2000. Newly development encapsulation-dehydration protocol for plant cryopreservation. *CryoLetters*, 21: 53-62.
- Sakai, A., Hirai, D. and Niino, T.** 2008. Development of PVS-based vitrification and encapsulation-vitrification protocols. In: Reed, B.M. (ed.) *Plant Cryopreservation: A Practical Guide*, Springer, Scotland, pp. 33-57.
- Sant, R., Taylor, M. and Tyagi, A.** 2006. Cryopreservation of *in vitro*-grown shoot-tips of tropical taro (*Colocasia esculenta* var. *esculenta*) by vitrification. *CryoLetters*, 27: 133-142.
- Santos, I. and Stushnoff, C.** 2003. Desiccation and freezing tolerance of embryonic axes from *Citrus sinensis* [L.] OSB. pre-treated with sucrose. *CryoLetters*, 24: 281-292.
- Sarkar, D. and Naik, P. S.** 1998a. Factors affecting minimal growth conservation of potato microplants *in vitro*. *Euphytica*, 102: 275-280.
- Sarkar, D. and Naik, P.S.** 1998b. Cryopreservation of shoot tip of Tetraploid Potato (*Solanum tuberosum* L.) clones by vitrification. *Annals of Botany*, 82: 455-461.

- Scandalios, J.G.** 1993. Oxygen stress and superoxide dismutase. *Plant Physiology*, 101: 7-12.
- SCBD:** Secretariat of the Convention on Biological Diversity, Netherlands Commission for Environmental Assessment. 2006. Biodiversity in Impact Assessment, Background document to CBD Decision VIII /28: Voluntary Guidelines on Biodiversity-Inclusive Impact Assessment, Montreal, Canada.
- SCBD:** Secretariat of the Convention on Biological Diversity, Netherlands Commission for Environmental Assessment. 2008. Understanding and Influencing the Process: A Guide to Understanding and Participating Effectively in the Ninth Conference of the Parties to the Convention on Biological Diversity (COP-9).
- Schmidt, L.** 2000. Guide to handling of tropical and subtropical forest seed. *Danida Forest Seed Centre*. Denmark.
- Schmidt, E., Lotter, M. and McClelland, W.** 2002. Trees and shrubs of Mpumalanga and Kruger National Park. *Jacana Education (Pty) Ltd.*, Johannesburg, South Africa.
- Schopfer, P., Plachy, C. and Frahry, G.** 2001. Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellins and abscisic acid. *Plant Physiologist*, 125: 1591-1602.
- Scocchi, A., Faloci, M., Medina, R., Olmos, S. and Mroginski, L.** 2004. Plant recovery of cryopreserved apical meristems-tips of *Melia azedarach* L. using encapsulation / dehydration and assessment of their genetic stability. *Euphytica*, 135: 29-38.
- Scottez, C., Godard, N., Joulie, C., Duron, M., Arnaud, Y. and Dereuddre, J.** 1991. Cryopreservation of pear shoot-tips after encapsulation-dehydration. In: Proceedings of the 28th Annual Meeting of the Society for Cryobiology, Leuven, Belgium, 7-12 July, 59.

- Scowcroft, W.R.** 1984. Genetic viability in tissue culture: Impact on germplasm conservation and utilisation, *International Board for Plant Genetic Resources*, Report (AGPG: IBPGR/84/152), Rome.
- Sershen, Berjak, P. and Pammenter, N.W.** 2010. Effects of cryopreservation of recalcitrant *Amaryllis belladonna* zygotic embryos on vigour of recovered seedlings: a case of stress ‘hangover’? *Physiologia Plantarum*, 139: 205-219.
- Sershen, Pammenter, N.W., Berjak, P. and Wesley-Smith, J.** 2007. Cryopreservation of embryonic axes of selected amaryllid species. *CryoLetters*, 28: 387-399.
- Sershen, Pammenter, N.W., Berjak, P. and Wesley-Smith, J.** 2012a. The effects of various parameters during processing for cryopreservation on the ultrastructure and viability of recalcitrant zygotic embryos of *Amaryllis belladonna*. *Protoplasma*, 249: 155-169.
- Sershen, Varghese, B., Pammenter, N.W. and Berjak, P.** 2012b. Cryo-tolerance of zygotic embryos from recalcitrant seeds in relation to oxidative stress - a case study on two *Amaryllid* species. *Journal of Plant Physiology*, 169: 999-1011.
- Shao, H.B., Chu, L.Y., Lu, Z.H. and Kang, C.M.** 2008. Primary antioxidant free radical scavenging and redox signalling pathway in higher plant cells. *Integrative Journal of Biological Sciences* 4: 8-14.
- Sharma, P., Jha, A.B., Dubey, R.S. and Pessarakli, M.** 2012. Reactive oxygen species, oxidative damage and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany*.
- Shibli, R. A., Smith, M.A.L. and Swatnawi, M.A.** 1999. Pigment recovery from encapsulated-dehydrated *Vaccinium pahalae* (ohelo) cryopreserved cells. *Plant Cell Tissue Culture and Organ Culture*, 55: 119-123.

- Shibli, R.A., Shatnawi, M. A., Subaih, W.S. and Ajlouni, M.M.** 2006. *In vitro* conservation and cryopreservation of plant genetic resources: A Review. *World Journal of Agricultural Science*, 2: 372- 382.
- Shirahanta, S., Kabayama, S., Nakano, M., Miura, T., Kusumoto, K., Gotoh, M., Hayashi, H., Otsubo, K., Morisawa, S. and Katakura, Y.** 1997. Electrolyzed-reduced water scavenges active oxygen species and protects DNA from oxidative damage. *Biochemical and Biophysical Research Communications*, 234: 269-274.
- Steinmacher, D.A., Saldanha, C.W., Clement, C.R. and Guerra, M.P.** 2007. Cryopreservation of peach palm zygotic embryos. *CryoLetters* 28: 13-22.
- Sutherland, J.R., Diekmann, M. and Berjak, P.** 2002. Forest tree seed health for the germplasm conservation. *IPGRI Technical Bulletin*, no.6. IPGRI, Rome.
- Suzuki, N., Koussevitzky, S., Mittler, R. and Miller, G.** 2012. ROS and redox signalling in the response of plants to abiotic stress. *Plant, Cell and Environment*, 35: 259-270.
- Sugimoto-Shirasu, K. and Roberts, K.** 2003. ‘‘Big it up’’: endoreduplication and cell-size control in plants. *Current Opinion in Plant Biology*, 6: 544–553.
- Thammasiri, K.** 1999. Cryopreservation of embryonic axes of jackfruit. *CryoLetters* 20: 21-28.
- Thihn, N.T., Takagi, H. and Yashima, S.**1999. Cryopreservation of *in vitro* grown shoot tips of banana (*Musa* spp.) by vitrification method. *CryoLetters*, 20: 163-174.
- Tommasi, F., Paciolla, C., de Pinto, M.C. and Gara, L.D.** 2006. Effects of storage temperature on viability, germination and antioxidant metabolism in *Ginkgo biloba* L. seeds. *Plant Physiology and Biochemistry*, 44: 359–368.
- Touchell, D.H., Dixon, K.W. and Tan, B.** 2002. Cryostorage of somatic tissues of endangered Australian species, In: *Cryopreservation of Plant Germplasm*. Towill,

- L.E. and Bajaj, Y.P.S. (eds) pp. 357-372, Springer Verlag, ISBN 7055-8630, Berlin Heidelberg, Germany.
- Turner, S.R., Senaratna, T. Bunn, E., Tan, B., Dixon, K.W. and Touchell, D.H.** 2001. Cryopreservation of shoot tips from six endangered Australian species using a modified vitrification protocol. *Annals of Botany*, 87: 371-378.
- Varghese, D., Berjak, P. and Pammenter, N.W.** 2009. Cryopreservation of shoot tips of *Trichilia emetica*, a tropical recalcitrant-seeded species. *CryoLetters* 30: 280-290.
- Varghese, B. and Naithani, S.C.** 2002. Desiccation-induced changes in lipid peroxidation, superoxide level and antioxidant enzymes activity in neem (*Azadirachta indica* A. Juss) seeds. *Acta Physiologiae Plantarum*, 24: 79–87
- Varghese, B., Sershen, Berjak, P., Varghese, D. and Pammenter, N.W.** 2011. Differential drying rates of recalcitrant *Trichilia dregeana* embryonic axes: A study of survival and oxidative stress metabolism. *Physiologia Plantarum*, 142: 326-338.
- Vertucci, C.W.** 1990. Calorimetric studies of the state of water in seed tissues. *Biophysiology Journal*, 58: 1463-1471.
- Vertucci, C.W. and Roos, E.E.** 1990. Theoretical basis of the protocol for seed storage. *Journal of Plant Physiology*, 94: 1019-1023.
- Vertucci, C.W. and Farrant, J.M.** 1995. Acquisition and loss of desiccation tolerance. In: Kigel, J. and Galili, G. (eds) *Seed Development and Germination*. Marcel Dekker, New York, pp. 237-271.
- Vertucci, C.W., Berjak, P., Pammenter, N.W. and Crane, J.** 1991. Cryopreservation of embryonic axes of a *homoiohydrous* (recalcitrant) seed in relation to calorimetric properties of tissue water. *CryoLetters*, 12: 339-350.

- Volk, G.M. and Walters, C.** 2006. Plant vitrification solution 2 lowers water content and alters freezing behavior in shoot tips during cryoprotection. *Cryobiology*, 52, 48-61.
- von Fintel, G.T.** 2006. Towards the long-term germplasm conservation of selected *Amaryllidaceae*. MSc Thesis. University of KwaZulu-Natal, Durban, South Africa.
- Walters, C., Pammenter, N.W., Berjak, P. and Crane, J.** 2001. Desiccation damage, accelerated ageing and respiration in desiccation tolerant and sensitive seeds. *Seed Science Research*, 11: 135-148.
- Walters, C., Farrant, J.M., Pammenter, N.W. and Berjak, P.** 2002. Desiccation stress and damage. In Black, M. and Pritchard, H.W. (eds) *Desiccation and survival in plants: Drying without dying*. Wallingford, CABI publishing. pp. 263-291.
- Walters, C., Wesley-Smith, J., Crane, J., Hill, L.M., Chmielarz, P., Pammenter, N.W. and Berjak, P.** 2008. Cryopreservation of recalcitrant (i.e. desiccation-sensitive) seeds. In: Reed B.M. (ed) *Plant Cryopreservation: A Practical Guide*. Springer, New York, pp. 465-484.
- Wang, X., Hua, T., Sun, D., Liu, B., Yang, G. and Cao, Y.** 2007. Cryopreservation of tissue-engineered dermal replacement in Me₂SO: toxicity study and effects of concentration and cooling rates on cell viability. *Cryobiology*, 55: 60-65.
- Wesley-Smith, J.** 2002. Investigations into the responses of axes of recalcitrant seeds of dehydration and cryopreservation. PhD Thesis, University of Natal (now University of KwaZulu-Natal), Durban, South Africa.
- Wesley-Smith, J., Vertucci, C.W., Berjak, P. and Pammenter, N.W.** 1991. Freezing rate, ultrastructural preservation and survival of embryonic axes of *Camellia sinensis*. *Electron Microscopy Society of Southern Africa*, 21: 175-176.

- Wesley-Smith, J., Berjak, P., Pammenter, N.W. and Vertucci, C.W.** 1995. Ultrastructural evidence for the effect of freezing in embryonic axes of *Pisum sativum* L. at various water contents. *Annals of Botany*, 75: 59-64.
- Wesley-Smith, J., Pammenter, N.W., Walters, C. and Berjak, P.** 2001. The effects of two drying rates on the desiccation tolerance of embryonic axes of recalcitrant jackfruit (*Artocarpus heterophyllus* Lamk.) seeds. *Cryobiology* 42: 196-206.
- Wesley-Smith, J., Walters, C., Berjak, P. and Pammenter, N.W.** 2004a. Non-equilibrium cooling of *Poncirus trifoliata* L. embryonic axes at various water contents. *CryoLetters* 25: 121-128.
- Wesley-Smith, J., Walters, C., Berjak, P. and Pammenter, N.W.** 2004b. The influence of water content, cooling and warming rates upon survival of embryonic axes of *Poncirus trifoliata*. *CryoLetters* 25: 129-138.
- Wesley-Smith, J., Berjak, P., Pammenter, N.W., and Walters, C.** 2014. Intracellular ice and cell survival in cryo-exposed embryonic axes of *Acer saccharinum*: an ultrastructural study of factors affecting cell and ice structures. *Annals of Botany* 113: 695-709.
- Wesley-Smith, J., Pammenter, N.W., Walters, C., and Berjak, P.** 2015. Why is intracellular ice lethal? A microscopical study showing evidence of programmed cell death in cryo-exposed embryonic axes of recalcitrant seeds of *Acer saccharinum*. *Annals of Botany* (in press).
- Withers, L.A.** 1983. Germplasm storage in plant biotechnology. In: Mantell, S.H. and Smith H. (eds) *Plant Biotechnology*. Cambridge: Cambridge University Press, pp. 187-218.
- Withers, L.A.** 1991. *In vitro* conservation. *Biological Journal of the Linnean Society*, 43: 31-42.

- Withers, L.A. and Engelmann, F.** 1998. *In vitro* conservation of plant genetic resources. In: Altman, A. (ed) *Agricultural Biotechnology*. Marcel Dekker, New York, pp. 57-87.
- Whitaker, C. Beckett, R.P. Minibayeva, F.V. and Kranner, I.** 2010. Production of reactive oxygen species in excised, desiccated and cryopreserved explant of *Trichilia dregeana* Sond. *South African Journal of Botany*, 76: 112-118.
- Wojtaszek, P.** 1997. Oxidative burst: an early plant response to pathogen infection. *Journal of Biochemistry*, 322: 681- 692.
- Yap, L.V., Hor, Y.L. and Normah, M.N.** 1999. Effects of sucrose preculture and subsequent desiccation on cryopreservation of alginate- encapsulated *Hevea Brasiliensis* embryo. IUFRO Seed Symposium 1998 "Recalcitrant seeds": Proceedings of the Conference, Kuala Lumpur, Malaysia, 12-15 October 1998: 140-145.
- Yap, L.V., Noor, N.M. Clyde, M.M. and Chin, H.F.** 2011. Cryopreservation of *Garcinia cowa* shoot tips by vitrification: the effects of sucrose preculture and loading treatment on ultrastructural changes in meristematic cells. *CryoLetters*, 32: 188-196.
- Zamecnik, J., Faltus, M., Bilavcik, A. and Kotkova, R.F.** 2012. Comparison of cryopreservation methods of vegetatively propagated crops based on thermal analysis. In: *Agricultural and biological sciences: Current frontiers in cryopreservation*. In Tech. Available from: <http://www.intechopen.com/books/current-frontiers-in-cryopreservation/comparison-of-cryopreservation-methods-of-vegetatively-propagated-crops-based-on-thermal-analysis>.
- Zamecnik, J., Faltus, M., Kotkova, R. and Hejnak, V.** 2011. Glass transition determination in *Allium* shoot tips after dehydration. *Acta Horticulturae*, 908: 33-38.

Zhao, M.A., Dhital, S.P., Fang, Y.L., Khu, D.M., Song, Y.S., Park, E.J., Kang, C.W. and Lim, H.T. 2005. Application of slow-freezing cryopreservation method for the conservation of diverse potato (*Solanum tuberosum* L.) genotypes. *Journal of Plant Biotechnology*, 7: 1-4.