Towards ameliorating some of the stresses associated with the procedural steps involved in the cryopreservation of recalcitrant-seeded germplasm

by

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Submitted in fulfilment of the academic requirements for the degree of Doctor of Philosophy in Science

in the School of Life Science

College of Agriculture, Engineering and Science

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Durban Westville

South Africa

February 2017

Preface

The research contained in this thesis was completed by the candidate while based in the Discipline of Biology, School of Life Science of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Durban Westville, South Africa. The research was financially supported by the National Research Foundation.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

Declaration 1: Plagarism

I, Cassandra Dasanah Naidoo, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

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

(iv) 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, their writing has been placed inside quotation marks, and referenced;

(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii) 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 dissertation and in the References sections.

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Cassandra Dasanah Naidoo

Declaration 2: Publications

My role in each paper and presentation is indicated. The * indicates corresponding author.

1. Cassandra Naidoo Berjak P, Pammenter NW, Varghese B*. 2016. The role of reactive oxygen species and anti-oxidants during pre-cooling stages of axis cryopreservation in recalcitrant *Trichilia dregeana* Sond. Botany 94: 391-403.

2. Sershen Naidoo*, Varghese B, Cassandra Naidoo, Pammenter NW. 2016. The use of plant stress biomarkers in assessing the effects of desiccation stress in zygotic embryos from recalcitrant seeds: challenges and considerations. Plant Biology 18: 433-444.

3. Cassandra Naidoo, Berjak P, Bharuth VB, Pammenter NW, Varghese B. Effects of post-cryopreservation decontamination on viability of embryonic axes of *Trichilia dregeana*. Microscopy Society of Southern Africa, Pretoria 2013.

4. Cassandra Naidoo, Berjak P, Bharuth VB, Pammenter NW, Varghese B. Overcoming oxidative and physical barriers towards the cryopreservation of desiccation-sensitive embryonic axes: a case study on *Lychee sinensis*. 1st International Conference of New Frontiers in Anhydrobiosis, France 2014.

5. Cassandra Naidoo, Berjak P, Bharuth VB, Pammenter NW, Varghese B. Comparative studies on oxidative state, ultrastructure and survival between temperate and tropical recalcitrant-seeded species in relation to cryopreservation. 11th Conference of the International Society for Seed Science, China 2014.

6. Cassandra Naidoo, Berjak P, Bharuth VB, Pammenter NW, Naidoo S, Varghese B. An ultrastructural and biochemically focused comparative study on mechanisms underlying survival during cryopreservation: a case study on four recalcitrant-seeded species. 7th International Workshop on Desiccation Sensitivity and Tolerance across Life Forms 2016.

The research reported on is based on the data I collected from optimising and analysing cryopreservation protocols. I designed the experiments, collected and analysed the data and wrote the first paper with input from my supervisors Prof. Berjak, Prof. Pammenter and Dr Varghese.

The second paper was written by Dr Sershen Naidoo, with my scientific contribution forming an aspect of the experimental work conducted on *T. dregeana* and *S. gerrardii*.

Conference presentations (3-6) were orally presented by myself, based on my own experimental work and analysis thereof, with guidance from my supervisors Prof. Berjak, Prof. Pammenter, Dr Varghese and Dr Naidoo.

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Cassandra Dasanah Naidoo

Abstract

There is an acute need for rapid and effective conservation efforts given the threats to plant biodiversity. Long term conservation of plant germplasm has far reaching implications for food security and biodiversity conservation in the face of climate change. Germplasm of recalcitrant-seeded species, unlike those of orthodox (desiccation tolerant) types, cannot be stored in the long-term using conventional methods (e.g. seed storage) due to the ongoing metabolic activity, high shedding water contents, desiccation sensitivity and often chilling sensitivity of recalcitrant seeds. Cryopreservation is currently the most promising long-term storage method for preservation of recalcitrant-seeded germplasm and generally involves storage of excised zygotic embryos in, or above, liquid nitrogen (-150°C to -196°C). For the present study, explants from four recalcitrant-seeded species, viz., *Trichilia dregeana*, *Quercus robur*, *Lychee sinensis* and *Strychnos gerrardii*, were used to characterise the stresses induced by the various procedural steps involved in the cryopreservation of recalcitrant-seeded embryos. The study went on to investigate how some of these stresses may be ameliorated in order to design more appropriate cryopreservation protocols. Cryopreservation of the zygotic explants from all the selected species has been previously attempted, with varying degrees of success. The procedural steps investigated included excision, cryoprotection, flash drying, cooling and retrieval from the cryogen. The stresses associated with each of these steps were characterised using a range of established stress biomarkers (viz. superoxide production, hydrogen peroxide production, total aqueous antioxidant capacity, respiratory activity and cellular ultrastructure). Selected steps were then modified using potential stress amelioration techniques in an effort to improve post-cryo survival. The stress amelioration methods included techniques to (i) reduce the damage incurred during explant excision, (ii) improve partial dehydration rates and (iii) ameliorate oxidative stress by supplying exogenous antioxidants (ascorbic acid and cathodic water) during specified steps of the protocol. Stress biomarkers measured at each of the procedural steps were compared within species, across procedural steps and within procedural steps, across species in order to elucidate, compare and contrast explant responses.

Stresses identified to occur at the different procedural steps were related to explant anatomical and physiological characteristics and ultimately explant viability; untreated

embryos served as the control in all cases. The relationship between (i) explant tissue architecture and physiological characteristics with viability and (ii) stress biomarkers and viability, during procedural steps were comparatively and correlatively analysed to establish the factors that either hampered or promoted explant survival. Comparative data interpretation of basal (control) and measured levels of reactive oxygen species (ROS) and aqueous antioxidants with explant viability did not indicate clear trends during procedural steps, which presented a challenge in elucidating the role of these biomarkers in survival. To overcome this, an alternate method of assessing the competence of the antioxidant system using a ratio of total ROS to total antioxidants (TAA:ROS) was investigated.

Previous studies on cryopreservation of recalcitrant germplasm have shown excision, desiccation and cooling to all contribute to explant loss of viability, with the stresses associated with each of these step being primarily related to oxidative metabolism. It had also been suggested that a cause of cell death during cryopreservation of recalcitrant-seeded embryos was the unregulated activity of ROS combined with dysfunctional endogenous antioxidant systems.

Analysis of explant anatomy, physiology and stress biomarkers using the methods stated above resulted in the following principle findings:

(i) ameliorative measures taken to abate physical and oxidative injury are necessary implementations in explant cryopreservation of the investigated species,

(ii) tissue architecture, particularly the position of meristematic regions and location and number of incision sites made during final explant excision affected physiological responses after procedural steps,

(iii) explants did not respond favourably to the cryoprotectants tested; therefore, cryoprotection is not recommended as an appropriate pre-treatment for cryopreservation of zygotic explants of the species investigated,

(iv) damage to shoot meristem cellular ultrastructure was not apparent after excision and post-excision exposure to antioxidants but varying degrees of damage did feature in explants (across species) after partial dehydration and cooling,

(v) a greater antioxidant capacity and reduced levels of superoxide production contributed to higher explant viability and,

(vi) higher TAA: ROS ratios corresponded with higher viability retention during all procedural steps.

While the protocols developed resulted in acceptable levels of post-cryo survival in two species, viz. *S. gerrardii* and *Q. robur, T. dregeana* and *L. sinensis* did not survive cryopreservation despite the stress amelioration techniques adopted. Future studies should therefore investigate the use of alternate explants (e.g. shoot meristems) for cryopreservation of *T. dregeana* and *L. sinensis* and aim to reduce the stresses associated with excision, partial dehydration and cooling. Additionally, the use of antioxidants such as ascorbic acid and cathodic water to ameliorate oxidative stress during the various procedural steps is recommended. This study has highlighted the fact that the importance of species-specific anatomical, physiological and biochemical factors in influencing zygotic embryo survival after cryopreservation and identified some of the stresses contributing to explant post-cryo viability loss in recalcitrant-seeded species. This research contribution has made significant advances towards elucidating and ameliorating some of the stresses associated with the cryopreservation of recalcitrant-seeded germplasm and yielded successful cryopreservation protocols for zygotic explants of two recalcitrant-seeded species.

Acknowledgements and dedication

First and foremost I am thankful for the presence and protection of God throughout my studies. For I can do all things through Him who strengthens me.

To my supervisor Norman, thank you for never failing to teach me something new. Your guidance, advice and support over the years made this journey more than just academically fulfilling, my research was a source of happiness under your supervision. You moulded my scientific thinking and for that I am immensely grateful.

To my co-supervisors Boby and Sershen, thank you both for the tireless hours of guidance and assistance. Boby, thank you for being with me every step of the way, until the very end. I am so grateful for your sacrifices and efforts to ensure I was the very best student I could be. Your dedication to my research and wellbeing is more than I could have asked for. Thank you for always doing your best for me. Sershen, you believed in me before I believed in myself...you are truly an inspiring scientist. Thank you for pushing me towards excellence.

To my parents, I could ask for no better support structure. I am deeply appreciative for your unfailing willingness to help me realise my dreams and the love and guidance you provided along the way. I could not have achieved my goals without you. To my brother Kody, thank you for your companionship throughout this journey. Our laughter and your encouragement throughout the long working hours made me push through the tough times...I am grateful to have been blessed with such a wonderful and caring sibling. To my grandparents, I am the product of the strong foundation you built our family on....I am proud to have my roots emerge from your struggles and hardship. You taught me that greatness stems from perseverance against all odds...I love you all.

To my friends who are more like my family, Udisha, Preyan, Yolanda, Prenay, Pregasan and Elliosha...thank you all for the love and motivation. I cannot thank you enough for giving me those precious moments away from the lab...I am truly grateful for the circle of support you all provided. To one of my best friends and colleagues Vishal, thank you for your assistance with the microscopical aspect of my research. But more so...thank you for making the endless working hours so enjoyable...your friendship means the world to me.

This thesis is dedicated in loving and fondest memory of my late supervisor and mentor Prof. Patricia Berjak.....

Of all the scientists who have become bearers of the torch of discovery in our quest for knowledge, to me your torch will forever shine the brightest of them all. For pushing me towards excellence and for being the unwavering source of strength behind me...I am eternally grateful...you were a once in a lifetime blessing and you remain loved, treasured and remembered my dearest Pat...the most brilliant and inspiring woman I will ever know.

Table of contents

Page

List of tables

Table **Page**

List of figures

Figure **Page**

List of abbreviations and symbols

Ψ_w	water potential
2 ip	6-(γ,γ-Dimethylallylamino) purine
$^{\cdot}O_2^-$	superoxide radical
$^1O_2$	singlet oxygen
$^{\cdot}OH$	hydroxyl radical
ABA	abscisic acid
ABTS•1	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
AC	activated charcoal
ANOVA	analysis of variance
APX	ascorbate peroxidase
AsA	ascorbic acid
AsC	ascorbate
ATP	adenosine triphosphate
BAP	6-benzylaminopurine
C	cooling
Ca^{2+}	calcium cation
$CaCl_2$	calcium chloride
CAT	catalase
CaMg	calcium magnesium
$Ca(OCl)_2$	calcium hypochlorite
CP	cryoprotection
Conc.	concentration
Cu^{2+}	copper cation
CW	cathodic water
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase

DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dmb	dry mass basis
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organisation of the United Nations
FD	flash dried/drying
Fe^{3+}/ Fe^{2+}	iron cation
Fe_2SO_4	iron suphate
FW	fresh weight
GDP	gross domestic product
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	oxidised glutathione
H_2	hydrogen
H_2O_2	hydrogen peroxide
$HO_2^{\cdot}$	hydroperoxyl radical
IBPGR	International Board for Plant Genetic Resources
IUCN	International Union for Conservation of Nature
$K_2S_2O_8$	potassium persulphate
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
LEA	late embryogenic abundant/accumulating
LPI	Living Planet Index
LN	liquid nitrogen
MAPK	Mitogen-Activated Protein Kinase

MDHA	monodehydroascorbate
MDHAR	monodehydroascorbate reductase
Mn	manganese
MS	Murashige and Skoog medium
NaDCC	sodium dichloroisocyanurate
NAD(P)H	reduced nicotinamide adenine dinucleotide phosphate
NaOCl	sodium hypochlorite
$(NH_4)_2SO_4$	ammonium sulphate
PCD	programmed cell death
PEG	polyethylene glycol
pH	negative logarithm of hydrogen ion concentration
pKa	negative logarithm of acid dissociation constant
PBS	phosphate buffered saline
PS	photo system
PVP	polyvinylpyrrolidone
PVS	plant vitrification solution
Rboh	respiratory burst oxidase homolog
RH	relative humidity
RNA	ribonucleic acid
ROS	reactive oxygen species
RW	rewarming
SANBI	South African National Biodiversity Institute
SOD	superoxide dismutase
SSA	Sub-Saharan Africa
rpm	revolutions per minute
TAA	total aqueous antioxidants
TEM	transmission electron microscopy

Tg°C	glass transition temperature
TTC/TTZ	2,3,5 triphenyl tetrazolium chloride
TPF	1,3,5 triphenyl formazan
Tween 20/80	polyoxyethylene sorbitan monolaurate
USDA	United States Department of Agriculture
UV	Ultra violet
V FD	vacuum flash dried/drying
WC	water content
Zn	zinc

Units of measurement

%	Percentage
°C	degrees celcius
°C min^{-1}	degrees celcius per minute
°C s-^{1}	degrees celcius per second
µg	microgram
µm	micrometre
µM	micomolar
µl	micolitre
µs	microsecond
µmol	micromole
d	day/s
g	gram
g g^{-1}	gram per gram
g *l*$^{-1}$	gram per litre
h	hour/s
l	litre
M	molar
mg	milligram
min	minute/s
m*l*	millilitre
ml *l*$^{-1}$	mil per litre
mm	millimetre
mM	millimolar
MPa	megapascal
m^{2} s^{-1}	square metre per second
mV	millivolts

nm	nanomolar
s	second/s
Tg°C	glass transition temperature
V	volts
v/v	volume/volume
w/v	weight/volume

CHAPTER 1: Introduction

1.1 Risks to Plant Biodiversity

Biodiversity is widely cited as being defined as the disparity amongst genes, species and ecosystems whereby ecosystem diversity further encompasses variability in the attributes of structure, composition and function of ecosystems (Franklin *et al*., 1981; Noss, 1990). The unprecedented rate of climate change and human encroachment has placed millions of plant and animal species at risk of extirpation. In the last 500 years, human activity has caused 869 species extinctions (International Union for the Conservation of Nature, 2007). According to IUCN (2007) the extinction rate of species is between 1 000 and 10 000 times higher than the natural rate of species loss. Implications of this extinction rate on global biodiversity are as follows: one in eight birds; one in four mammals; one in five plants and one in three amphibians are at risk of extinction. Further to this, 75% of the genetic diversity of agricultural crops has already been lost (Food and Agricultural Organisation of the United Nations, 1999a). Plant pathogens are also predicted to reduce crop yields by up to 16% globally, and the impact will have a major influence on food security especially in Sub-Saharan Africa (Royal Botanic Garden KEW, 2016). If continued uncontrolled, anthropogenic activities will result in a global temperature rise of more than 3.5°C during this century, and it is estimated that 8% of the 8 300 known animal breeds will become extinct and 22% of these species will be at risk of extinction. Scientists also predict that up to 70% of all known species will be at risk of extinction (FAO; IUCN, 2015).

Variation and variability of plant species are vital for sustaining key functions and processes of the agricultural biodiversity ecosystem for food production and security (FAO, 1999b). The increase of human impact on a global scale necessitates the urgent need to identify biomes of the greatest plant biodiversity and concurrently, the most threatened species therein. It is unfeasible to completely eradicate the principal pressures on global biodiversity which commonly include - but are not limited to - deforestation, habitat fragmentation, habitat loss and degradation, over exploitation and unsustainable use of wild species populations, encroachment on valuable wildlife habitats, excessive nutrient load and other forms of pollution, invasive alien species, wild fires, logging, hunting and human-induced

climate change (Living Planet Report, 2010; IUCN, 2011; Abdu-Raheem & Worth, 2013). Therefore, securing biologically important resources for the future and ensuring robust environmental stability depends heavily on current conservation strategies.

Agriculture plays a key role in sustaining economic growth and food security in Sub-Saharan African (SSA) countries, where it constitutes for 30% of the Gross Domestic Product (GDP) and creates employment for approximately 75% of the population (Munthali *et al*., 2012). However, SSA is considerably behind North African and Asian regions in agricultural productivity (Moyo *et al*., 2015) which is a reflection on the degradation of the natural resource base. There are similar concerns in Southern African countries which house 582 national parks and nature reserves covering 6% of the region, within which only 34% of plants are protected (Southern Africa Resource Document, 2016). In South Africa alone, 2577 (13%) species of plant taxa are threatened with extinction and a further 2232 (11%) are listed under various categories of conservation concern (Abdu-Raheem & Worth, 2013). The combined proportion of threatened taxa and those listed under conservation concerns is currently at 24% of South African flora which translates to roughly one in four species that are threatened, with 20 national vegetation types listed as critically endangered (Raimondo, 2011; South African National Biodiversity Institute, 2015).

The efforts undertaken towards conserving plant biodiversity in South Africa thus far has been somewhat progressive, and in terms of agriculture, efforts in increasing soil fertility, usage of better fertilisers and management and use of better seed varieties have been made. However, the escalating pressure on natural resources combined with the under-performance of the agricultural sector calls for higher levels of proficiency in current and future initiatives towards securing long-term preservation of plant species for food security and biodiversity conservation in its entirety. Therefore, endeavours towards preservation of threatened and economically and medicinally important plant species is a primary research focus.

1.2 Preservation of plant genetic resources

Plant genetic resources include a conglomerate of seed varieties, traditional and modern cultivars of planting accessions, crop wild relatives, breeding lines, genetic stocks and weedy types, all of which forms the biological basis for improvement and selection through breeding

for food security while directly or indirectly supporting the livelihood of all individuals (International Plant Genetics Resource Institute, 1993; Upadhyaya *et al*., 2008; FAO, 2016). Plant genetic diversity represents the basis for agricultural development and serves to bolster resilience in an ecosystem, i.e., genetic adaptability acts as a buffer against environmental change by reducing vulnerability to pressures such as drought, climate change, coastal erosions etc. (Esquinas-Alcázar, 1993; IUCN, 2010; Abbas & Qaiser, 2011).

Plants provide a source of phytochemicals which are used as active pharmaceutical ingredients (Samy & Gopalakrishnakone, 2010) and are vital for plant breeding programmes throughout the world (Panis & Lambardi, 2005). Plant resources are widely utilised for food, fibre, feed for domesticated livestock, clothing, energy, shelter and medicinal purposes around the globe, and for these reasons all countries are fundamentally interdependently linked in conservation of and accessibility to plant germplasm. Conservation of plant germplasm in a sustainable manner has far reaching implications for food security, job creation, poverty alleviation, environmental protection and climate change mitigation (Upadhyaya *et al*., 2008). There is an acute need for rapid, decisive and effective conservation efforts in the face of widespread erosion of plant diversity.

1.2.1 *In situ* conservation

The preservation of plant genetic resources can be done within their natural habitats (*in situ*), under conditions different from their natural habitats (*ex situ*) or using both strategies complementarily (Cohen *et al*., 1991; Hammer & Teklu, 2008). The implemented conservation method(s) is dependent on the target species and the specific need for germplasm conservation which is ultimately based on logical, scientific and economic criteria. *In situ* conservation of germplasm usually involves conservation of genetic material in natural reserves or a properly managed ecological continuum, where populations continue to evolve in the environments which have influenced the development of their distinctive traits, i.e., perpetuation of the natural progression of evolutionary processes (Benson, 2008; Hammer & Teklu, 2008). Also referred to as Genetic Reserve Conservation, this approach involves the location, management and monitoring of genetic diversity in natural wild populations within defined areas designated for active, long-term conservation, where large

ecosystems can be protected as conservation reserves with minimum intrusion and disruption by humans (Maxted & Bennet, 2001).

In situ conservation offers the distinct advantages of permitting selective and adaptive processes to occur which gives rise to genes that are able to respond to changing environmental stresses (Hummer, 1999), as well as host-parasite co-evolution which is likely to result in material resistant to pests and diseases (Hawkes, 1991; Kasso & Balakrishnan, 2013). It also acts as a source for genes not as yet captured by *ex situ* methods and as "live laboratories" for the study of genes that are progenitors to new and current crop cultivars (Montnegro de Wit, 2016). It is the recommended method of conservation for tropical fruits, wild species related to domestic crops and forest tree species with short seed longevity (Montenegro de Wit, 2016). However, *in situ* conservation has the major limiting factor of significant loss of preserved species in the event of natural and man-made disasters (Li & Pritchard, 2009; Paunescu, 2009), and the restricted spectrum of diversity that can be enclosed within natural reserves due to space and land availability constraints (Hawkes, 1991). Of great concern is the fate of long-lived species, e.g., cycads and many temperate tree species, as renewal–replacement cycles take decades to centuries to complete (Queensland Herbarium, 2007). It is therefore prudent to combine both *in situ* and *ex situ* strategies, but in certain cases *ex situ* conservation is the only viable method for long term conservation.

1.2.2 *Ex situ* conservation

Ex situ conservation encompasses active collection methods such as seed storage, *in vitro* cultivation, field gene banks and botanical gardens and base collections of material for potentially indefinite periods (Maunder *et al*., 2004). This includes the storage of an array of germplasm types including meristems, shoot tips, embryos (both zygotic and somatic), embryonic explants, protoplasts, cell suspension culture, seeds, pollen, buds and DNA (Panis & Lambardi, 2005; Radha *et al*., 2012), of which seed storage in the short (3-5 years) and medium term (30 years or longer) is the most common *ex situ* conservation method for forest trees (FAO, 1993). Storage of orthodox seeds is the primary *ex situ* conservation method employed by genebanks (Engelmann, 1998) and approximately 90% of the reported 6.1 million germplasm accessions are stored as seed in 1300 genebanks worldwide (Walters & Engels, 1998).

Maintaining genetic diversity as seeds that are attained – usually from a range of cultivars – is considered to be the most expedient, widely-used, cost-effective and researched means of *ex situ* conservation (Justice & Bass, 1978; Engels & Visser, 2003). Importantly, preservation of genetic stock in the form of seeds must ensure maintenance for an indefinite period while retaining the highest possible viability (Vertucci & Roos, 1990) and therefore optimisation of seed storage conditions is imperative. Factors such as the chemical composition, physiology, shedding water content (WC) and physical status of water within seeds must be considered for each species separately, but in general, seeds must be maintained under conditions of reduced temperature, relative humidity (RH) and oxygen to prolong longevity (Justice & Bass, 1978; Vertucci & Roos, 1993). Storage methods largely depend on seed storage physiology, where recalcitrant (desiccation/chilling sensitive, large, high shedding WC) and orthodox (desiccation/chilling tolerant, small, low shedding WC) seeds necessitate storage protocols that differ in relative humidity, temperature and gas exchange conditions (FAO, 1993).

Two chief requirements for long-term storage of orthodox seeds are: partial drying i.e., seed moisture content (fresh mass basis) of ≈ 3% for oily seeds and ≈ 5% or slightly higher for starchy seeds, and low storage temperature (-18°C to -20°C; Bonner, 2008). While desiccation and maintenance at low temperature is ideal for long-term conservation of most woody plant species that produce seeds tolerant to dehydration and chilling, a major shortcoming of this approach is the applicability to only orthodox seeds. This type of storage is not appropriate for a variety of species such as many forest angiosperms that produce recalcitrant, immature or sterile seeds; vegetatively propagated plants that do not produce seeds, e.g., banana, clonally propagated crops such as fruit, nut, root and tuber vegetables, and cultivars that require maintenance of a unique true-to type genomic constitution but produce highly heterozygous seeds that are susceptible to deterioration due to seed-borne pathogens (Engels & Visser, 2003; Panis & Lambardi, 2005; Taha *et al*., 2007; Paunescu, 2009; Radha *et al*., 2012).

In vitro cultivation refers to the maintenance of cultures in the growing state, e.g., actively growing cultures or minimal growth cultures for the purpose of *ex situ* conservation of plant genetic resources (Johnson, 2002). Actively growing cultures involve the ongoing transfer of

in vitro grown plant material on to fresh medium at regular intervals while minimum growth involves manipulating certain physical environmental conditions (e.g. light and temperature) and components of the tissue culture environment (via growth medium manipulations) in order to slow down growth *in vitro* (Staden *et al*., 2008). Both have the advantages of preserving large amounts of material in small areas and provision of bulk material for multiplication and distribution of species (Withers, 2001). However, this technique is not without limitations as there is the prospect of losing accessions due to contamination, human error and somaclonal variation (spontaneous mutations that take place during tissue culture with an increased occurrence upon frequent sub-culturing), when working with actively growing cultures (Larkin & Scowcroft, 1981; Withers, 2001). The primary risk with minimal growth cultures is the susceptibility of tissue to viability loss caused by the stress imposed by growth retardation (Withers, 2001).

In vitro conservation is primarily a biotechnological approach, a facet of which involves cryobanking (cryopreserved collections of genetic resources), and is one of the most complex and specialized forms of *ex situ* conservation of plant resources for their retention and restoration in culture collections and germplasm repositories (Maunder *et al*., 2004; Benson, 2008). Owing to various problems and limitations with both genetic reserves and field genebanks, cryopreservation or freeze-preservation is considered to be the only realistic, safe and cost-effective tool for the long-term conservation of plant genetic resources of recalcitrant-seeded species (Engelmann, 2000; Withers, 2001; Engels & Visser, 2003; Panis & Lambardi, 2005; Normah & Makeen, 2008; Paunescu, 2009; Radha *et al*., 2012). Cryopreservation of recalcitrant-seeded species forms the focus of the current study and is discussed in greater detail in Section 1.4.

1.3 Seed storage behaviour: comparative responses between the orthodox and recalcitrant state

Seed post-harvest physiology is an important factor to consider before establishment of a storage protocol. Regardless of the type of germplasm extracted from seeds for long-term storage, the responses to any conservation method will depend heavily on post-harvest seed behaviour and their storage characteristics. Both factors differ considerably from species to species including those closely related within a genus (Hong & Ellis, 1996). Early

considerations of seed storage behaviour in terms of storage method and longevity did not account for responses to the environment (Ewart, 1908; Altman & Dittmer, 1972; Harrington, 1972). Roberts (1973) differentiation between orthodox and recalcitrant seeds was not based on longevity as such, but rather looked at behaviour within the context of contrasting physiological responses to moisture content and temperature. As alluded to earlier, orthodox seeds are those that can be dried to very low WC and in this state be successfully stored at sub-freezing temperatures, while recalcitrant seeds do not undergo maturation drying in their final developmental phase, tolerate very little post-shedding desiccation (Chin & Krishnapillay, 1989; Bewley & Black, 1994; Vertucci & Farrant, 1995) and are often chilling-sensitive (Berjak *et al*., 1989; Bonner, 2008). Bonner (1990) further categorised seeds within each of these types based on the response to storage environment and adaptation to natural habitats. Seeds were categorised as true orthodox, sub-orthodox, tropical-recalcitrant and temperate-recalcitrant, the latter two categories being especially significant to this study and will be discussed in detail further below. Another category of seed behaviour was introduced by Ellis *et al*. (1990): the 'intermediate' seed category which falls between the extremes of orthodoxy and recalcitrance, where seeds exhibit tolerance to desiccation but not to the extent of orthodox seeds and often lose viability more rapidly at lower temperatures typically employed for storage of orthodox seeds.

Due to the considerable variation in seed responses to desiccation and storage within and between species it is not feasible to accurately and consistently describe seed behaviour under discrete behavioural groups and it was proposed that this categorisation should not be rigidly confined (Berjak & Pammenter, 1994, 1996; Pritchard, 2004; Berjak & Pammenter, 2008;). Rather, those authors suggested that post-harvest seed behaviour can be better described as an "open-ended continuum" subtended by extremes of orthodoxy and acute recalcitrance at either end with varying gradations of recalcitrant behaviour falling in between, depending on the natural habitat to which they are adapted. This manner of categorisation has its foundations in the physiological status of seeds at various water potentials and the properties of water at different hydration levels commensurate with different water potential ranges (Vertucci, 1993; Vertucci & Farrant, 1995). This accommodates for the marked differences in post-harvest behaviour across species and exemplifies the need for species-specific cryopreservation protocols which account for the extent to which germplasm can tolerate

desiccation. The response to removal of tissue water is pivotal to the placement of seeds along the continuum, however one should note that factors such as provenance, seed maturity, chemical composition of seeds and seed morphology also affects seed longevity and demands careful consideration (Daws *et al*., 2004; 2006; Bonner, 2008; Bharuth, 2012).

Orthodox and recalcitrant seed characteristics and behaviour is an extensive topic and within the context of this research these terms will be discussed further with regards to two practical aspects in seed storage: (i) effects of desiccation on seed viability and (ii) response of seed longevity to the storage environment, both of which are contrasting in recalcitrant and orthodox seeds (Hong & Ellis, 1990).

1.3.1 Orthodoxy

According to Vertucci and Leopold (1986), desiccation tolerance can be firmly associated with the properties of water in seeds at certain hydration levels corresponding to specific water potentials. Each hydration level is subtended by specific water potentials (ψ_w), within which water has distinct properties. Upon the removal of water from any hydration level, the metabolic and chemical processes that are facilitated by the WC within the tissue is no longer functional and thus protective mechanisms will cease to function, allowing for the accrual of desiccation induced damage. Seeds that have the ability to withstand almost complete loss of cellular water are termed orthodox (Vertucci & Farrant, 1995). For seeds to be defined as orthodox (Berjak *et al*., 1990), two requirements that must be satisfied are desiccation tolerance and amenability to storage at low temperatures. As explained by Roberts (1973), orthodox seeds acquire desiccation tolerance during development and can be dried down to moisture contents of 10% (fresh weight) or less without accumulating damage. Orthodox seeds do not display desiccation tolerance throughout all stages of development, the early stages of development being the least tolerant to desiccation. It is well appreciated that desiccation tolerance and maintenance thereof in dry orthodox seeds is controlled by the interplay of mechanisms and processes during their development (Berjak & Pammenter, 2002). However, the extent to which desiccation can be tolerated and the rate of post-harvest water loss differs significantly across species (Hong & Ellis, 1996; Pammenter & Berjak,

1999) and must also be considered within the context of the manner of drying (Vertucci & Farrant, 1995).

Orthodox seeds can be maintained in the dry state for predictable periods under defined conditions such that longevity, within limits, increases logarithmically with decreasing storage moisture content and temperature in a quantifiable manner as defined by the improved seed viability equation (Ellis & Roberts, 1980). Characteristically, orthodox seeds acquire the ability to withstand drying (maturation drying) after the acquisition of the ability to germinate, during which period the maximal dry weight of the seed is attained (Ellis & Pieta Filho, 1992). These seeds are shed at low WC which is in equilibrium with the relative humidity of the microclimate (Berjak & Pammenter, 2002; Hay & Probert, 2013). While seed composition influences equilibrium WC at any given relative humidity, all orthodox seeds can tolerate dehydration to WC approximately less than 5% on a dry mass basis (dmb) regardless of maturation drying being complete prior to shedding (Berjak & Pammenter, 2000).

Bonner (1990) further classified orthodox seeds based on longevity at sub-freezing temperature. "True orthodox" seeds are those dried to moisture contents < 10% and can be stored at sub-freezing temperatures for relatively long periods in this state, whereas "sub-orthodox" seeds can be stored under the same conditions but has a shorter storage life span, postulated as a consequence of lipid content or thin fruit/seed coats. Orthodox seed longevity seemingly extends with decreasing WC (Justice & Bass, 1978). Ellis (1998) and Sastry *et al*. (2007) demonstrated storage longevity of orthodox seeds may be more greatly influenced by moisture content than temperature. Those authors showed that seeds could remain viable at high temperatures (50 – 65°C), provided moisture content ranged between 1.7 – 3% under hermetically sealed (ultra-dry storage) conditions. While ultra-dry storage can improve seed longevity, it should be noted here that seeds have a finite lifespan and below the critical WC, further removal of water from seeds cannot extend longevity (Vertucci & Roos, 1990).

Survival in the desiccated state is the outcome of systematically co-ordinated multi-genic expression of tolerance mechanisms which include physical characteristics of cells and intracellular constituents; insoluble reserve accumulation; intracellular de-differentiation;

metabolic ‘switching off’; presence and effective operation of putatively protective substances including late embryogenic accumulating proteins (LEAs), production of sucrose and other oligosaccharides; effective anti-oxidant systems; and the presence and operation of repair systems during rehydration (Vertucci & Farrant, 1995; Pammenter & Berjak, 1999; Berjak & Pammenter, 2004). It is accepted that orthodox seeds possess and fully express a suite of mechanisms that allow for survival in the desiccated state, and some of these mechanisms are discussed further below in the context of seed storage.

Orthodox seeds usually maintain vigour and viability from harvest to the next growing season or for decades if stored at -18°C (International Board for Plant Genetic Resources, 1976) unless debilitated by xerotolerant storage fungi (Berjak *et al*., 1989). However, even under the most satisfactory storage conditions, orthodox seeds do have a finite lifespan and will eventually succumb to deterioration induced by seed ageing (Walters, 1998; Berjak, 2006; Fu *et al*., 2015). This can be caused by depletion of food reserves or alteration of their chemical composition; free-radical mediated lipid peroxidation; enzyme inactivation; membrane alteration (aged seeds have the propensity to lose cell constituents upon imbibition), cytoskeleton damage; and genetic damage such that cell DNA is unable to duplicate and divide (Roos, 1994; Murthy *et al*., 2003; Fu *et al*., 2015). Nevertheless, viability and genetic integrity of orthodox seeds can be maintained for decades if stored under the recommended storage conditions i.e. 3 – 7% seed WC and hermetically sealed in foil bags, aluminum cans or glass jars held at temperatures of -18°C ± 3°C or lower (Botanic Gardens Conservation International, 1997; FAO, 2011). Such conservation practices have proved reliable for preserving valuable genetic resources and providing planting stock for subsequent seasons, and have been endorsed by internationally recognised institutions involved with plant germplasm conservation (KEW Gardens, UK; FAO and Bioversity International, Italy).

1.3.2 Recalcitrance

Recalcitrant seeds do not undergo maturation drying as the final phase of development and do not fully acquire (Berjak *et al*., 1990) or express (Berjak & Pammenter, 2008; Berjak & Pammenter, 2013a) the mechanisms that confer desiccation tolerance (Farrant *et al*., 1993). Consequently, recalcitrant seeds tolerate very little post-shedding desiccation and are often sensitive to chilling, rendering them unstorable for useful periods by any conventional

method applied to orthodox seeds (Berjak *et al*., 1990; Hong & Ellis, 1990; Engelmann, 1998; Pammenter & Berjak, 1999). This presents a major obstacle towards long-term storage of many recalcitrant-seeded tropical, sub-tropical, tree and shrub species (Engelmann, 1998). South Africa, being of sub-tropical climate, encompasses mega-diverse flora and is home to three of the four recalcitrant-seeded species focussed on in this study.

A discerning characteristic between the orthodox and recalcitrant state in seeds is metabolic, chemical and mechanical damage associated with desiccation to different hydration levels (Fig. 1.1; Vertucci & Farrant, 1995), where orthodox seeds are thought to possess the appropriate tolerance mechanisms to counteract deleterious effects of water removal (Pammenter & Berjak, 1999). In desiccation-sensitive seeds, the expression and interaction of the above mentioned factors is incomplete or severely impaired, leading to metabolism-linked desiccation damage — often escalated by slow loss of water (Pammenter *et al*., 1998) — and eventually cell death (Vertucci & Leopold, 1986; Pammenter & Berjak, 1999; Walters *et al*., 2001).

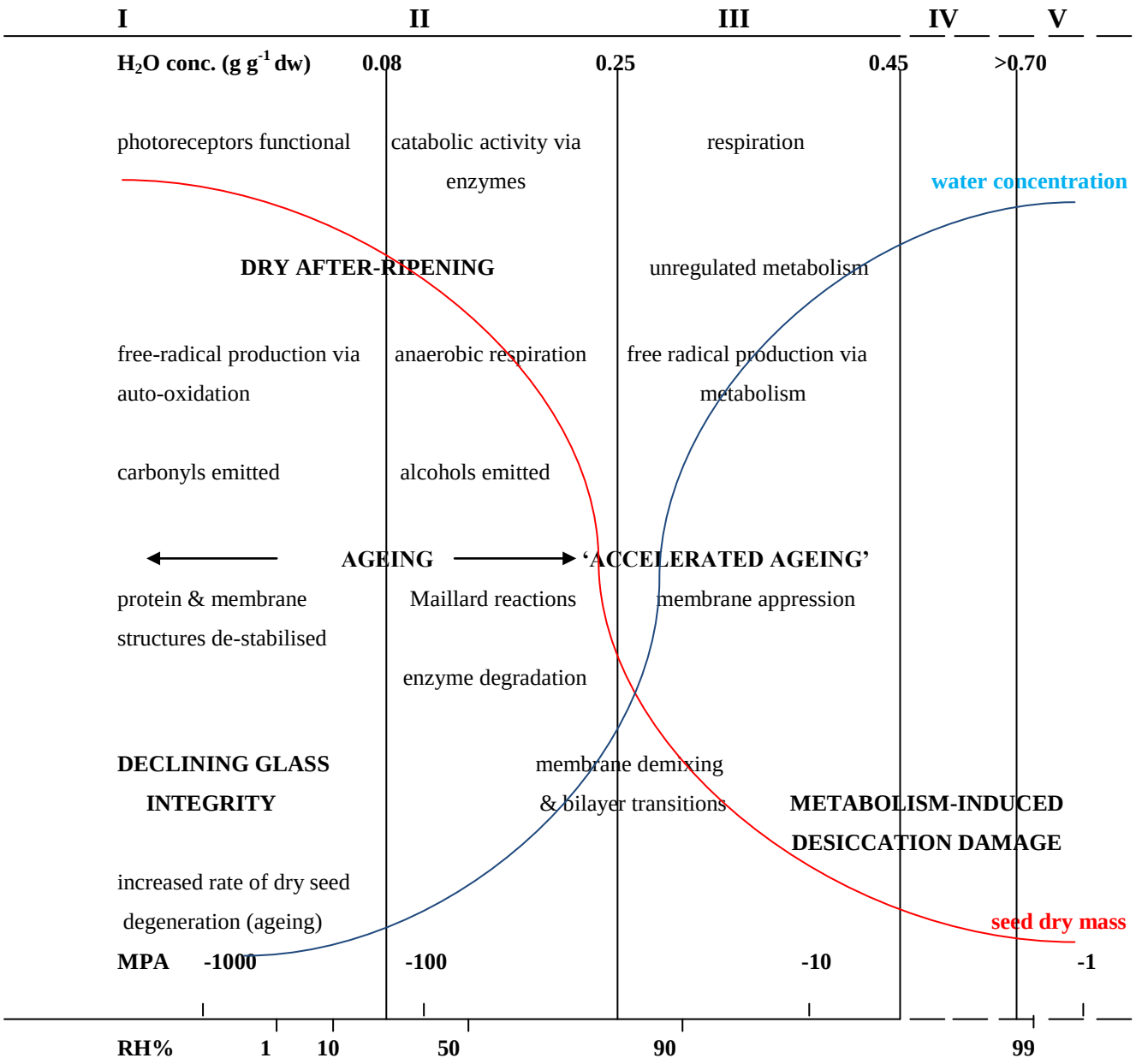

Fig. 1.1: A schematic representation of processes that are associated with hydration levels III to I. WC ranges (dmb) are reflected at the top of the figure, and equivalent water potentials (ψ) and relative humidities (%) are shown at the bottom (Berjak, 2006; Berjak *et al.*, 2007). Red and blue lines depict seed dry mass and water concentration respectively.

Ultimately, changes acquired to tolerate desiccation occur during embryogenesis but are not expressed completely in non-orthodox seeds (Berjak *et al.*, 1989; Vertucci & Farrant, 1995); therefore recalcitrance is a product of seed development (Berjak & Pammenter, 2004).

Recalcitrant seeds are sensitive to desiccation during development and post-shedding. However, a slight decline in WC prior to shedding has been noted in several temperate species, e.g., *Acer pseudoplatanus* (Hong & Ellis, 1990), *Acer hippocastanum* (Tompsett & Pritchard, 1993), *Quercus robur* (Finch-Savage & Blake, 1994) and some tropical species, e.g., *Machilus thunbergii* (Lin & Chen, 1995), suggesting acquisition of desiccation tolerance to a certain extent (Berjak & Pammenter, 2008). However, this decline in WC could be attributed to the characteristic accumulation of dry matter after fertilization in recalcitrant seed development (Finch-Savage & Blake, 1994). When recalcitrant seeds or recalcitrant-seeded germplasm are further artificially dehydrated to low WC (the levels of which are drying-rate dependent) associated with lower hydration levels, viability declines as a consequence of the appropriate protective and adaptive mechanisms being absent (Sun, 2000; Berjak & Pammenter, 2002). When WC and ψ_w continually drop through hydration level III, vital intracellular events become increasingly curtailed, resulting in unregulated metabolism (Vertucci & Farrant, 1995; Walters *et al*., 2005; Berjak, 2006). Removal of water in this hydration level causes perturbations in normal cellular function and if the seed tissues do not possess the mechanism(s) to counteract the induced stress – which is the case with recalcitrant material – either of the following types of damage may be incurred: (i) mechanical damage due to a reduction in cell volume and (ii) 'metabolism-induced damage', which involves aqueous-based degradative processes, and is influenced by both metabolic processes and rate of dehydration. In some instances, the removal of water can effect both types of damage (Sershen *et al*., 2016). In hydration level II (WCs generally ≤ 0.25 g g^{-1}; ψ approaching -100 MPa) desiccation damage *sensu stricto* is held to occur. This refers to damage ensuing when the primary stress is water removal, i.e., structure-associated (bound or non-freezable) water that is required to maintain intracellular integrity, is removed (Pammenter *et al.*, 1994; 1998; Pammenter & Berjak, 1999; Walters *et al.*, 2005; Berjak, 2006; Berjak *et al.*, 2007).

As with orthodox seeds, Bonner (1990) further categorised recalcitrant seeds into tropical and temperate varieties. Tropical-recalcitrant seeds predominantly originate from moist tropical ecosystems such as marshy or aquatic environments (wetlands, humid forests) and tolerate very little water loss while being markedly sensitive to low temperatures (Berjak *et al*., 1989; Bonner, 1990). Temperate-recalcitrant seeds commonly originate from dry colder regions and

can tolerate exposure to relatively low temperatures but may have similar or relatively lesser sensitivity to desiccation as tropical types (Berjak *et al*., 1989; Bonner, 1990). It is also accepted that the habitat of seed origination, development, maturation and shedding will influence the extent to which seeds are sensitive to desiccation and chilling, where seeds would only acquire properties offering a selective advantage in terms of survival (Berjak & Pammenter, 2008). For example, there is no reason for seeds to undergo maturation drying if the existing habitat is conducive to seedling establishment (Berjak *et al*., 1989). However, the extensive variability in post-harvest behaviour across recalcitrant seeds is a consequence of more than temperate or tropical origin. The fundamental cause of variation is the constant ongoing metabolic state prevalent in recalcitrant seeds due to a hydrated condition upon shedding. Apart from the shedding WC showing variation in itself across species and at times within a species across different seasons, ongoing metabolism inexorably changes the developmental status of seeds, thus behaviour even within the same species is incomparable from one season to the next (Berjak *et al*., 1989; Song *et al*., 2003; Malik *et al*., 2005). Notably, variation in desiccation sensitivity is also attributed to markedly different responses in the rate of water loss from tissue, as exemplified by Farrant *et al.* (1989).

Seed shedding WC also varies amongst and within recalcitrant-seeded species and across seasons, which can influence viability retention in response to desiccation. For seeds shed at WC close to the high range (>1.5 g g^{-1}) typical of tropical recalcitrant seeds, even the slightest loss of water is lethal, whilst those shed at lower WC will tolerate further desiccation but not without experiencing some deleterious metabolic and physiological events (Berjak & Pammenter, 2008).

The variation in shedding WC with and across species influences the quantification of desiccation sensitivity in a number of temperate and tropical recalcitrant-seeded species (Berjak *et al*., 1989: Farrant *et al*., 1989; Vertucci & Farrant, 1995; Pammenter & Berjak, 1999; Malik *et al*., 2005; Walters *et al*., 2008). However, Vertucci and Farrant (1995) suggested that common metabolic events occur at a particular water potential range during dehydration (Fig. 1.1), and it is pertinent to consider activities associated with specific hydration levels when aiming to investigate and ameliorate desiccation-induced damage. Dry orthodox seeds can be conserved under sub-zero to cryogenic temperatures based on the

required period of longevity, quantitative dynamics of temperature and seed aging kinetics (Pritchard & Dickie, 2003). For recalcitrant seeds, however, hydrated storage at reduced but above freezing temperatures is feasible only for short to medium term storage. Long-term conservation efforts must employ cryostorage, such that pre-cooling conditioning, freezing and retrieval from the cryogen is conducted in a manner that precludes lethal desiccation damage and ice formation (Pammenter & Berjak, 2000; Walters *et al*., 2008; Wesley-Smith *et al*., 2015). The size of recalcitrant seeds (small surface area: volume) however, restricts sufficiently rapid drying which is necessary to avoid lethal metabolism-linked damage occurring at WC around 0.8 g g^{-1} dmb (Pammenter *et al.*, 1994; 1998; Varghese *et al*., 2011). Studies have shown though that the excised embryonic/zygotic axis (zygotic explant) is ideal for cryopreservation since it represents genetic diversity of the species while also constituting a fraction of the volume and mass of the entire seed (Berjak & Pammenter, 2004). The rapidity at which the explant can be dried to sufficiently low WC appropriate for cryopreservation affords the opportunity to bypass metabolism-linked damage (Pammenter *et al*., 2002) thus facilitates the option for *in vitro* conservation (Hong & Ellis, 1990; Engelmann, 1991; Pammenter & Berjak, 1999; Berjak & Pammenter 2004; 2008). As in the present study, the excised embryonic axis is the zygotic explant of choice for cryopreservation of a number of recalcitrant-seeded species. To provide context for the present study the principals of cryopreservation and practical aspects of designing cryogenic protocols for the long-term storage of recalcitrant-seeded germplasm are discussed further below.

When undertaking cryopreservation of the zygotic explant, an understanding of the events occurring during freezing is essential to implement the best procedures that preclude or minimise the formation of injurious ice crystal formation. Section 1.4 describes freezing events and the possible damage that can be incurred during freezing and thawing which provides a basis for the cryopreservation techniques used for zygotic explants of selected species.

1.4 Principals of Cryopreservation

1.4.1. Mechanisms of freezing

Cryopreservation is defined as the storage of structurally intact, viable cells, tissues, organs and organisms at ultra-low temperatures in perpetuity (Mazur, 1970; Pegg, 1995), and is the only feasible method for long-term preservation of germplasm from recalcitrant-seeded species (Benson, 2008; Berjak & Pammenter, 2008; 2013a). It is essential to take cognisance of theoretical aspects of freezing biological tissues before attempting to design and implement cryogenic protocols. When cellular systems are exposed to a cryogen such as liquid nitrogen vapour (~140°C), liquid nitrogen (-196°C) or nitrogen slush (-210°C), ice formation is inevitable. As a result, profound changes in the biophysical and chemical properties of the surrounding *milieu* occur and cells physically respond to these changes (Mazur, 1970; 2004). The changes induced by freezing are largely influenced by the specific water properties of the tissues and cells being cryopreserved. Therefore, the water status of recalcitrant zygotic explants in relation to the biological effects of cooling is of paramount importance. Once the mechanisms of freezing are noted, one can then address the problems with tissue cryopreservation, which are essentially established as two types of injury: (i) direct and indirect mechanical damage through ice crystal formation and re-crystallisation during warming and (ii) toxicity via changes in the composition of the liquid phase via exposure to cryoprotectants (Pegg 1995; 2001; discussed further in Section 1.4.2). The relative contribution of mechanical damage and toxicity to limiting survival is dependent on cell type, drying, cooling and warming rates (Pegg, 2005). An overview of these principals and fundamental mechanisms involved in cryopreservation of recalcitrant zygotic explants will aid in understanding practical aspects of cryogenic protocols discussed later on.

The properties of water dictate the formation of the frozen, thawed and vitrified states in cryopreserved material (Benson, 2008). There are two key events concerning the crystallisation of ice during the freezing process, viz., ice nucleation and its subsequent growth (Meryman, 1966). These events are more meaningfully discussed within the context of three integral temperatures, i.e., 0°C, -40°C and the glass transition temperature (Tg°C), during which thermodynamic phase transitions of water occur which have important implications for the success of cryopreservation (Meryman, 1966; Meryman & Williams,

1985; Benson, 2008). Water is widely accepted to freeze at 0°C (Meryman & Williams, 1985), but in fact two conditions have to be satisfied for freezing to occur, i.e., water molecules must prevail in a system that is energetically favourable for crystallisation to occur and that system must provide a basis suitable for molecules to associate with each other (Mazur, 1970; Benson, 2008). Thus, water supercools (defined as lowering the temperature of a gas or liquid to below its freezing point without the phase transition to a solid) to freezing points far below 0°C (Meryman, 1966).

Water will supercool until the lowest freezing point is reached, which in most biological systems occurs at -40°C. This is the temperature at which homogenous ice nucleation occurs. Nucleation is the first step of a new thermodynamic phase and can be described as molecular bombardment of a minute ice crystal suspended in an aqueous solution (Meryman, 1966). Water molecules continuously bombard the ice crystal and will become part of it and contribute to growth when the molecule strikes at the right location with sufficient energy and the correct orientation (Benson, 2008). Homogenous ice nucleation is the random aggregation of molecules resulting in the development of a critical sized nucleus at -40°C (Meryman & Williams, 1985). A critical sized nucleus forms when conditions in the system are such that ice crystals have an equal chance of losing or gaining water molecules from their surface depending on the vapour pressure and convexity of their surface, and therefore exists at a critical size as there is equal propensity to grow or vanish (Meryman, 1966). More commonly, when crystallisation occurs at a temperature above which random aggregation of molecules occur, heterogeneous nucleation occurs which refers to ice formation catalysed by foreign particles (Meryman & Williams, 1985). When water transitions to ice, an ordered matrix is created concomitantly with a release of energy known as the latent heat of fusion (Meryman, 1966). One of the consequences of ice nucleation is the increased concentration of solutes within the system when liquid water is removed to form ice. Concentrated solute molecules impede mobility and interaction between water molecules which inhibits the further development of ice crystals (Benson, 2008). Above the temperature of -40°C, the freezing point of water is increasingly depressed to a eutectic point, which is the point at which the system solidifies and no further changes can occur as all water has frozen.

The glass transition temperature (Tg°C), is the second critically important event in cryopreservation (Meryman & Williams, 1985). It can be described as the temperature at which cells are dehydrated such that the remaining liquid cannot form a crystalline state due to insufficient energy of molecules in a highly viscous or glassy state (Benson, 2008). Below this temperature, no diffusion takes place and so all biological activities are suspended. The glassy, vitrified or amorphous state may also be described as the solidification of a liquid without it becoming solid and the restricted molecular motion occurring in this phase is crucial for long-term stability in cryostorage (Benson, 2008). The process of re-crystallisation can occur above Tg, where many small innocuous crystals can grow and form bigger, lethal ones. This is also a common event during the thawing and warming (rewarming) of tissue retrieved from the cryogen, but can be avoided or minimised if the rate of cooling is comparable to the rate of warming, i.e., rapid cooling followed by rapid warming (Mazur, 2004).

1.4.2 Freezing damage

The outline of mechanisms of freezing discussed above serves as the framework to discuss damage during cryopreservation in terms of ice formation and re-crystallisation. Early studies on freezing damage suggested that physical injury by ice crystals was the principal cause of lethality, thus the removal of intracellular water capable of forming ice crystals during freezing and re-warming was seen as crucial (Meryman & Williams, 1985; Normah *et al*., 1986). Later studies showed that there are direct and indirect effects of freezing, i.e., direct physical tearing of the cell by ice crystals or indirect freeze-induced dehydration leading to toxicity damage (Pearce, 2001; Mazur, 2004). There are two classical approaches to cryopreservation where one effects freezing (conventional cryopreservation) and the other theoretically prevents it (vitrification). The application of the first approach is more likely to cause damage in the physical sense due to intracellular and extracellular ice formation, the mode of induced damage being different in each. The application of vitrification based protocols is fraught with damage caused by toxicity (Pegg, 1995). Vitrification of cells is achieved in three ways, i.e., increasing solute concentration, decreasing WC and reducing temperature to below the glass transition temperature. All three manipulations can be injurious to recalcitrant-seeded zygotic explants (Pammenter & Berjak, 2014). Particularly, the WC necessary for vitrification to occur is often lethal to recalcitrant zygotic explants

which seldom remain viable below the lower limit (Pammenter *et al*., 2003). Since no cryopreservation procedure involved vitrification of tissue in this study, principles and practices of vitrification-based protocols will not be discussed further.

The rate of cooling greatly influences the location of ice crystallisation (Wesley-Smith *et al*., 2014; 2015). Slow cooling (approximately 10°C s^{-1}) causes almost exclusive formation of extracellular ice, often resulting in osmotic dehydration (Meryman, 1966). When ice is formed, solute concentration increases in the remaining liquid and it was postulated that the principal mechanism of freezing injury is caused via the secondary effect of ice formation, i.e., increased concentration of damaging solutes in the remaining liquid (Pegg, 2001). On this premise, Lovelock and Bishop (1959) explained that cryoprotectants such as glycerol with properties of high solubility in water and low cellular toxicity (Polge *et al*., 1949) played a vital role in survival by penetrating cells and reducing the mole concentration of detrimental solutes. Another significant role of cryoprotectants during cryopreservation is the increase in concentration of intracellular solutes that depress the freezing point of cells (Wolfe & Bryant, 1999; Fuller, 2004). However, cryoprotectant application and exposure is a component of cryopreservation that has to be carefully optimised as it can have lethal effects (Best, 2015) but this will be discussed under practical considerations of cryopreservation. In short, slow cooling causes injury through the individual and or combined effects of excessive cell shrinkage, reduction in temperature and exposure to increased solute concentration (Farrant & Morris, 1973).

In contrast, when the rate of cooling is increased (approximately 200°C min^{-1}) it creates more opportunities for nucleating events (Mazur, 2004). During rapid cooling, freezing occurs faster than osmotic transfer of water out of the cells which results in intracellular ice formation within the cytoplasm (Mazur, 2004). Intracellular ice is almost always lethal (Mazur, 2004) due to mechanical distortion of intracellular structures or cell membrane rupture due to an increase in volume from ice formation (Chaudhury, 2005), but it has been reported that ice crystals formed intracellularly below a critical size of 0.05 and 0.1 µm does not affect viability (Shimada & Asahina, 1975). Furthermore, it was later shown by Wesley-Smith *et al*. (2015) that the size, location and number of intracellular ice crystals play an influential role in survival during freezing in embryonic explants of recalcitrant-seeded *Acer*

saccharinum. In terms of damage in relation to cooling rates, survival is facilitated when an optimal rate can be achieved such that the cooling rate is rapid enough to minimise excessive cell volume reduction and solution effects but not so rapid that extreme intracellular ice formation is permitted (Mazur *et al*., 1969; Mazur, 2004).

Cooling rates and warming rates are interlinked such that optimisation of one cannot occur without consideration of the other (Pegg, 2001). Rewarming is one of the most important processes that affect post-cooling viability (Normah, 2008). During rewarming of tissue, damage occurs during the re-crystallisation of ice which is dependent on the rate at which heat is introduced back into the system (Meryman, 1966). When small ice crystals coalesce and form much larger ones, it is particularly detrimental to plasma membranes (Mazur, 1984). When slow cooling rates are used, it is accepted that the rate at which tissue is rewarmed is inconsequential to survival (Meryman, 1966). However, it is also known that slow rewarming promotes structural changes in the ice which may affect cell viability (Chaudhury, 2005) and is especially damaging if used in combination with fast cooling rates, but less detrimental to slow-cooled tissue (Wesley-Smith *et al*., 2004). Researchers have generally opted for rapid rewarming for the cryopreservation of embryonic explants from recalcitrant seeds (Sershen *et al*., 2007; Normah & Makeen, 2008; Berjak *et al*., 2011; Hajari *et al*., 2011; Sershen *et al*., 2012a; b) on the premise that ultra-rapid cooling rates are predominantly used for cooling of embryonic explants.

1.5 Procedural steps involved in cryopreservation of seed germplasm

Some of the theoretical and practical considerations for procedural steps involved in cryopreservation of seed germplasm, more specifically recalcitrant seeds, are summarised in Fig. 1.2. Furthermore, the stresses and difficulties associated with many of these procedural steps are discussed here to provide the basis for preventative and ameliorative measures investigated in this study.

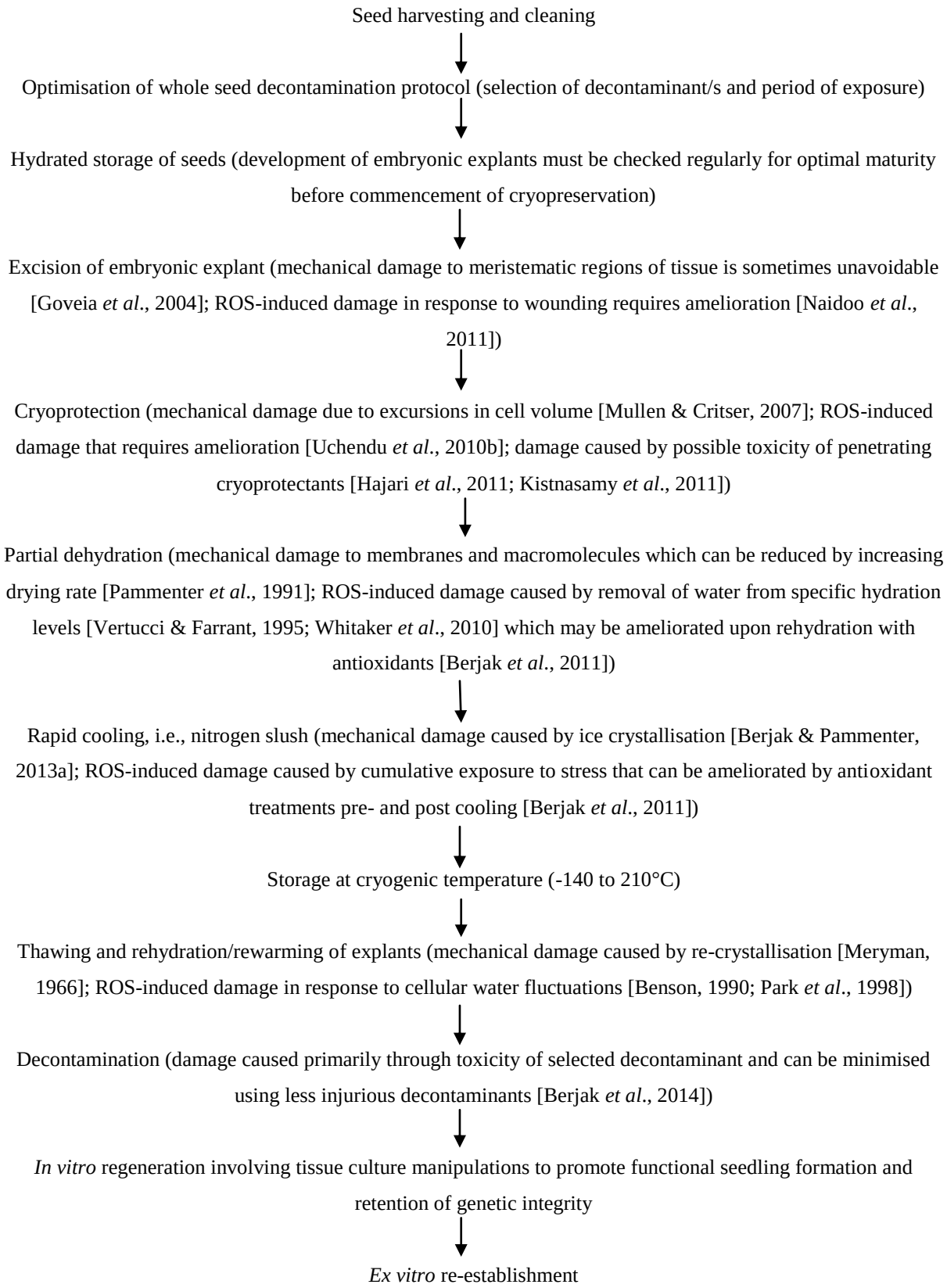

Fig. 1.2: Sequence of procedures and associated problems typifying cryostorage of recalcitrant zygotic explants.

1.6 Seed harvesting, cleaning and hydrated storage

As reviewed above, recalcitrant seeds are shed metabolically active, hydrated and desiccation sensitive. The inability of these seeds to undergo metabolic switch-off underlies the limited period for which seeds can be stored even for the short to medium term. According to Pammenter *et al*. (1994), recalcitrant seeds are exposed to an initially mild but increasingly severe period of water stress which disrupts co-ordinated metabolism thereby perpetuating ROS mediated oxidative damage. This process underlies tissue death in hydrated storage. Ongoing metabolism progresses seed maturation from development to germination and with the onset of germination the lifespan of seeds in storage is curtailed (Berjak & Pammenter, 2013a). Storage lifespan of recalcitrant seeds depends largely on specific species characteristics, even under the most ideal conditions of hydrated storage. Germinative metabolism is ongoing in hydrated storage and some tropical varieties may only survive a few weeks (Farrant *et al*., 1989) while other chilling tolerant temperate varieties can survive between 2-3 years (Pritchard *et al*., 1996). In the present study, the investigated species have a range of storage lifespans prior to the onset of germination, i.e. *L. sinensis* (1-2 months), *S gerrardii* (2-3 months), *T. dregeana* (3-6 months) and *Q. robur* (12-18 months). Seeds of *Q. robur*, being the only species of temperate origin, have been documented to last even up to 2-3 years in storage at an optimum temperature of -1°C (Suszka & Tylkowski, 1981).

Seed collection techniques must ensure genetic diversity within the sample population. There are guidelines that should be followed during harvesting of seeds that are considered general protocol (Luna & Wilkinson, 2009). Some of the stipulations from these guidelines that were incorporated into seed collection during this study were the collection of seeds from more than 50 individuals, equal collection of seeds from all parts of the tree, collection throughout the fruiting season and assessment of insect damage and empty seeds prior to collection. After collection, seeds should be cleaned to remove excess dirt and debris. The final preparation and surface sterilisation of recalcitrant seeds prior to hydrated storage would be species specific, and is described in detail for each investigated species in Chapter 2. Ideally, recalcitrant seeds should be kept fully hydrated at relative humidities maintained between 80-90%, with provision made for constant gas exchange (FAO, 1985). The temperature for recalcitrant seed storage is determined according to species and their native climate but is generally above chilling temperature for tropical recalcitrant-seeded species. The specified

hydrated storage conditions are conducive to proliferation of seed associated mycoflora on and within seeds (Mycock & Berjak, 1990). Therefore, it is vital that decontamination protocols include systemic fungicides or controlling agents that curtail fungal activity and limit the spread of infection, thereby extending storage lifespan. Effective short-medium term storage is imperative for the maintenance of high quality, bacterial and fungal free recalcitrant seeds for the purpose of ongoing experimentation.

1.6.1 Zygotic explant excision

The physiological structure of an embryonic explant is essentially a root-shoot continuum (Berjak, 2006), consisting of a heterogeneous composition of cells (Gonzalez-Arnao *et al*., 2008). Earlier studies in cryopreservation of embryonic explants did not necessarily quantify post-cryo survival in terms of seedling production, with many authors reporting survival in terms of root production and callus development as an indication of successful cryopreservation. The implications of this inconsistency have become increasingly more apparent as more recalcitrant seeds with fleshy cotyledons attached to the embryo fail to produce shoots upon excision of the cotyledons from the explant, e.g., *Trichilia dregeana* (Kioko *et al*., 1998; Goveia, *et al*., 2004; Goveia, 2007; Naidoo *et al*., 2011); *Ekebergia capensis* (Perán *et al*., 2006; Hajari *et al*., 2011); *Trichilia emetica* (Goveia, 2007; Naidoo, 2012) and *Protorhus longifolia* (Naidoo, 2012). For these species, the complete excision of the cotyledons flush with the explant is necessary to obtain an appropriate size for rapid dehydration and cryogenic cooling, but this leads to damage to meristematic zones (Pammenter *et al*., 2011). The difficulty is influenced by the topography of the explant, i.e., the location of the meristem relative to the wound site (Goveia *et al*., 2004; Ballesteros *et al*., 2014). Physical damage to the meristem(s) can occur through mechanical injury imposed by the scalpel used for removal of cotyledons but more importantly, excision imposes the first of the oxidative stresses inflicted during the processing for cryostorage (Goveia, 2007; Berjak *et al*., 2011; Berjak & Pammenter, 2013a). The superoxide ($^{\cdot}O_2^-$) burst which occurs in response to wounding (Roach *et al*., 2008; Whitaker *et al*., 2010; Berjak *et al*., 2011; Naidoo *et al*., 2011) also negatively affects viability of the meristem (Naidoo *et al*., 2011; Berjak & Pammenter, 2014).

While the production of reactive oxygen species (ROS), particularly $\cdot O_2^-$, is associated with wounding (Ross *et al*., 2006) extension of the deleterious effects to the meristem is largely dependent on the topography of the explant (Ballesteros *et al*., 2014). In instances where explants can be removed in a non-injurious manner from the seed or where the shoot meristem is located at a distant proximity from the wounding site, shoot production is not affected, as exemplified typically by monocots and endospermous dicots such as *Syzygium cordatum*, *Phoenix reclinata*, *Landolphia kirkii* and various amaryllid species (Pammenter *et al*., 2011). Progress to ameliorate excision induced damage has been made. Benson *et al*. (2007) suggested the use of hypodermic needles to reduce physical trauma to the explant during excision. Naidoo *et al*. (2011) incorporated the potent antioxidant dimethyl sulphoxide (DMSO) into a pre-culture medium, prior to excision of cotyledons, and subsequently excised cotyledons flush with explant surface using hypodermic needles beneath an antioxidant solution containing DMSO. This technique was successfully applied to explants of three species, viz., *T. dregeana*, *T. emetica* and *P. longifolia* (time of exposure to antioxidants was optimised for each) resulting in shoot development after complete excision of cotyledons which had never previously been achieved.

Overcoming physical and oxidative damage induced by excision such that shoot production is facilitated is fundamental to attaining seedling development after all subsequent steps of cryopreservation. After each cryogenic step, the viability achieved will determine the proportion of explants still retaining the ability to survive upon retrieval from cooling. Furthermore, damage incurred at each step predisposes explants to further damage in subsequent steps, so it often observed that explants might retain full viability after excision but lose substantial viability upon exposure to the second or third step due to the effect of cumulative stress (Sershen *et al*., 2016).

1.6.2 Partial dehydration

It has been established that partial dehydration enables cryopreservation of recalcitrant explants (Bajaj, 1985), although careful modulation of the conditions under which explants are dried is crucial (Pammenter *et al*., 2002). Partial dehydration should entail the most rapid drying possible to the absolute minimum WC that can be tolerated without significant viability loss. Below this WC, subcellular damage would result in cell death (Bajaj, 1985;

Pammenter & Berjak, 2000). Exposure to cryogenic temperature necessitates dehydration of tissues to WC approaching the level of non-freezable water to minimise — but ideally preclude — lethal ice crystallisation (Pammenter & Berjak, 1999; 2013). There is a 'school of thought' pertaining to the combination of drying and cooling rates where it is suggested that explants can be dehydrated to higher WC, therefore reducing the period of desiccation stress, while still limiting lethal ice crystal formation, provided that the cooling rate is sufficiently slow (Wesley-Smith *et al*., 2014). Regardless of the extent to which explants must be dehydrated or the rate at which drying occurs, removal of water from specific hydration levels is intrinsically linked to structural and metabolically derived damage that is often irreversible (Walters *et al*., 2002a; Berjak & Pammenter, 2013a).

When explants are dried to a WC range between 0.45 and 0.70 g g^{-1} [corresponding to hydration levels V and VI], the induced stress is comparable to that of limited water supply and high transpirational demand (Walters *et al*., 2001) but upregulation of genes that code for antioxidants and protective molecules necessary for general abiotic stresses can still occur (Illing *et al*., 2005). When explants are dried to intermediate WC levels, i.e., between 0.25 and 0.45 g g^{-1} respiration and protein synthesis occur in line with metabolically active cells, and production and function of protective molecules and processes that are aqueous based are facilitated. This WC range is not damaging in itself *per se*, rather it is the time exposure of explants to intermediate WC levels (Pammenter *et al*., 1998; Varghese *et al*., 2011) that will determine the quantity of ROS production and hence, oxidative damage that will occur. However, restricted fluidity of this phase limits the efficiency of corresponding detoxifying processes (Vertucci & Farrant, 1995; Bailly, 2004; Berjak, 2006). Desiccation damage *sensu stricto* as defined by Walters *et al*. (2001) describes the withdrawal of water from intracellular structures and macromolecules when tissue is dehydrated to limits below which all intracellular water is unfreezable ($\leq$ 0.28 g g^{-1} DW), and this perturbation of bound water is lethal to desiccation sensitive tissue (Pammenter *et al*., 1993).

As alluded to above, dehydration in recalcitrant tissue is accompanied by an imbalance between ROS production and antioxidant mechanisms (Varghese & Naithani, 2002; Kranner *et al*., 2006; Varghese *et al*., 2011), particularly if the drying rate is not sufficient enough to bypass the intermediate WC range rapidly. The responses of oxidative metabolism to

desiccation are largely influenced by seed shedding WC, the rate of water loss, the provenance and the developmental stage of the seed (Daws *et al*., 2004; 2006). Ultimately, the consequence of continued respiration during dehydration is the leakage of high-energy intermediates from the mitochondria and plastids which form ROS (Hendry, 1993; Smirnoff, 1993; Halliwell & Gutteridge, 1999). Reactive oxygen species invariably react with proteins, lipids and nucleic acids (Halliwell & Gutteridge, 1999) at WC that do not allow efficient activity of antioxidant enzymes responsible for their detoxification, metabolic switch off or de-differentitation of organelles, all of which are processes necessary to combat metabolically derived damage during desiccation (Pammenter & Berjak, 1999; 2000).

Water removal also causes mechanical changes to the cell structure. Desiccation damage in the strict sense refers to those events or processes that occur in response to dehydration and do not resume normal function upon rehydration of the cell. Removal of water from recalcitrant explants has the following implications that impose mechanical and physical strain. The first sign of desiccation is seen in the loss of turgidity in cells and upon further water loss the cell starts shrinking (Vertucci & Farrant, 1995; Walters *et al*., 2002). Biophysical changes start occurring when water is removed from macromolecular and membrane surfaces (Pammenter & Berjak, 2000). Cytoskeletal disassembly occurs at fairly high water potentials and further desiccation induces dismantling of the mitochondria and chloroplasts and possibly the endoplasmic reticulum, dictysomes and polysomes all contributing to a decrease in the rate of protein synthesis. Complete resumption of cytoskeletal activity or normal re-assembly and function of organelles rarely occur in desiccation sensitive material upon rehydration which contributes to cell death. In orthodox seeds, the increase in dry matter augments stabilisation of the subcellular environment which can reduce mechanical stress. Increased reserves also serve to decrease cytoplasmic volume imposing metabolic quiescence and spatially separating membranes that would otherwise interact (Vertucci & Farrant, 1995). It has been proposed that these mechanical stresses in recalcitrant embryos can be attributed to the lack of reserve deposition (Vertucci & Farrant, 1995).

Desiccation of explants, while necessary for cryopreservation, is a severe stress to overcome and this stress may be more pronounced in explants from tropical recalcitrant seeds. In many

situations, cryopreservation is unsuccessful because of the high proportion of explants that die at the dehydration step, and not necessarily because freezing damage is lethal. As mentioned previously, the rate of drying is crucial to bypass intermediate WC at which mechanical and physical damage occurs rapidly, and different drying techniques are explored in the present study to reduce the damage incurred during this step (which will be discussed further). Another persistent problem associated with drying seed explants is the lack of even dehydration across heterogeneous tissue within the explant. Kioko *et al*. (1998) showed that the rate of water loss in the root and shoot apical region in embryonic explants of *T. dregeana* is disparate, where the shoot dries more rapidly. Wesley-Smith *et al*. (2001a) reported that the cells of the root cortex of *Artocarpus heterophyllus* dried more rapidly than those of the procambial cylinder. These reports imply that under the same rate and drying conditions for the same period, there is an uneven distribution of water in recalcitrant explants (Ballesteros *et al*., 2014). Since it is general practice to measure bulk embryo WC, it commonly follows that the calculated WC is not a true reflection of the root/shoot apices which is possibly lower than the minimal WC at which viability is retained. Uneven drying is reportedly linked to explant size and tissue topography which influences drying rate and intensity, both factors playing a role in desiccation damage. The implication of uneven drying for cryopreservation is that hydrated tissues will be less susceptible to dehydration damage but more likely to incur ice crystal formation upon cooling (Ballesteros *et al*., 2014), thus completely precluding successful seedling establishment. This was the case described by Ballesteros *et al*. (2014) for rapidly dried explants of *Castanospermum australe* where 70% of explants were either above (associated with ice crystallisation damage) or below (associated with desiccation damage *sensu stricto*) the WC amenable for cooling. This exemplifies the situation in most cases, where reported survival after cooling represents a mere fraction of explants that were exposed to cooling at the correct WC.

1.6.3 Cryoprotection

Cryoprotective agents are primarily utilised in cryopreservation to eliminate ice formation upon cooling of tissue to cryogenic temperatures (Best, 2015), with the major downfall of cryoprotectants being toxic at high concentrations (Benson, 1990). When including cryoprotectants in a protocol, efforts are made towards developing strategies to eliminate ice formation and minimizing toxicity. This dictates that cryoprotectants must be non-toxic to

tissue at the concentrations required for their efficacy (Benson, 2007). Cryoprotectants are either penetrating, such as those used in this study, i.e., glycerol and DMSO, or non-penetrating such as sucrose, polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP).

The principal modes of action of cryoprotectants are dependent on their characteristic properties of being penetrative or non-penetrative (Finkle *et al*., 1985). Low molecular weight, penetrating/colligative compounds protect firstly by lowering the temperature at which the cell freezes, i.e., depressing the freezing point (Benson, 1990). Secondly, colligative cryoprotectants increase the osmolality of the cell prior to freezing, thus reducing the level of water to be frozen out to attain osmotic equilibrium (Meryman & Williams, 1985). Larger, non-penetrating cryoprotectants act by dehydrating the cell such that there is a reduction in the amount of water within the cell that can come together and form ice upon freezing (Benson, 1990). Cryoprotectants such as trehalose, proline, DMSO and glycerol also act by stabilising membranes (Benson, 1990). As damaged membranes can act as a site for the initiation of free radical activity, these cryoprotectants indirectly prevent free radical formation and, in the case of DMSO, can itself act as a free radical scavenger (Yu & Quinn, 1994). Cryoprotectants have also been used to achieve the metastable, vitrified state, i.e., the solidification of liquid without crystallisation (Benson, 2007) by structuring water in cells such that it is less likely to freeze (Wolfe *et al*., 2002). An *a priori* requirement for vitrification is an increase in cell viscosity which occurs upon the concentration of cell solutes. This is achieved by using very high concentrations of cryoprotectants, such as those used in Plant Vitrification Solution 2 (PVS2; Sakai, 2004) or by using comparably lower concentration of cryoprotectants and dehydrating cells lower than 0.25 g g^{-1} dmb, resulting in second order water transitions (Volk & Walters, 2006). These methods of achieving vitrification upon cooling pose a problem when cryopreserving recalcitrant-seeded zygotic embryos. Firstly, high concentrations of cryoprotectants are usually toxic (Steponkus *et al*., 1992). Secondly, the WC which is necessary to sufficiently increase solute concentration within the cell is often lethal to desiccation sensitive zygotic explants, however in some instances vitrification can be achieved and cryopreservation is successful (Sershen *et al*., 2007). For this reason, cryoprotectants are not applied in this study for the purpose of achieving vitrification but rather for the colligative protective actions mentioned above.

1.6.4 Cooling

Damage through osmotic dehydration and ice crystallisation during cooling has already been discussed (Section 1.4.2). While it is necessary to design cryopreservation protocols that obviate ice damage, factors such as oxidative stress also influence post-cryogenic survival (Kaczmarczyk *et al*., 2012). It is now necessary to consider free radical activity at ultra low temperatures as it has critical implications for survival of the explant post-cooling.

Cryopreservation imposes a series of osmotic and temperature related stresses on zygotic explants (Reed *et al*., 2012), and at the final step of cooling, cumulative stress induces severe oxidative damage. Levitt (1962) postulated four phases during cooling where damage may be incurred, i.e., the moment of freezing; in the frozen state; the moment of rewarming and the post rewarming period. During freezing of biological tissue, Singh and Miller (1985) noted a loss in physical compartmentalisation and metabolic uncoupling. The disruption in metabolism negatively affects antioxidant systems and often leads to the uncontrolled production of toxic free radical species and their by products (Benson & Bremner, 2004). For example, it has been shown that the compromised ability of antioxidants to scavenge reactive oxygen species (ROS) and reduction in antioxidant recycling under ultra-low temperature conditions severely damages the chloroplast through lipid peroxidation, inactivation of photosynthetic proteins and loss of pigments (Wise, 1995; Wise & Naylor, 1987). Each of these contributes to limiting survival and recovery from cryopreservation. Mitochondrial enzymes and ATP-synthesis are disrupted during freeze-thaw cycles, redox balance is disturbed and the functionality of antioxidant enzymes (e.g., ascorbate peroxidase, glutathione reductase and catalase) is compromised in response to cold/chilling stress (Tapell, 1966; Guy, 1990).

In addition to ROS-mediated damage, membranes lose functional retention, where the balance between intra and extracellular solutes is disrupted and this causes irreversible loss of cellular integrity leading to cell death (Anchordoguy *et al*., 1987; Benson & Bremner, 2004). Membrane instability during cooling can be caused by four types of injury: expansion-induced lysis which describes over expansion of cells due to increased extracellular osmotic pressure during thawing; reduced osmotic responsiveness where warming does not induce an osmotic change due to a slow cooling rate and the cells remain dehydrated; altered osmotic

behaviour where there is leakage of water and solutes from the membrane into the surrounding *milieu*; and intracellular ice formation where rapid cooling perpetuates the formation of ice crystals causing membrane disruption (Steponkus, 1984).

To obviate ROS-induced injurious events, which may be activated during freezing but only expressed upon rewarming, Benson and Bremner (2004) proposed minimising the amount of water present in the tissue, limiting oxygen levels and inclusion of a ROS scavenger/antioxidant during steps of cryopreservation. These suggestions were tentatively included in this research.

1.7 Redox metabolism in plant tissue

1.7.1 ROS, oxidative stress and oxidative damage

Oxygen is a fundamental requirement for maintenance of an efficient bioenergetic state in all aerobic organisms (Benson 1990; Apel & Hirt, 2004; Andreyev *et al*., 2005; Halliwell, 2006). Oxygen is, thermodynamically, a powerful oxidising agent (Halliwell, 2006) and the cost of aerobic metabolism is invariable oxidative perturbations which alter cellular redox state and induce ROS-mediated stress responses (Benson & Bremner, 2004). All oxygen radicals are ROS, but not all ROS are oxygen radicals (Halliwell, 2006). Reactive oxygen species' is not a monolithic term, it is a collective descriptor that incorporates oxygen radicals and non-radical derivatives of oxygen (Halliwell & Gutteridge, 2006; Murphy *et al*., 2011). As ROS production is a consequence of normal aerobic metabolism (Benson, 1990), it is necessary to delineate the role of oxygen in ROS reactions.

Ground state, diatomic oxygen (triplet molecular oxygen) is a paramagnetic biradical having two outermost unpaired valence electrons with the same quantum spin number (separate orbitals with parallel spins [Benson, 1990; Benson & Bremner, 2004]). To form a radical species, triple state oxygen has to react with a pair of electrons with parallel spins such that free electron orbitals are filled. Electrons frequently have opposite spins and so the reactivity of triplet oxygen with most molecular species is restricted based on its electronic behaviour (Benson, 1990; Apel & Hirt, 2004). Singlet oxygen, produced by the electronic excitation of molecular oxygen (resulting in spin inversion) by energy transfer is highly reactive and consequently can be sequentially reduced by electron transfer reactions to generate the ROS

hydrogen peroxide (H_2O_2), $^{\cdot}O_2^-$ and the hydroxyl radical ($^{\cdot}OH$; Smirnoff, 1993; Apel & Hirt, 2004; Benson & Bremner, 2004), the latter two being free radical species. Free radical reactions occur when conventional molecular bonds are broken such that electrons split homolytically (Benson, 1990) or via the addition or loss of an electron to a neutral molecule (Benson & Bremner, 2004), hence creating free radicals capable of independent existence (Halliwell, 2006). The characteristic unpaired electron/s of free radical entities must abstract a second electron from stable molecules, thus perpetuating a cascade of self-propagating free radical species (Benson, 1990; Fridovich, 1998) which participate immediately in new chemical reactions (Benson & Bremner, 2004). Free radical activity fundamentally depends on two factors: (i) the dependence of functional metabolism on electron movement and (ii) the essential involvement of oxygen in respiration (Benson, 1990). Any event causing disruption of metabolism by undermining tight metabolic coupling and cellular compartmentalisation, which strictly controls free radical activity under normal conditions propagates the formation of destructive free radicals. Oxidative stress and —pending the severity — the resultant oxidative damage are events that have been defined to occur when the pro-oxidant anti-oxidant balance is disturbed in favour of the former (Sies, 1991). Before these concepts are discussed, it is necessary to detail where and how ROS are produced in plants, and how an overproduction of ROS can lead to an oxidative stress-induced cell death.

Reactive oxygen species (free radical species and non-radical derivatives) are usually localised in cellular compartments and are often produced in the mitochondria via over reduction of the electron transport chain (Moller, 2001; Turrens, 2003; Andreyev *et al*., 2005); chloroplasts via Mehler reactions and antenna pigments (Asada & Takahashi, 1987; Foyer *et al*., 1994); peroxisomes (Corpas *et al*., 2001); apoplast via NADPH oxidases, amine oxidases, cell-wall bound peroxidases (Mittler, 2002); cytoplasm and endoplasmic reticulum via detoxifying reactions catalysed by cytochromes (Urban *et al*., 1997) and in nitrogen-fixing nodules (Becana *et al*., 2000). In plants, ROS have many biologically significant roles and are efficiently modulated by the reactive oxygen gene network that controls ROS production and perception (Mittler *et al*., 2004). They are produced as by-products of innumerable metabolic pathways, especially those involved in respiration and photosynthesis (Benson, 1990; Apel & Hirt, 2004), in defence pathways against pathogens (Apostol *et al*., 1989; Knight & Knight, 2001), cell signalling, notably in regulators of growth and

development and programmed cell death (Mittler *et al*., 2004), hormone regulation (Kwak *et al*., 2006), ageing (Tian *et al*., 2008) and in response to a variety of biotic and abiotic stresses (Apel & Hirt, 2004). Reactive oxygen species play a dual role in seed physiology, i.e., as signalling molecules in growth and development pathways and as accumulated toxic products under stressed conditions (Bailly *et al*., 2008). The duality of ROS functions will be discussed further in Section 1.7.2.

Under typical cellular homeostasis conditions, the basal steady state rate of ROS production is relatively low (240 µM s^{-1} $^{\cdot}O_2^-$; 0.5 µM H_2O_2 in chloroplasts [Asada, 1994; 1997; Polle, 2001]). This steady state is modulated by the reactive oxygen gene network, which controls the array of detoxification and elimination systems (Mittler *et al*., 2004) comprising enzymatic and low molecular weight antioxidant compounds that are responsible for protection against oxidative damage (Noctor & Foyer, 1998) by counterbalancing excessive ROS production (Lushchak, 2014). Stressful events that disrupt cellular homeostasis such as drought, desiccation, chilling, heat shock, heavy metal exposure, nutrient deprivation, pathogen attack and high light stress etc. (Bowler *et al*., 1992; Allen, 1995; Noctor & Foyer, 1998; Dat *et al*., 2000; Desikan *et al*., 2001) increases the rate of ROS production above the steady state (240-720 µM s^{-1} $^{\cdot}O_2^-$; 5-15 µM H_2O_2 [Asada, 1994; 1997; Polle, 2001]). In the absence or dysfunction of protective antioxidative mechanisms, as exemplified by recalcitrant seeds (Berjak & Pammenter, 2008), an over accumulation of ROS can occur, therein lies the onset of oxidative stress.

Oxidative stress reflects a situation where steady state ROS concentration is transiently or chronically enhanced, disturbing the balance between systemic manifestation of ROS and a biological system's ability to readily detoxify ROS/reactive intermediates or to repair the consequent damage to cellular constituents (Lushchak, 2011). Steady state ROS levels fluctuate within a range under normal conditions (Lushchak, 2014), but ROS accumulation above a threshold level results in cellular alterations which is a progenitor of "acute" or "chronic" oxidative stress (Halliwell, 2006; Bailly *et al*., 2008; Lushchak, 2014). There exists three scenarios that illustrate perturbations in ROS homeostasis as described by Lushchak (2014). Acute oxidative stress occurs when the antioxidant mechanisms are not overwhelmed and defensive resources permit the system to return to steady state ROS levels after

minutes/hours of the applied stress. Chronic oxidative stress describes a situation where ROS levels do not return to steady state levels but stabilise at a new, slightly higher basal level, in which case stress is not prolonged. Chronic intoxication by ROS activity leads to the final scenario where ROS levels stabilise much higher than the steady state and this is called the quasi-stationary level (Lushchak, 2014).

At very low concentrations, ROS does not affect cell viability and antioxidant activity. Upon slightly elevated increases in concentration, ROS can effect an increase in antioxidants. This is a response related to resilience against oxidative stress and stress in general and typifies development of an adaptive response. The relationship between an increase in ROS and the consequent increase in antioxidants/related enzymes can be described as hormesis (Calabrese, 2008; Ristow & Schmeisser, 2011), a term referring to the adaptive response of cells and organisms to a moderate and usually intermittent, stress. In a state of oxidative stress, cellular activities will involve: (i) enhancement of steady state level ROS-modified components, (ii) activation of ROS regulated transcription factors and (iii) ROS induced inactivation of antioxidants. As detailed above, ROS have important implications for plant stress and survival, with each having specific reaction preferences, rate and site of production, targets for damage, kinetics and diffusion and degradation characteristics (Murphy *et al*., 2011). Each of these factors will be discussed within the physiological context in which each ROS is being generated.

1.7.2 ROS signalling networks and the duality of ROS

It is accepted that the direction of many biological reactions are dependent upon the "redox state" (Schafer & Buettner, 2001) and that an applied stress must be quantifiable in the "redox environment" and not merely qualified as observational effects. This has immense value in establishing the oxidative state required to initiate or perpetuate a specific cascade of cellular signalling (Schafer & Buettner, 2001). Production, scavenging and toxicity of ROS in seeds and plants have been well documented (Benson, 1990; Hendry *et al*., 1992; Chaitanya & Naithani, 1994; Fridovich, 1998; Varghese and Naithani, 2002; Halliwell & Gutteridge, 2004; Halliwell, 2006; Berjak & Pammenter, 2008 and references therein, Sershen *et al*., 2012c). All relevant literature cited thus far suggest that under ideal circumstances detoxification mechanisms tightly control ROS production rather than eliminate them from

the system completely. On this premise, it is accepted that ROS play normal physiological roles in seed and plant systems, of which a pivotal function is that of signalling (Bailly, 2004; Baxter *et al*., 2013).

Signalling or signal transduction is fundamentally an intricate system of communication that governs cellular activities, functions and co-ordination of actions which allows for the correct perception and response of cells to their microenvironment. Signalling pathways are essential for the transmission and flow of information from the cell's exterior to its interior environment (Bailly *et al*., 2008). The ROS signalling network is an evolutionary transduction network that utilises ROS as signalling molecules to influence gene expression and signalling pathways to control a plethora of biological processes (Suzuki *et al*., 2011). Such a network is proficient in core regulation of cellular responses under conditions where cells are able to readily and effectively detoxify ROS in various cellular compartments (Mittler, 2002; Mittler *et al*., 2004; Bhattacharjee, 2005). Signalling events are influenced by redox reactions and therefore the redox state (Tripathy & Oelmüller, 2012) and is intimately linked with the regulatory capabilities of ROS, the interaction between different pools of ROS and the communication between various compartments where ROS is produced and localised (Torres *et al*., 2006). Signalling occurs as a consequence of the alteration of the redox state which can be brought about by the contrasting, simultaneous processes of ROS production and scavenging (Mittler *et al*., 2004: Foyer & Noctor, 2005). Rapidly altered ROS levels often propagates a signal which potentially transmits over long distances causing a cascade of cell-to-cell communication events occurring within and between cells and various organelles (Mittler *et al*., 2011).

The ROS signalling network facilitates two activities, i.e., maintenance and regulation of steady state non-toxic levels of ROS and transient accumulation of specifically compartmentalised ROS which act as signals (Suzuki *et al*., 2011). Properties and mobility of ROS, i.e., small, short radius for diffusion and several mechanisms in place for rapid production and removal, make them ideally suited for versatile signalling roles (Hancock *et al*., 2001; Pitzschke *et al*., 2006; Mittler *et al*., 2011), and each ROS having a signalling function, viz., H_2O_2, $^{\cdot}O_2^-$ and $^{\cdot}OH$ interacts with a specific cellular target to activate each signalling event (Tripathy & Oelmüller, 2012). Respiratory burst oxidase homologues

(Rboh's) or the enzyme NADPH oxidases play a central role in ROS signalling in plants and are responsible for the genetically controlled release of ROS (Laloi *et al*., 2004; Torres & Dangl, 2005; Suzuki *et al*., 2011). Stress stimuli induces early response signals including increased flux of Ca^{2+} into the cytosol, activation of mitogen-activated protein kinases (MAPK; reviewed by Pitzschke & Hirt, 2006) pathways and protein phosphorylation (Benschop *et al*., 2007; Baxter *et al*., 2013). Phenotypic changes in the growth, development and survival of cells are amongst long-term responses following early signal regulatory mechanisms (Torres & Dangl, 2005; Gapper & Dolan, 2006).

Many aspects of ROS signalling in plants requires clarity, however there are distinct functions of ROS in plants that is so forth outlined. Reactive oxygen species play a central signalling role in ABA mediated stomatal closure and this role of H_2O_2 was specifically illustrated by Pei *et al*. (2000). For example, ROS signalling pathways are closely linked to a number of protein kinases that are associated with regulation of root hair development (Pitzschke *et al*., 2006); ROS signalling is also involved in stress hormone production, acclimation and programmed cell death (PCD; Apel & Hirt, 2004). Reactive oxygen species produced by NADPH oxidases play a significant role in plant pathogen defence and these ROS are closely linked to other plant signalling molecules such as salicylic acid and nitric oxide (Torres & Dangl, 2005). In seeds specifically, ROS have been noted to play signalling roles in dormancy and seed germination (Bailly, 2004; El-Maarouf-Bouteau & Bailly, 2008). Hydrogen peroxide, nitric oxide, $^{\cdot}O_2^-$ and OH^- have been documented to accumulate during germination events in several species (Gidrol *et al*., 1994; Caro & Puntarulo, 1999; Schopfer *et al*., 2001; Müller *et al*., 2009; Kranner *et al*., 2010; Sershen *et al*., 2016) and an increase in their production has been frequently noted during germination *sensu stricto* involving activation of a regulatory system controlled by extrinsic (such as environmental conditions) and intrinsic factors (e.g., dormancy; Bailly *et al*., 2008). The primary role of ROS as signalling molecules during germination is to regulate the redox state by inducing oxidation of proteins and triggering particular gene expression for completion of germination (Bailly *et al*., 2008). Also, there are well established interactions of ROS with plant hormones ABA, GA and ethylene, all of which play prominent roles in seed dormancy (Finkelstein *et al*., 2002; Finkelstein *et al*., 2008). Bailly (2004) proposed the role of ROS as signals which precipitated a shift from dormancy to non-dormancy in seeds and this role was further

established by El-Maarouf-Bouteau and Bailly (2008) and Oracz *et al*. (2009). In light of these roles it is accepted that ROS act directly or as messengers in hormonal networks as signalling molecules (Bailly *et al*., 2008).

1.8 The use of stress biomarkers in optimising plant cryopreservation protocols

It has been well established that variation is the hallmark of seed recalcitrance, with this manifesting in terms of topography and morphology, water activity, developmental status and maturity, desiccation and chilling sensitivity, storage behaviour and longevity, sensitivity to cryoprotectants and decontaminant toxicity, inherent fungal and microbial activity, endogenous levels and capacity of antioxidants, and rate of water loss during desiccation. All these factors impact explant responses to the procedural steps of cryopreservation. Behavioural patterns of explants from each investigated species will ultimately determine one major outcome, i.e., survival or death. The successful cryopreservation of any species requires knowledge on: (i) what underlies damage prior to, during, and upon retrieval from cooling and (ii) what ameliorative measures may facilitate survival. On this premise, stress biomarkers were selected to be measured/observed after each step of cryopreservation in treated and control zygotic explants. Biochemical markers offer scope for analytical quantification of cumulative stress during cryopreservation and the associated manipulations. As such, reactive oxygen species ($^{\cdot}O_2^-$ and H_2O_2); total aqueous antioxidant capacity, respiratory activity and cellular ultrastructure were selected as stress biomarkers. These were quantitatively measured (qualitatively for ultrastructural investigations) commensurate with viability after each step of cryopreservation. The significance of these biomarkers will be reviewed below.

1.8.1 Stress biomarker: Superoxide ($^{\cdot}O_2^-$)

Superoxide anion radicals, a progenitor ROS containing one unpaired electron are intermediates generated on the pathway that utilises four electrons to reduce oxygen to water (Fridovich, 1998). In plants, it is commonly the most reactive — albeit not most destructive — ROS and is usually the first to be generated (Fridovich, 1998; Gill & Tuteja, 2010). Superoxide is selectively reactive, and diffuses great distances within cells before reacting with suitable targets (Fridovich, 1998). As such, it is potentially more damaging than other ROS which only react within a small radius from the site of generation (Fridovich, 1998).

The hydroperoxyl radical ($HO_2^{\cdot}$) is the conjugate base of $^{\cdot}O_2^-$ and has a pKa (index to express the acidity of weak acids) of 4.8. Superoxide is therefore unstable in protic solvents such as water which contains transient hydrogen atoms but gains stability upon an increase in pH (Fridovich, 1998). A moderately reactive ROS, $^{\cdot}O_2^-$ has a half life of approximately 2-4 μs and travels a distance of 30 nm (assuming the diffusion co-efficient is 10^{-9} m^2 s^{-1}; Moller *et al*., 2007). In plant cells, 1-2% of oxygen consumption leads to the generation of ROS (Puntarulo *et al*., 1988; Gill & Tuteja, 2010). In green plant parts exposed to light conditions, chloroplasts and peroxisomes are considered to be the main sites of ROS generation (Foyer & Noctor, 2003); in non-green parts under dark conditions, mitochondria are key producers of ROS (Moller, 2001). Superoxide production occurs predominantly in the mitochondria and chloroplast, but generation is not limited to these sites. NAD(P)H oxidases, known as respiratory burst oxidase homologs in plants (Rboh), are localised on plasma membranes (Kaur *et al*., 2014) and prominently release $^{\cdot}O_2^-$ under the control of growth factors and cytokines (Sagi & Fluhr, 2006). In the mitochondrial respiratory chain, redox centers/carriers leak electrons which reduce oxygen to $^{\cdot}O_2^-$ (Turrens, 2003). Mitochondria possess at least nine known sites of $^{\cdot}O_2^-$ generation (Andreyev *et al*., 2005) and being a precursor ROS, $^{\cdot}O_2^-$ is actively involved in the propagation of oxidative chain reactions (Turrens, 2003). In chloroplasts, the Mehler reaction describes the electron pathway responsible for oxygen photoreduction, where membrane-bound thylakoids are the principal electron acceptors of photo system I (PS I) and $^{\cdot}O_2^-$ is the primary reduced product in this reaction centre (Scandalios, 1993; Apel & Hirt, 2004; Asada, 2006). Superoxide generated in chloroplasts that are photosynthetically active lead to the activation of genes fundamental to signalling pathways as a vital part of the early antioxidant response as illustrated in *Arapidopsis thaliana* leaves (Scarpeci *et al*., 2008). The rate of $^{\cdot}O_2^-$ production in the chloroplast depends on prevailing photon intensity, direct UV radiation or the leakage of light energy on oxygen atoms from chlorophyll upon saturation of caretenoid pigments (Wise, 1995). There are two mechanisms via which $^{\cdot}O_2^-$ gives rise to further potent oxidants: (i) protonation to the hydroperoxyl radical and (ii) the *in vivo* Haber-Weiss reaction where $^{\cdot}O_2^-$ oxidises the 4Fe-4S centre of dehydrases and increases 'free iron'. Released iron then reacts with H_2O_2 to generate destructive hydroxyl radicals via the Fenton reaction (Fridovich, 1997; Moller *et al*., 2007).

At neutral pH, $\cdot O_2^-$ can oxidise polyphenols, thiols, ascorbate, catecholamines, leukoflavins, tetrahydropterins and sulphites (Fridovich, 1998) but does not significantly attack polyunsaturated lipids, DNA or carbohydrates (Fridovich, 1997; Fry, 1998). The key defence against $\cdot O_2^-$ induced damage in cells is the enzyme superoxide dismutase (Halliwell, 2006) which will be discussed in Section 1.7.5.

With specific regard to recalcitrant-seeded zygotic explants, $\cdot O_2^-$ activity has been measured during various procedures associated with cryopreservation. A brief overview of the research that has been undertaken includes studies in *Shorea robusta* where loss of viability of stored seeds were associated with increased lipid peroxidation and $\cdot O_2^-$ activity (Chaitanya & Naithani, 1994). An increase in $\cdot O_2^-$ production was correlated with increasing desiccation sensitivity in recalcitrant-seeded explants of *Antiaris toxicaria* (Cheng & Song, 2008). In *Castanea sativa,* transient $\cdot O_2^-$ bursts were associated with explant excision and desiccation stress (Roach *et al*., 2008) and these bursts were thereafter established to have a signalling role in wound response, regeneration and growth events (Roach *et al*., 2010). In *T. dregeana* prolonged $\cdot O_2^-$ production was associated with excision, dehydration and rewarming (Whitaker *et al*., 2010). In zygotic explants of the same species, $\cdot O_2^-$ production was suggested to act as a biochemical trigger for germination after rapid desiccation (Varghese *et al*., 2011), and was also assessed in relation to viability during the various steps of cryopreservation (Naidoo, 2012; Naidoo *et al*., 2016). Berjak *et al*. (2011) measured $\cdot O_2^-$ levels in zygotic explants of *S. gerrardii* in response to desiccation and during *in vitro* germination and provided a means for amelioration of unregulated production of this radical. Superoxide production was measured and compared after slow and rapid drying of recalcitrant-seeded zygotic explants of *Q. robur* and related to viability by Pukacka *et al*. (2011). Sershen *et al*. (2016) used $\cdot O_2^-$ production as a stress biomarker and measured levels of this radical in conjunction with other stress biomarkers in zygotic explants of *Amaryllis belladonna*, *Haemanthus montanus, T. dregeana* and *S. gerrardii* after partial dehydration to specified WC. The results from this study will be discussed with consideration of the findings reported by these authors in Chapter 4.

1.8.2 Stress biomarker: Hydrogen peroxide (H_2O_2)

Hydrogen peroxide, a non-radical, two electron product of oxygen is a normal aerobic metabolite which has multi-faceted roles in redox signalling and oxidative stress in biological cells (Bailly, 2004; Sies, 2014). Initially considered as a toxic threat to cellular homeostasis, the role of H_2O_2 was later discovered to be central to signalling processes involved in growth and development, i.e., cell elongation (Foreman *et al*., 2003), differentiation (Tsukagoshi *et al*., 2010) and responses to environmental stimuli (Dat *et al*., 2000; Gapper & Dolan, 2006); H_2O_2 is additionally necessary for communication with external abiotic and biotic stimuli (Sies, 2014). Nevertheless, the cellular targets of H_2O_2 are numerous and widespread and so interactions with this ROS can invariably lead to malignant cell damage and in worst cases, cell death (Bienert *et al*., 2006). The biological effects of H_2O_2 in plant cells depend largely on concentration, site of production and developmental stage (Gechev & Hill, 2005).

Hydrogen peroxide is a relatively stable molecule with both reducing and oxidising properties (as a consequence of each oxygen molecule having an intermediate oxidation number of -1; Bienert *et al*., 2006). It exists in high concentrations in plants (µM-mM range) with intracellular levels depending largely on compartmentalisation/localisation (Puntarulo *et al*., 1988; Cheeseman, 2007). It has a half-life of 1ms in biological systems and travels a distance of 1 µm in 1 ms (diffusion co-efficient: 10^{-9} m^2 s^{-1}; Puntarulo *et al*., 1988). It rarely reacts with lipids, DNA and carbohydrates (Fry, 1998) but is interactive with the amino acid L-cysteine that can result in protein crosslinking (Moller *et al*., 2007). The direct generation of H_2O_2 is a consequence of many important metabolic reactions and is central to a plethora of physiological functions such as the action of oxygenases in glyoxysomes and peroxisomes (Petrov & Breusegem, 2012); defence against pathogen attack via direct inhibition of growth of plant pathogenic organisms (Apostol *et al*., 1989; Wojtaszek, 1997); photosynthetic reactions including photorespiration in chloroplasts (Asada, 1992; Ishikawa *et al*., 1993); resistance acquisition; cell wall strengthening; senescence; phytoalexin production; stomatal opening and cell cycle; β-oxidation of fatty acids (Kuźniak & Urbanek, 2000); electron transport in the mitochondria and lignin synthesis in the apoplast (Schopfer, 1996). Hydrogen peroxide is also produced circuitously, i.e., through cell wall peroxidases which oxidise NADH and thus catalyses the formation of $^{\cdot}O_2^-$ and cell wall oxidases which subsequently catalyse the oxidation of NADH to NAD^+ thus reducing oxygen to $^{\cdot}O_2^-$ (Bolwell *et al*., 2002;

Bhattacharjee, 2005). Since the prominent source of H_2O_2 in cells is the spontaneous or SOD-catalysed dismutation of $^{\cdot}O_2^-$ (Benson, 1990; Sies, 2014), these processes indirectly generate H_2O_2. Oxalate oxidases and amine oxidases have also been suggested to generate H_2O_2 at the apoplast (Bolwell & Wotjaszek, 1997).

Hydrogen peroxide is continuously produced as a by-product of aerobic metabolism and photosynthesis and as such is commonly located in the mitochondria and chloroplast (Rasmusson *et al*., 1998; Andreyev *et al*., 2005; Asada, 2006; Bienert *et al*., 2006). Other prominent sites of production are peroxisomes, the cytosol and apoplastic space (Sharma *et al*., 2012). Structurally, H_2O_2 is very similar to water and cellular transport is widely accepted to occur through free diffusion or aquaporins across the lipid bilayer of membranes (Henzler & Steudle, 2000; Biernet *et al*., 2006), which is a definitive characteristic of this species. The permeation of H_2O_2 across membranes at rates comparable to that of diffusional water flow has important implications for intracellular concentrations of the species and of other compounds it might subsequently interact with (Henzler & Steudle, 2000). Hydrogen peroxide is detoxified chiefly by catalase (Moller *et al*., 2007) which will be discussed in Section 1.7.4.

An important concept to consider in oxidative stress is the relationship between the measure of a particular ROS and the assessment of resultant damage as indicated by markers of damage such as protein carbonyls, lipid peroxidation products and the breakdown products of compromised DNA (Halliwell & Gutteridge, 2007). Specifically concerning H_2O_2, it is unclear as to the level at which activity is considered toxic, and this fluctuates depending on the abiotic and biotic stresses, the efficacy of the antioxidant system and the species. In light of the prominent role H_2O_2 plays in signalling, cellular levels are generally high albeit under strict modulation by antioxidants. Therefore, high cellular concentrations are not always an indication of insipient oxidative damage and must be assessed in parallel with antioxidant levels (Kranner & Birtić, 2005; Murphy *et al*., 2011).

Within the context of cryopreservation of recalcitrant-seeded zygotic explant, studies have elucidated links between measures of H_2O_2 and associated procedures thereof. Many of the studies mentioned in Section 1.7.2 has measured H_2O_2 levels along with $^{\cdot}O_2^-$ in relation to

excision, desiccation and other procedural steps of cryopreservation (Cheng & Song, 2008; Roach *et al*., 2010; Pukacka *et al*., 2011; Naidoo, 2012; Naidoo *et al*., 2016).

1.8.3 Stress biomarker: Total aqueous antioxidants (TAA)

Regulation of ROS and their detoxification occurs either by avoidance of reactions that generate free radical species such as hydroxyl radicals, that have no known scavengers, or the evolution of efficient strategies to control concentrations of ROS intermediates and transition metals such as Fe^{2+} and Cu^{2+} (Apel & Hirt, 2004). The complexity of ROS regulation stems from its dual role in toxicity and signalling. Competent antioxidant mechanisms will therefore function in fine modulation of ROS at levels necessary for signalling and expulsion of excessive ROS during exposure to stress (Mittler, 2002). The functionality of the antioxidant complex is determined by aspects of compartmentalisation of ROS formation and commensurate antioxidant localisation; synthesis and transport of antioxidants; induction of antioxidant defence and networking between different antioxidant systems (Blokhina *et al*., 2003). It is thought that oxidative stress/damage in recalcitrant-seeded germplasm during cryopreservation is largely influenced by a deficiency or total absence of one or all of these mechanisms (Berjak & Pammenter, 2008; Berjak *et al*., 2011).

A crucial factor in determining germinability and survival of seeds or their embryos in stressed conditions is dependent on the efficiency with which defences are mobilised. Plants contain several enzymatic antioxidants (superoxide dismutase [SOD]; ascorbate peroxidase [APX]; glutathione peroxidase [GPX]; catalase [CAT], detoxifying lipid peroxidation products (glutathione-S-transferases, phospholipid-hydroperoxide glutathione peroxidase) low molecular mass non-enzymatic antioxidants (ascorbate, glutathione, phenolic compounds, tocopherols, flavanoids, alkaloids and carotenoids) and enzymes required for regeneration of active forms of antioxidants in the ascorbate (monodehydroascorbate reductase and dehydroascorbate reductase) - glutathione (glutathione reductase) pathway (Arrigoni, 1994; Noctor & Foyer, 1998; Blokhina *et al*., 2003; Apel & Hirt, 2004). Enzymatic and non-enzymatic antioxidants, while equally important, have different mechanisms of action, i.e., enzymatic antioxidants function by breaking down and removing free radicals whilst the latter functions by interrupting free radical chains (Flora, 2009). One of the principal modes of enzymatic ROS detoxification is the ascorbate-glutathione cycle

(Polle, 2001; Apel & Hirt, 2004) which will be briefly detailed here. A brief overview of SOD and catalase, two enzymes linked to the detoxification of the selected ROS biomarkers in this study, will follow.

Glutathione (γ-glutamylcysteinylglycine) is a cytoplasmatic, ubiquitous water-soluble thiol present in the chloroplast and cytosol in relatively high concentrations (Alscher, 1989; Kranner *et al*., 2006) in plant tissue and occurs in two distinct redox forms, i.e., reduced glutathione (GSH) and oxidised glutathione (GSSG; Apel & Hirt, 2004). Reduced and oxidised glutathione exist interchangeably in the system. The oxidised form of glutathione (GSH) exists as GSSG and oxidised ascorbate exists as monodehydroascorbate (MDHA) and dehydroascorbate (DHA). During the ASC-GSH cycle, the oxidised forms of these antioxidants (GSSG, MDHA and DHA) can be converted to their reduced states thereby regenerating glutathione and ascorbate in the cellular system. For efficient ROS scavenging, high ratios of reduced to oxidised GSH and ASC are required and the reduced states of these antioxidants are maintained by glutathione reductase (GR), MDHA reductase and DHA reductase. Glutathione reductase uses NADPH as an electron source to reduce GSSG to GSH while ROS and other oxidants oxidise GSH to GSSG (Noctor *et al*., 1998). Reduced glutathione is a potent detoxifier and redox buffer and its efficiency as an electron acceptor or donor is conferred by its chemical reactivity, water solubility and stability (Potters *et al*., 2002).

Detoxification of $^{\cdot}O_2^-$, a stress biomarker used in this study, is carried out by SOD. Superoxide dismutase is a multimeric metalloprotein (Scandalios, 1993) that catalytically removes $^{\cdot}O_2^-$ by dismutation, resulting in the formation of H_2O_2 and water (Benson, 1990). The removal of $^{\cdot}O_2^-$ is considered to be more urgent than the formation of H_2O_2 as the former causes more extensive damage to cells (Fridovich, 1997). Plants have multiple isozymes (enzymic) of SOD and each molecular form has a separate metabolic role in the removal of $^{\cdot}O_2^-$ (Scandalios, 1993). Superoxide dismutases are cytosolic and are secreted from the cell while also being localised to specific sub-cellular organelles. They have different metal ion cofactors such as Cu/ZnSOD; MnSOD or FeSOD (Scandalios, 1993), each of these being active at different sites of $^{\cdot}O_2^-$ generation. For example, MnSODs are expressed in the mitochondrial matrix whereas Cu/ZnSODs are expressed in mitochondrial intermembrane

spaces (Turrens, 2003) and plastids in plant cells (Fridovich, 1998). All SODs scavenge $\cdot O_2^-$ by the mechanism of catalysing a disproportionate reaction of $\cdot O_2^-$ dismutation to a rate very close to that of diffusion (McCord & Fridovich, 1969; Scandalios, 1993; Turrens, 2003).

Detoxification of H_2O_2, the second ROS assessed in this study as a stress biomarker, is predominantly carried out through the catalytic reaction by enzyme catalase which is found throughout plant cells but is chiefly located in peroxisomes (Moller *et al*., 2007; Sies, 2014). In the chloroplast, the ascorbate / glutathione cycle is responsible for scavenging H_2O_2 during photorespiratory reactions as catalase is absent in this organelle (Asada, 1992). Glutathione peroxidases are present in the chloroplast, cytosol, endoplasmic reticulum and mitochondria while the peroxiredoxin system is active in the chloroplast, cytosol, mitochondria and nucleus; both function to detoxify excessive production of H_2O_2 at these sites (Moller *et al*., 2007).

Antioxidants, measured individually or in totality (as in this study), have often been used as stress biomarkers during cryopreservation of recalcitrant-seeded zygotic explants or associated procedural steps thereof. Antioxidants have been previously measured in zygotic explants of *T. dregeana* (Varghese *et al*., 2011; Naidoo, 2012; Naidoo *et al*., 2016; Sershen *et al*., 2016) *S. gerrardii* (Berjak *et al*., 2011; Sershen *et al*., 2016) and *Q. robur* (Hendry *et al*., 1992; Pukacka *et al*., 2011) after various steps of cryopreservation, with many of these focused on desiccation. Antioxidants have also been measured in other recalcitrant-seeded species such *C. sativa* (Roach *et al*., 2010) in response to desiccation, in *S. robusta* (Chaitanya & Naithani, 1994) in relation to moisture content and viability loss, and in *Livistona chinensis* (Wen *et al*., 2011) in response to desiccation and freezing. When assessed in conjuction with ROS, it is a useful measure of the efficiency of ROS detoxification in recalcitrant-seeded zygotic explants. In this study, endogenous aqueous antioxidants were quantified as a single measurement and used as a stress biomarker in response to ROS generation and cryogenic stress in recalcitrant-seeded zygotic explants.

1.8.4 Stress biomarker: Respiratory competence

The 2,3,5 triphenyltetrazolium chloride (TTZ) test was developed in Germany in the early 1940's by Georg Lakon (Vankus, 1997) and has been widely accepted as a "snapshot"

indication of viability that gives the maximum number/percentage of respiring cells. In this study it was used on the meristematic tissue of embryonic explants to establish metabolic status and the potential of explants to form functional seedlings (Vankus, 1997; Atia *et al*., 2011; Grzybowski *et al*., 2012; Ntuli *et al*., 2013; Shrivastava, 2013).

In seed tissue, respiratory metabolism results in the oxidation of food reserves, the production of numerous intermediates which form the basis of synthesis of protoplasmic components (proteins, nucleic acids and lipids) and ATP production as a source of energy for these biochemical reactions (Abdul-Baki & Anderson, 1972). Therefore active respiration is an indication of living tissue. In this context, the TTZ test measures the use of dehydrogenase enzymes during respiratory processes (Vankus, 1997) and viability can be recorded as a qualitative or quantitative estimation. Dehydrogenases react with substrates that release hydrogen ions which are subsequently released into the soluble TTZ salt solution. These ions enzymatically reduce the TTZ to a red, stable, non-diffusible, water-insoluble compound called 1,3,5 triphenyl formazan (TPF) which accumulates in respiring tissue. Dead or necrotic tissue will not stain as the lack of respiration prevents the production of formazan. Decrease in mitochondrial dehydrogenases is associated with viability loss in seed tissue (Shaban, 2013). Respiratory activity has been measured qualitatively in seeds (Porter *et al*., 1947) and in recalcitrant-seeded zygotic explants in response to desiccation and hydrated storage, e.g., *Avicennia marina, Q. robur*, *T. dregeana, T. emetica* and *S. madagascariensis* (Ntuli *et al*., 2013). Sershen *et al*. (2016) reported respiratory competence in recalcitrant-seeded zygotic explants of *A. belladonna, H. montanus, T. dregeana* and *S. gerrardii* in response to desiccation using quantitative methods. The present study assessed respiratory competence by the biochemical estimation of dehydrogenase activity described by Sershen *et al*. (2016).

1.8.5 Stress biomarker: Cell ultrastructure

Ultrastructural studies give important clues of the cellular state and can be very useful to support biochemical data. Berjak and Pammenter (2000) reported on various organelles and cytoskeletal structures found within plants/seeds and how their presence and development is indicative of cellular activities. Cryopreservation procedures physically affect tissue, i.e., where injurious mechanical stress is endured by explants, especially after drying (associated with volume reduction when water is lost; Iljin, 1957) and cooling (ice crystallisation; Berjak

& Pammenter, 2013a). Damage is incurred on a biochemical and physiological level during cryopreservation and the weighted contribution of damage to tissue on each of these levels is yet to be determined. The percentage recovery of surviving explants after cryopreservation is typically low because many explants lose viability after desiccation, therefore explants are dead prior to cryogen exposure (Pukacki & Juszczyk, 2015; Naidoo *et al*., 2016). To elucidate physiological responses at an ultrastructural level to the steps selected for cryopreservation in this study, i.e., post-excision soaking, partial dehydration and cooling and rewarming, necessitated microscopical investigations on soaked, dried and frozen and rewarmed explants in relation to fresh material.

The presence and development of certain organelles in zygotic explants during cryopreservation can relay valuable information on the cellular state in response to stress. For example, the degree of development of mitochondria and endoplasmic reticulum indicate respiratory activity and membrane synthesis respectively. These are important organelles to observe when establishing the extent of cryo-related stresses in recalcitrant embryos (Chandel *et al*., 1995; Wesley-Smith *et al*., 2001a; b) and have also been observed in relation to the acquisition and loss of cryotolerance in recalcitrant *L. chinensis* (Wen *et al*., 2011). Vacuoles are the centres of intracellular turnover and increased vacuolation of cells usually indicates a stress response (Berjak & Pammenter, 2000). Golgi bodies are responsible for carbohydrate metabolism and the products of their activity play an important role as cell wall precursors. Therefore the presence of well developed golgi bodies correspond to high intracellular activity which is also a response to externally applied stress (Berjak & Pammenter, 2000; Young *et al*., 2008; Liu *et al*., 2015). The integrity of the cytoskeleton is vital to maintain organisation and transport of organelles within the intracellular *milieu* and disruption usually impacts negatively on viability (Berjak & Pammenter, 2000).

Studies regarding the impact of cryopreservation procedures on ultrastructure and viability in shoot meristems and embryonic explants have been undertaken. Some important advancments in understanding the basis of post-cryo viability loss in recalcitrant germplasm have been made via ultrastructural studies. Perán *et al*. (2006) used microscopy to show that the cotyledonary insertions were contiguous with the shoot apex in explants of recalcitrant-seeded *E. capensis*, and that removal of cotyledons by excision from the explant could

underlie failure of shoot development after cryopreservation. Kioko *et al*. (2006) reported ultrastructural assessments made on zygotic explants of recalcitrant seeded *T. emetica* after rapid and slow dehydration, and different hydrated storage conditions. Wesley-Smith *et al.* (2001a) looked at ultrastructural integrity after rapid and slow drying of explants of recalcitrant-seeded *Artocarpus heterophyllus*. Sershen *et al*. (2012a) demonstrated the benefits of glycerol cryoprotection over sucrose cryoprotection in cryopreserved explants of recalcitrant-seeded *Amaryllis belladonna,* where high viability corresponded to minimal vacuolation and normal ultrastructure. Sershen *et al*. (2012b) illustrated the impact of glycerol and sucrose cryoprotection on respiration, protein synthesis and viability using ultrastructural assessments. Gebashe (2015) showed the derangement of plastids in shoot meristems of recalcitrant-seeded *T. dregeana* when treated with PVS2 treatment as part of the cryopreservation protocol. Wesley-Smith *et al*. (1992) used ultrastructural evidence to study the effects of cooling rates during cryopreservation of explants of the intermediate *Camellia sinensis*. Wesley-Smith *et al*. (2015) showed that ice formation during cooling is not always lethal provided ice crystals are small and localised within the cytoplasm in explants of recalcitrant-seeded *Accer saccharinum*. Those authors also showed autophagic decomposition of cells in explants of *Acer saccharinum* after cryogen exposure and identified the cells from which regrowth of roots (ground meristem and procambium) and shoots (peripheral and pith meristems) occurred.

It is expected that mechanical injury during drying of recalcitrant explants occurs as a consequence of vacuolar collapse in the absence of insoluble material (Berjak & Pammenter, 2013a). As partial dehydration is an essential component of any cryopreservation protocol, it is necessary to ascertain the extent to which ultrastructural integrity of the tissue is lost and how this affects viability. In terms of cooling, it is well established and widely accepted that large ice crystal formation causes lethal physical damage to tissue (Meryman, 1968). This forms the premise into efforts made to obtain the vitrified intracellular state during the freezing process. Since recalcitrant zygotic explants do not, in most cases, respond well to cryoprotection which is a principal mode of achieving cellular viscosity, drying rate and time to achieve optimal WC become instrumental to survival during cryopreservation (Berjak & Pammenter, 2013b). Ultrastructural examination of explants after retrieval from cooling will

give insight on the state of cellular integrity in accordance with the viability retained by each species.

The above mention biomarkers afford a measure of looking at stress after procedural steps during cryopreservation. Exogenously applied antioxidants were used as an ameliorative treatment towards achieving a state of oxidative balance. Ascorbic acid and cathodic water were the substances selected for this purpose and details of these antioxidants are reviewed below.

1.9 L-Ascorbic acid: endogenous functions and exogenous applications

Approximately 70 years ago, the 6-carbon sugar with unknown function was first named Hexuronic acid that was later on named L-threo-hexenon-1,4-lactone or ascorbic acid (AsA; Davies *et al*., 1991; Potters *et al*., 2002; De Tullio & Arrigoni, 2003). All plants are capable of synthesising AsA via the Smirnoff-Wheeler pathway, and it is now undeniable that no plant can survive without it (Smirnoff *et al*., 2001). Ascorbic acid, a highly abundant metabolite plays a significant role in growth, development and stress physiology (Conklin, 2001). It accumulates in millimolar concentrations in photosynthetic and non-photosynthetic tissues and organelles, i.e., cytosol, chloroplast, vacuoles, mitochondria and cell walls (Anderson *et al*., 1983). The concentration of AsA in chloroplasts is high (2-50 mM in chloroplast stroma; Foyer, 1993; Smirnoff, 2000) indicating a central role in photosynthesis and removal of H_2O_2 since catalase is absent in this organelle (Smirnoff, 2000). The powerful reducing ability and acidic properties of AsA is due to the reactive ene-diol group at C2/3 and the ionisation of the hydroxyl group at C3 (pKa = 4.17) respectively (Smirnoff, 1996).

Ascorbic acid is extensively documented for its role as the primary compound for ROS detoxification in the aqueous phase due to its ability to donate electrons in a variety of enzymatic and non-enzymatic reactions (Blokhina *et al*., 2003). Apart from its antioxidant role (which will be discussed further), AsA has functions in a quite a few processes such as photosynthesis, photo protection, cell wall growth and cell expansion, pathogen defence, redox signalling, metal and xenobiotic detoxification (Loewus, 1980; Smirnoff, 2000; Conklin, 2001), activation of biological defence mechanisms, resistance to environmental stress, synthesis of ethylene, gibberellins, anthocyanins, oxylate, tartrate and hydroxyproline

(Smirnoff & Wheeler, 2000), as a co-substrate for the activity of many 2-oxoacid-dependent dioxygenases and as an enzyme co-factor and electron transporter in trans membranes (Arrigoni & De Tullio, 2000; 2002). In addition to these activities, there is overwhelming evidence that illustrate a profound negative effect on plant growth, defences and responses to environmental stimuli/triggers when there is a 50-70% depletion in the ascorbate pool (Foyer & Noctor, 2011).

Ascorbic acid is a vinylgous carboxylic acid where electron pairs are transmitted between the hydroxyl and carbonyl double (vinyl) bond (Flora, 2009). It is implicated as a principle protectant against stress by being energetically and preferentially favourable for oxidation by ROS during aerobic metabolism to preserve more important biomolecules and organelles, thus often being referred to as a "sacrificial antioxidant" (Conklin, 2001; Flora, 2009). The essence of AsA as a potent reductant is based on the following properties, viz.: the poor reactivity of the radicals that form upon interaction with this compound; its ubiquitous presence in adequate amounts in the cell; its ability to react with a variety of free radicals/ ROS and its efficient turnover/regeneration capacity (Arrigoni & De Tullio, 2002; Halliwell & Gutteridge, 2006). These characteristics enable AsA to act as an efficient biological antioxidant which finely regulates ROS without completely eliminating it (Arrigoni & De Tullio, 2000).

The regeneration and reactions of ascorbate and glutathione are linked via the Halliwell-Asada cycle (Potters *et al*., 2002). These reactions are briefly delineated as follows: oxidation of ascorbate results in monodehydroascorbate radicals (MDHAR), which disproportionate to form ascorbate and dehydroascorbate (DHA, unstable above pH 7) which is catalysed by the enzymes ascorbate oxidase and ascorbate peroxidase (Loewus, 1980; Asada, 1994). Under steady-state conditions, the ascorbate pool remains 90% reduced (Foyer, 1993) and is maintained as such by dehydroascorbate reductase (DHAR; which uses glutathione as a reductant) and monodehydroascorbate reductase (MDHAR; which uses NADP (H) as a reductant). In addition to the replenishment of the ascorbate pool, AsA regenerates the lipophilic antioxidant α-tocopherol from the α-chromanoxyl radical which is important for membrane protection in the non-aqueous phase (Flora, 2009).

In desiccation tolerant higher plants, AsA is the first line of defence against oxidative stress while in recalcitrant seeds it is documented that AsA and ASC peroxidase decrease during the desiccation stage (Arrigoni *et al*., 1992; Pukacka & Ratajczak, 2010). Ren *et al*. (2015) showed that the addition of ascorbic acid to cryoprotectants doubled survival ratio in seedlings of *Arabidopsis thaliana*. It is postulated that oxidative stress in recalcitrant systems is exacerbated during cryopreservation as a consequence of diminished activity of AsA. Uchendu *et al*. (2010a; b) reported on the improved re-growth of cryopreserved blackberry (*Rubus*) shoot tips as a consequence of reduced oxidative damage after the use of tocopherol, ascorbic acid, lipoic acid, glutathione and glycine betaine during cryopreservation protocols. Reed *et al*. (2012) supported the use of antioxidant chemicals such as melatonin and AsA during cryopreservation and reported significantly improve survival of cryopreserved shoot tips. More recently, Berjak *et al*. (2011) used cathodic water to ameliorate stresses and improve survival during cryopreservation of zygotic explants of *Boophane disticha* and *S. gerrardii* (discussed further in Section 1.7.5.3). The selected antioxidant treatment throughout cryopreservation procedures for this study was a combination of AsA in a cathodic water solution.

1.10 Cathodic water: properties and application in cryopreservation

Electrolysed water, originally developed by Shimizu and Hurusawa (1992) has been shown to have significant antibacterial and antioxidative properties (Shirahata *et al*., 1997; Hanaoka *et al*., 2004; Hsu, 2005; Huang *et al*., 2008; Berjak *et al*., 2011; Naidoo *et al*., 2016). Reduced "cathodic" water is generated by electrolysis, with the cathode and anode in separate chambers, of a solution containing dilute electrolyte(s). Cathodic water can be distinguished from anodic water (produced at the anode) by its high pH (10.00 – 11.50), high dissolved hydrogen content and low oxidation-reduction potential (-800 to -900mV) (Huang *et al*., 2008). It has been shown to have antioxidant properties *in vitro* in terms of its free radical scavenging ability ($^{\cdot}O_2^-$, H_2O_2, $^{\cdot}OH$ and singlet oxygen) and its role in protecting DNA, RNA and proteins from oxidative damage (Shirahata *et al*., 1997). Hanaoka (2001) reported an increase in $^{\cdot}O_2^-$ dismutation by antioxidants in the presence of cathodic water as a consequence of increased dissociation activity of water. Cathodic water itself did not show superoxide dismutation activity when applied on its own (Hanaoka, 2001). However, cathodic water was able to reduce H_2O_2 levels due to the activity of its constituent –

activated, dissolved H_2 which is the ideal scavenger of dissolved O_2. Berjak *et al.* (2011) provided evidence of the efficacy of cathodic water supplied at appropriate steps of cryopreservation including retrieval of recalcitrant zygotic explants of *S. gerrardii* from liquid nitrogen, and also showed its positive effects on endogenous antioxidant activity in explants of *B. disticha*. The use of cathodic water in the present investigation is based on its proposed antioxidant abilities either as a powerful reductant and/or as an enhancer of endogenous antioxidant activity. Based on the previously established effectiveness of cathodic water to facilitate viability and reduce ROS levels in recalcitrant-seeded tissues during cryopreservation (Berjak *et al*., 2011; Naidoo, 2012; Gebashe, 2015; Naidoo *et al*., 2016), this electrolysed solution was used in combination with ascorbic acid as an exogenous antioxidant treatment.

1.11 This study

Rationale and motivation:

Many studies have shown that a range of physical, physiological and biochemical stresses preclude either shoot or root development or both in cryopreserved germplasm from recalcitrant seeds (Benson, 1990; Pence, 1990; Engelmann, 1998; Berjak *et al*., 1999b; Kioko, 2002; Kioko *et al*., 2006; Naidoo, 2010; Naidoo *et al*., 2011; Pammenter *et al*., 2011; Gebashe, 2015). Furthermore, studies have shown the following:

i. the procedural steps involved in cryopreservation of recalcitrant-seeded germplasm can inflict different types and degrees of damage, based on the species and explant type (Chaudhury & Malik, 1999; Wen *et al*., 2010: Berjak *et al*., 2011; Sershen *et al*., 2016);
ii. the potential for oxidative stress exists for almost all the procedural steps involved in the cryopreservation of recalcitrant-seeded germplasm (Benson, 1990; Roach *et al*., 2010; Uchenda *et al*., 2010a; b; Whitaker *et al*., 2010) and
iii. amelioration of the stresses, particularly those inflicted by imbalances in redox metabolism, inflicted during the various procedural steps can improve post-cryo survival in recalcitrant-seeded germplasm (Song *et al*., 2004; Berjak *et al*., 2011; Naidoo *et al*., 2011; Uchendu *et al*., 2010a; b; Reed *et al*., 2012).

This motivated the present study which investigated different approaches towards ameliorating some of the stresses associated with the procedural steps involved in the

cryopreservation of recalcitrant-seed germplasm. Based on the findings of a number of studies (cited earlier) that suggest that the levels of stress experienced and hence, survival achieved during cryopreservation of recalcitrant-seeded germplasm is based on the species and explant type, the study adopted a comparative approach, involving four species (viz. *T. dregeana*, *Q. robur*, *S. gerrardii* and *L. sinensis*) that differ in terms of explant morphology, anatomy and post-cryo survivability.

Aims: The over-arching aims of the study where to, (i) elucidate, compare and contrast some of the stresses associated with the various procedural steps involved in cryopreservation across the four species selected for investigation, and (ii) investigate approaches towards ameliorating some of these stresses, particularly those associates with uncontrolled ROS production.

Objectives: The specific objectives of the study were as follows:

i. to quantify and compare explant viability at each procedural step,
ii. to quantify and compare explant ROS production, and antioxidant and respiratory activity in relation to viability at each procedural step,
iii. to assess the effects of post-excision soaking, partial dehydration and cooling and rewarming on shoot meristem ultrastructural integrity and relate this to viability following each of these steps,
iv. to compare the effects of the various procedural steps across the zygotic explants of the four species using the stress biomarkers investigated (e.g. ROS production and antioxidant activity) and
v. to assess the effects of stress ameliorative approaches such as exogenous antioxidant protection on post-cryo survival in the four species.

Methodological approach:

i. **Development of species specific cryopreservation procedures:** As mentioned above, cryogenic procedures can cause physical damage and induce physiological changes in explants, both of which influence survival. The extent of damage caused by these events is largely dependent on morphological/anatomical explant characteristics, which differ across species. The development of strategies to

cryopreserve explants considered their individual tissue architecture and physiological responses in order to select the most suitable techniques for *in vitro* procedures, i.e., germination media selection and decontamination protocols; and cryo-preparative steps i.e., explant excision, post-excision soaking and partial dehydration with the aim of viability retention/improvement. Procedures conducted during these steps were based on previous methods used for explants of the species specific to the present study or on explants from other recalcitrant-seeded species (Berjak *et al*., 1990; Fu *et al*., 1993; Pammenter *et al*., 2002; Peràn *et al*., 2006; Berjak *et al*., 2011; Chmielarz *et al*., 2011; Naidoo *et al*., 2011).

ii. **Measures to counteract oxidative stress:** The application of an exogenous antioxidant solution during procedural steps of cryopreservation was used as a measure to scavenge excessive ROS production. This implementation was necessary to counteract an imbalance that might occur in the oxidative system due to ROS inducing steps of cryopreservation and the possible dysfunctionality of the endogenous antioxidant system in recalcitrant-seeded germplasm (Pammenter & Berjak, 1999). Methodology followed for composition of the exogenous antioxidant solution and the steps of cryopreservation during which it is applied to explants are those used in Berjak *et al*. (2011) and Naidoo (2012).

iii. **Characterisation of stress using determinants of survival:** Cryopreservation procedures carried out on recalcitrant-seeded explants induces direct physical and physiological changes which influence survivability (Chandel *et al*., 1995; Pammenter *et al*., 1998; Liang & Sun, 2002; Walters *et al*., 2008; Xia *et al*., 2014). These procedures concomitantly influence ROS production, antioxidant activity (Roach *et al*., 2008; Whitaker *et al*., 2010; Berjak *et al*., 2011; Naidoo *et al*., 2011; Varghese *et al*., 2011), respiratory competence (Leprince *et al*., 1999; Pammenter & Berjak, 1999; Ntuli *et al*., 2013; Sershen *et al*., 2016) and ultrastructural integrity (Wesley-Smith *et al*., 2001a; Wesley-Smith *et al*., 2004; Sershen *et al*., 2012a; b) which indirectly affect survivability. Stresses associated with cryopreservation procedures are induced by physical manipulation of the explant and can be exacerbated by oxidative imbalances that may occur as a consequence of structural damage/alterations, for e.g., the oxidative burst of $^{\cdot}O_2^-$ upon wounding of the explant during excision (Roach *et al*., 2008); ROS activity associated with the time of exposure of explants to intermediate

WC during partial dehydration (Pammenter *et al*., 2008; Varghese *et al*., 2011). The impact of physiological alterations to the explant can also reflect in the respiratory competence and the state of cellular ultrastructure during procedural steps.

On this basis, stress biomarkers selected for this study, i.e., $^{\cdot}O_2^-$, H_2O_2, TAA, respiratory competence and ultrastructure were assessed at each procedural step to determine if any of these variables individually or interactively play a role in survival of explants. Biochemical estimations of ROS, antioxidants and respiratory competence were modified on published methodologies (Misra & Fridovich, 1972; Harding & Benson, 1995; Re *et al*., 1999; Gay & Gebicki, 2000). Ultrastructural studies used transmission electron microscopical analyses (Reynolds, 1963; Sabatini *et al*., 1964; Spurr, 1969) and were conducted on shoot meristems of explants. The production of shoots by excised zygotic explants from recalcitrant-seeded species has been a consistent hindrance in obtaining seedling development during cryopreservation (Kioko, 2002; Kioko *et al*., 2006; Perán *et al*., 2006; Hajari, 2011; Naidoo, 2012; Naidoo *et al*., 2016). This study selected shoot meristems for ultrastructural assessment as this meristematic region is likely to be more sensitive than root meristems to cryopreservation procedures and is therefore more likely to incur stress. Microscopical analyses of shoot meristems were undertaken to facilitate a deeper understanding of the implications of cryopreservation procedures at the cellular level.

1.12 Species of interest

The species selected for investigation here exhibit differences in their origin, embryo and seed anatomy and explant type used for cryopreservation. These differences are established to influence post-cryo survivability (Ballesteros *et al*., 2014). The four species are described in greater detail below in terms of their ecology, economic importance and post-harvest seed physiology.

1.12.1 *Trichilia dregeana* Sond.: *Trichilia dregeana* (Meliaceae) is commonly known as the Forest Mahogany in KwaZulu-Natal and is widely distributed in the southern and

eastern parts of Africa (Pooley, 1993). The specific name, *dregeana*, honours the German collector and botanical explorer, Johan Franz Dregè who collected specimens of this species when visiting South Africa. It is very similar to *T. emetica* in terms of overall appearance but there is a difference in height (10-40m) and seed size, which is noticeably bigger in *T. dregeana*. A further distinguishing feature of *T. dregeana* is the leaves, which are shiny, have an almost hairless under-surface and are pointed, rather than rounded at the tip. The fruit and seeds of this species are consumed by many birds while the bright scarlet aril is eaten by baboons (Schmidt *et al*., 2002).

The wood of *T. dregeana* is also used in the rural carving industry and the seeds can be cooked as a vegetable once the aril has been removed (van Wyk & Gericke, 2000). The oil contained in the seeds can be used for industrial purposes (Mulholland & Taylor, 1980; Schmidt *et al*., 2002). Five limonoids have been isolated from the seeds, which include trichilin A and dregeanin, both of which are known to have insect repellent properties (Hutchings *et al*., 1996) and may contribute to the medicinal properties of this species. The bark is exploited mainly for its purgative effects and is also used in concoctions to treat backache and kidney ailments (Hutchings *et al*., 1996). The use of leaves for the treatment of syphilis is quite common in Nigeria, and aqueous leaf extracts have been shown to have some antimicrobial properties (Hutchings *et al*., 1996).

In 1990, Choinski reported that the seeds of *T. dregeana* were recalcitrant and chilling-sensitive. Since then, many investigations have been conducted resulting in in-depth characterisation of the seeds (Han *et al*., 1997; Kioko *et al*., 1998; Goveia *et al*., 2004; Goveia, 2007), as well as attempts to cryopreserve various explants (Kioko, 2002; Goveia, 2007). Some of the published work on this species includes strategies for their hydrated storage (e.g., Berjak *et al*., 2004); the potential for sub-imbibed storage (Drew *et al*., 2000); the effect of dehydration on the nucleoskeleton (Anguelova-Merhar *et al*., 2003); the possible association of dehydrin type molecules with their desiccation sensitivity (Han *et al*., 1997); the effect of developmental status on the explant choice for cryopreservation (Goveia *et al*., 2004); the effect of partial dehydration and cooling on survival of *T. dregeana* embryonic explants (Kioko *et al*., 1998); the role of ascorbic acid during desiccation of explants (Song *et al*., 2004); the production of ROS by excised, desiccated and cryopreserved explants

(Whitaker *et al*., 2010); the effect of differential drying rates of embryonic explants on survival and oxidative stress metabolism (Varghese *et al*., 2011) and the ameliorative effects of antioxidants to balance ROS activity and facilitate survival in excised embryonic explants (Naidoo *et al*., 2011; Naidoo *et al*., 2016; Sershen *et al*., 2016). In light of numerous unsuccessful attempts to cryopreserve explants of *T. dregeana*, this species was selected for investigation as it is known to incur physical and oxidative damage during the various steps of cryopreservation and is representative of some of the many challenges faced during cryogenic procedures applied to tropical-recalcitrant embryos.

The zygotic explant for this species has cotyledonary segments contiguous with the shoot apex. This embryo is unique in comparison to those of the other species, where the shoot meristem of this explant is most likely to incur physical injury during excision (Goveia *et al*., 2004).

1.12.2 *Quercus robur* L.: *Quercus robur*, commonly known as English, French or pendunculate Oak belongs to Fagaceae and is distributed within most of Europe, and temperate regions of China, North Africa and North America (Eaton *et al*., 2016). Fruits from the *Q. robur* tree are called acorns (pedunculate) and these ripen and shed during autumn (Eaton *et al*., 2016). In Latin, "*robur"* literally means strength or hard timber, and accordingly *Quercus robur* trees are exploited in the Forestry industry for the long-lasting and durable heartwood in their barks which is in high demand for interior and furniture work (Logan, 2005). Trees are also useful for the production of tanning leather, food for livestock, small mammals and birds (acorns are rich in starch), and for the provision of habitats for insects and wildlife (Tansley, 1952; Logan, 2005).

There have been many studies that have investigated aspects of *Q. robur* seeds and explants that have relevance to the present contribution. Finch-Savage (1992a; b) pioneered some of the early work bearing to conservation, where those authors investigated (i) the differential desiccation sensitivity of explant and cotyledonary tissue and determined critical WC below which explants will not survive desiccation and (ii) the rapidity of viability loss in explants and cotyledons during desiccation. Hendry *et al*. (1992) looked at ROS activity during declining WC and noted differences in the molecular defences, specifically antioxidants,

between explant and cotyledonary tissue. Finch-Savage *et al*. (1992) also investigated water status and endogenous ABA levels during embryonic explant development. Poulsen (1992), Chmielarz (1997) and Berjak *et al*. (1999b) investigated cryopreservation of *Q. robur* embryos, where post-cryo seedling development was only achieved by Berjak *et al*. (1999b). Berjak *et al*. (1999b) also reported on the highly detrimental effects of decontamination, desiccation and cooling on root and shoot apices of explants. Chaudhury & Malik (1999) also reported cryopreservation of embryonic explants to be unsuccessful. Mycock *et al*. (2000) looked at the impact of desiccation on microtubule and microfilament systems and the ability of the cytoskeletal components to reconstitute after desiccation. In 2011, Chmielarz *et al*. proposed a cryopreservation protocol for plumules of *Q. robur* and Plitta *et al*. (2014) validated this protocol by reporting no incidence of harmful effects on DNA integrity after procedural steps applied to plumules.

Quercus robur is considered to be a species of temperate origin and was included in this study as some success has been achieved in terms of its cryopreservation. The zygotic explant is unique, where the shoot meristem/plumule is partially concealed within the cotyledonary body and no physical injury occurs to this region during excision.

1.12.3 *Lychee sinensis* Sonn.: Commonly known as Litchi of the family Sapindaceae, this species was first described by a Frenchman, Pierre Sonnerat in 1782 (Groff, 1943). The trees can be described as small, dense, perennial evergreens reaching a height of 4-12 m (Morton, 1987). Fruits are borne in loose clusters of 3-20 and are rounded to ovate in shape depending on the cultivar (Morton, 1987). *Lychee sinensis* is adapted to warm tropical/sub-tropical climates and requires only a chilling period (temperatures below 20-22°C) to flower, pending the cultivar. Trees are widely distributed throughout Europe and Asia, certain parts of America, Swaziland and South Africa (FAO, 2007). *Lychee sinensis* does not reproduce well from seeds and seed cultivation is therefore primarily for selection/breeding purposes or for rootstocks. All parts of the tree are over-exploited for its numerous uses, i.e., these seeds are used as a food source consumed fresh, canned, dried or processed into juice or wine; as a source of honey; for its durable timber; usefulness in agroforestry and for a number of medicinal properties. The fruit, peel, flowers and seeds are used to treat many medical ailments such as for cough relief, gastralgia, tumours and enlargement of glands; analgesic

action; as a remedy for smallpox eruptions and diarrhoea; relief of intestinal troubles and throat ailments (Morton, 1987) etc.

There are published studies that have addressed and/or reported cryobiological investigations on the embryonic explant (Chaudhury & Malik, 1999; Sudarmonowati, 2000) as well as studies on the effect of moisture content and temperature on seed viability during desiccation (Fu *et al*., 1990). The zygotic explant of this species, unlike any of the others, has the entire embryo embedded within endospermous tissue. It is the smallest explant across species (at least 10 times smaller than any of the other explants) and incurs no physical damage upon excision to either the shoot or root meristem.

1.12.4 *Strychnos gerrardii* N.E. Br.: *Strychnos* (family Loganiaceae) is commonly known as the Forest Black Bitterberry or Black Monkey orange. The family is distributed widely throughout the world, with nine species represented in South Africa (South African National Biodiversity Institute, 2005). Trees of *S. gerrardii* are found along the coastal and dune forests of KwaZulu-Natal and reach heights of 10 - 25 m (Pooley, 1993). The leaves are shiny, hairless, fairly thin and taper at the tip and the base. Flowering occurs between November and January, and fruiting from April to November (Pooley, 1993). Fruits contain multiple endospermic seeds and have a substantial woody pericarp/exocarp that changes from green to pale yellow upon ripening (Pooley, 1993). Despite the mild toxicity of seeds of this species, the fruit pulp is eaten by humans and monkeys. The species with toxic seeds, known for producing strychnine (*S. nux-vomica*) and curare poison (*S. toxifera*), are found in India and South America, respectively (Pooley, 1993; Philippe *et al*., 2004).

A few studies have been carried out on the branches (Itoh *et al*., 2005) and leaves (Hoet *et al*., 2006) of another closely related species, *S. spinosa* to determine its chemical properties. Preliminary studies carried out by Khuzwayo (2002) indicated that the seeds of *S. gerrardii* show recalcitrant behaviour. More specifically, with regard to explant cryopreservation, Goveia (2007) optimised *in vitro* culture conditions, partial dehydration and decontamination procedures for *S. gerrardii* and showed that short-term seed storage enhanced *in vitro* germination after cryopreservation procedures before exposure to LN, although incomplete development (100% root elongation only) was achieved after retrieval from LN. Berjak *et al*.

(2011) reported breakthrough results when those authors showed the ameliorative effects of using cathodic water as a powerful reductant during various steps of cryopreservation, as exemplified by the highest post-cryo seedling survival of explants to date for this species (70%).

The zygotic explant excised from this species is unlike any of the other explants in the lack of hardiness of its tissue. The cotyledonary segments are papery thin and easily detach from the shoot meristem of the relatively small embryo. However, to further reduce the size of this explant, the cotyledons were halved horizontally such that no physical injury is incurred by the shoot or root meristem.

The primary reason for selecting this species in this study is the documented capacity of these explants to produce functional roots and shoots after cooling and drying (Berjak *et al*., 2011), thus completing the selection for this comparative study of recalcitrant-seeded species that display varying degrees of survival after cryopreservation.

1.13 Structure of dissertation

Chapter 1 features an in-depth literature review on the theory, practice and principles of cryopreservation and provides context for the rationale, aims and objectives of this study which are also explained. The four species investigated are also described.

Chapter 2 details the materials and methods employed in this study. This section includes a description of the explants investigated, methodology employed during the procedural steps of cryopreservation carried out, novel techniques used to alleviate some of the stresses associated with cryopreservation, and the assays used to assess the various stress biomarkers.

Chapter 3 features results on the physiological responses of the explants to the various procedural steps of cryopreservation and the corresponding effects on selected stress biomarkers. The results show how the development of cryopreservation protocols for explants across species required specific optimisations at procedural steps such as explant excision and partial dehydration, and the application of ameliorative treatments to counteract oxidative damage. Physiological responses to treatments applied are reported in the form of

explant viability and images (transmission electron microscopy) of shoot meristem cellular ultrastructure. Changes in stress biomarkers during each procedural step are compared within treatments/species across steps and within steps across treatment/species. Correlative analysis of the relationships between explant physiology and post-cryo viability, and stress biomarkers were undertaken. The data presented and analyses carried out are designed to address the aim of elucidating, comparing and contrasting stresses associated with the various procedural steps of cryopreservation.

Chapter 4 features a discussion on the roles of explant anatomy and physiology potentially played in determining post-cryo survival in the four species investigated, in relation to the stress biomarkers investigated. The results are interpreted by drawing on the broader literature on seed biology, plant germplasm conservation and stress physiology.

Chapter 5 presents the principle conclusions of the study, specifically in terms of the factors contributing to explant viability loss during cryopreservation of the four species. Recommendations for future avenues of research on cryopreservation of recalcitrant-seeded germplasm are also offered.

CHAPTER 2: Materials and Methods

This chapter outlines materials used and methods employed in this study for (i) the various procedural steps involved in the cryopreservation of recalcitrant-seeded zygotic explants and (ii) the materials and methods associated with the various biomarkers measured/assessed during these steps. All chemicals used in this study were procured from Sigma Aldrich (Germany), unless stated otherwise.

2.1 Plant Material

2.1.1 Seed procurement, decontamination and short term storage

***Trichilia dregeana*:** Mature fruits of *T. dregeana* were harvested between April and July over multiple years (2013-2015) to complete different aspects of the study. Open fruits were collected from trees located in the Glenwood (29.8708°S, 30.9905°E) and Westville (29.8317°S, 30.9303°E) suburbs of Durban, South Africa. The seeds were detached from capsules and the seed coat and aril were subsequently removed before hydrated storage (described below). A batch of seeds, immediately after collection and removal of seed coat, were used to determine the fresh weight and embryo shedding WC.

Whole seeds were decontaminated prior to hydrated storage by treatment with a 1% sodium hypochlorite (NaOCl) solution containing a few drops of the wetting agent, Tween 20/80® for 20 min on an orbital shaker (Labcon, Instrulab CC, Maraisburg, South Africa) at 120 rpm. Seeds were subsequently treated with an anti-fungal 'cocktail' of 0.5 ml l^{-1} Early Impact (active ingredients, triazole and benzimidizole; Zeneca Agrochemicals, South Africa) and 2.5 ml l^{-1} Previcur N (active ingredient, propamocarb-HC; AgrEvo, South Africa) and placed on an orbital shaker at 120 rpm for 240 min. The active ingredients contained in the decontamination treatment have previously been shown to be effective in curtailing fungal proliferation during storage of recalcitrant seeds (Calistru *et al*., 2000; Berjak *et al*., 2004). Decontaminated seeds were rinsed three times with sterile distilled water before being placed on paper towel for 24 h to dry back to their original batch fresh weight. They were then used immediately or prepared for hydrated storage.

Hydrated storage was carried out for seeds of *T. dregeana*, *L. sinensis* and *S. gerrardii*. This entailed lightly dusting seeds with Benomyl 500 WP (active ingredient, benzimidizole; Villa Protection, South Africa) and placement of seeds as a monolayer on a plastic mesh. In white, translucent 5 *l* plastic buckets, the mesh was suspended 200 mm above paper towel saturated with water to which a few drops of commercial domestic bleach had been added (Kioko, 2002; Naidoo, 2010; Hajari, 2011). Bucket lids were lined with paper towel (to prevent condensate dripping back onto the seeds) before the buckets were sealed and stored at 16°C. Containers were not entirely air-tight and allowed for minimal gas exchange with the atmosphere while maintaining high moisture content. Both plastic mesh and buckets were previously decontaminated by soaking them in a 1% NaOCl solution for 1 h, and subsequently wiped with 70% ethanol prior to use. Buckets were opened intermittently (minimum of once a week) to aerate seeds and to check for fungal proliferation or germination in storage. Seeds that were decontaminated or displayed ongoing germination were immediately discarded.

Strychnos gerrardii: The globose fruit come into season between August and September. Fruits are typically hard and woody with a bluish green colour when young; these were manually collected over 2 years (2014-2015). Only once fruit had ripened, indicated by the woody shell of the fruit turned yellowish-orange, were the seeds extracted. The fruits were cracked open with a metal vice grip and the hard, tetrahedral seeds were removed from the yellow pulp using a scalpel and nylon mesh. Each fruit, size pending, generally contained 10-15 seeds.

Seeds were decontaminated with the anti-fungal cocktail used for *T. dregeana* (described above) for 120 min and prepared for storage under hydrated conditions (described above). Seeds from this species were not used immediately. *Strychnos gerrardii* is likely to exhibit climacteric behaviour, where ripening occurs after fruit have been harvested. This has been exemplified by the ongoing development of the embryonic axis during storage of the seeds, approximately 20-35 d after hydrated storage. For the purpose of a consistent developmental stage of the seeds, experimental work was conducted after storage for 20-35 d (Berjak *et al*., 2011) after which time the explant was fully mature.

Quercus robur: Acorns were collected in Kórnik, Poland during October over multiple years (2012-2015) and air-freighted to South Africa. The maturity and germinability of acorns were determined by the ease with which the cups are removable, the colour of cup scars and the colour of the pericarp. Only acorns having a darkened pericarp and cups that were easily removable were selected and stored. Upon removal, the cup scars were bright, an indication of vigorous and viable acorns, as opposed to dark cup scars that are usually typical of rotting or diseased acorns.

The major problem with *Q. robur* seeds is contamination by fungus *Ciboria batschiana,* which is localised under the pericarp of the acorn seed and not on the surface. Remediation of this type of contamination is usually achieved through thermotherapy ([1]pers. Comm. M. Michalak), which unnecessarily increases the WC of the seeds and was avoided in this study. Therefore, seeds were not surface decontaminated but were lightly dusted with Benomyl and stored in large plastic buckets lined with plastic bags. *Quercus robur* is classified as a temperate-recalcitrant seeded species (Finch-Savage *et al*., 1994b) and seeds are acclimatised to colder temperatures (between 6 and 8°C) in the natural habitat (Vitasse *et al*., 2014). Seeds were therefore stored at 6°C in a constant temperature room and were used immediately or taken out periodically as required for experimentation.

Lychee sinensis: Fruits were sourced from Woolworths (Woolworths Holdings Limited, South Africa) in December over multiple years (2013-2015). Different varieties of *L. sinensis* are grown in South Africa but the fruits bought from Woolworths were harvested exclusively from Mauritian variety trees. The outer coat and fruit pulp were removed exposing the oval, brown, shiny seeds. Seeds were surface decontaminated using a 5% solution of sodium dichloroisocyanurate, commonly known as NaDCC (active ingredient: hypochlorous acid) for 30 min followed by 3 rinses with sterile distilled water. Seeds were prepared for hydrated storage as described above for *T. dregeana* and *S. gerrardii* or used immediately as required.

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2.1.2 Seed morphology and explant selection

Seeds of all species used in this study exhibit recalcitrant seed storage behaviour, and whether of temperate or tropical origin, have typical characteristics of being large with high shedding WC. The morphology, topography and tissue composition (endospermic or non-endospermic) of seeds from each species differ, and these differences have implications on the physical and oxidative damage that may be incurred by the embryo. The mature embryo, consisting of the precursor tissues for leaves (cotyledons), stems (hypocotyls) and embryonic roots (radicle) was the explant of choice for all species. As most recalcitrant seeds cannot be cryopreserved due to their large size and mass (Pammenter & Berjak, 1999; Berjak & Pammenter, 2013a), the embryo is the smallest explant that represents the genetic diversity of each species. Seed morphology and embryo description are as follows:

Trichilia dregeana: Seeds are non-endospermic and dicotyledonous. The zygotic embryo (4-5 mm in length) forms a continuous body with the cotyledons. Cotyledons are attached to the shoot meristem region of the explant (Fig. 2.1b: Inset). The explant consisted of the embryo and an excised portion of the cotyledons (Fig. 2.1b: Inset).

Strychnos gerrardii: Seeds are endospermic and dicotyledenous. The zygotic embryo is partially embedded in the endosperm, and consists of an axis (3-4 mm in length) and attached papery-thin cotyledons (Fig. 2.2b). The explant comprised of the zygotic embryo with an attached portion of cotyledons (Fig. 2.2b) horizontally excised away from the explant to minimise size and therefore increasing surface area: volume which is the basis for good post-cryo survival (Pammenter *et al.*, 1994; 1998).

Lychee sinensis: Seeds are endospermic with a fleshy and succulent aril (Nacif *et al*., 2000; Fig. 2.3a). The embryo of this seed is miniscule (1-3 mm) and completely embedded within the endosperm (Fig. 2.3b). The embryo is too small to be removed completely without incurring any damage. Therefore, the zygotic explant consisted of the embryo excised with a limited amount of endosperm surrounding it (Fig. 2.3b) as complete removal of the endosperm was not possible.

Quercus robur: Seeds are non-endospermic and dicotyledenous. The seed is covered completely by the pericarp, which is formed by hardening of the ovary walls (Bonner & Vozzo, 1987; Fig. 2.4a & b). The seed has a polarised embryo with a defined shoot apex and root cap which is continuous with the cotyledons. The zygotic explant is the embryo (4-6 mm in length) which is excised such that the plumules are enclosed within the split end of the cotyledons at the shoot apex, taking care not to expose this region to direct physical damage (Fig. 2.4c).

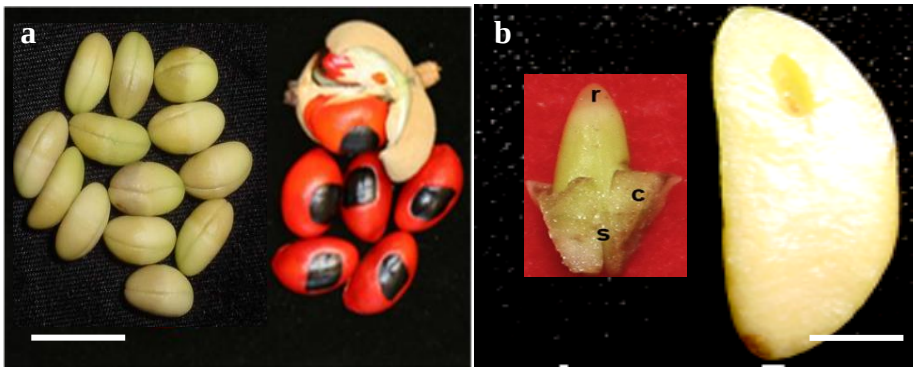

Fig. 2.1a & b: Cleaned *T. dregeana* seeds and whole seeds with seed coat (a). Longitudinally cut section of a *T. dregeana* seed (b). **Inset:** Excised zygotic explant with cotyledonary segments attached. Root meristem (r), Shoot meristem (s), Cotyledon (c). **Bar = 5mm**.

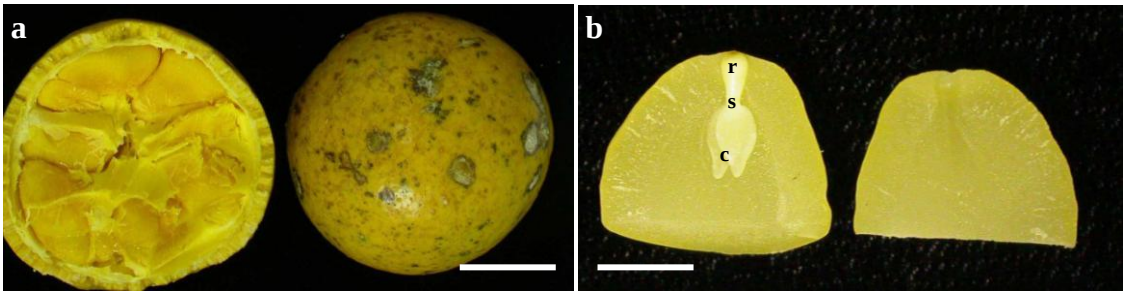

Fig. 2.2a & b: Cracked open mature fruit of *S. gerrardii* showing whole seeds within pulp (a). Longitudinal section of a *S. gerrardii* seed (top half containing the explant) showing the zygotic explant (b). Root meristem (r), Shoot meristem (s), Cotyledon (c). **Bar = 5mm**.

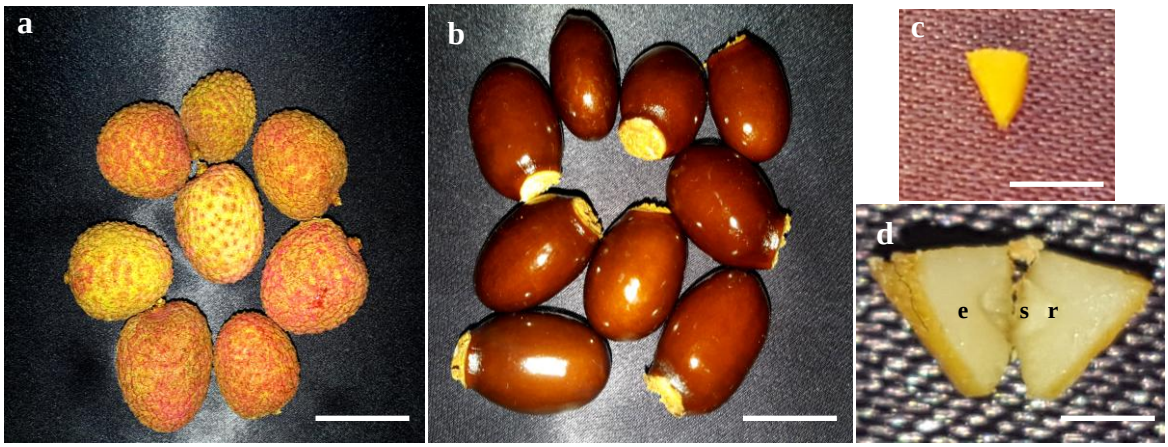

Fig. 2.3a-c: Mature fruits (a) and whole, cleaned seeds (b) of *L. sinensis*. **Bar = 5 mm.** Excised zygotic explant of *L. sinensis* (c) showing complete enclosure of the root and shoot meristem by endosperm (e) and split explant exposing the embryo (d). Root meristem (r), Shoot meristem (s). **Bar = 0.5 mm**.

Fig. 2.4a-c: Longitudinal section of *Q. robur* seed (a). Root meristem (r), Shoot meristem (s), Cotyledon (c). **Bar = 5mm**. [2]Mature *Q. robur* seeds (b). **Bar = 5mm**. Excised zygotic explants from *Q. robur* seeds showing enclosed shoot meristems (c). **Bar = 10 mm**.

[2] *Quercus robur* images (b) & (c) provided by Dr Pawel Chmielarz, Institute of Dendrology, Polish Academy of Sciences, Kòrnik, Poland.

2.2 Cryopreservation procedure: methods applied to each step

Before describing the methods used in each of the procedural steps involved in the cryopreservation of the species investigated, it is necessary to detail methods of explant WC determination, explant viability assessment *in vitro* and cathodic water generation. These methods apply to more than one of the procedural steps investigated. Figure 2.5 illustrates the order of, and parameters assessed at, each step.

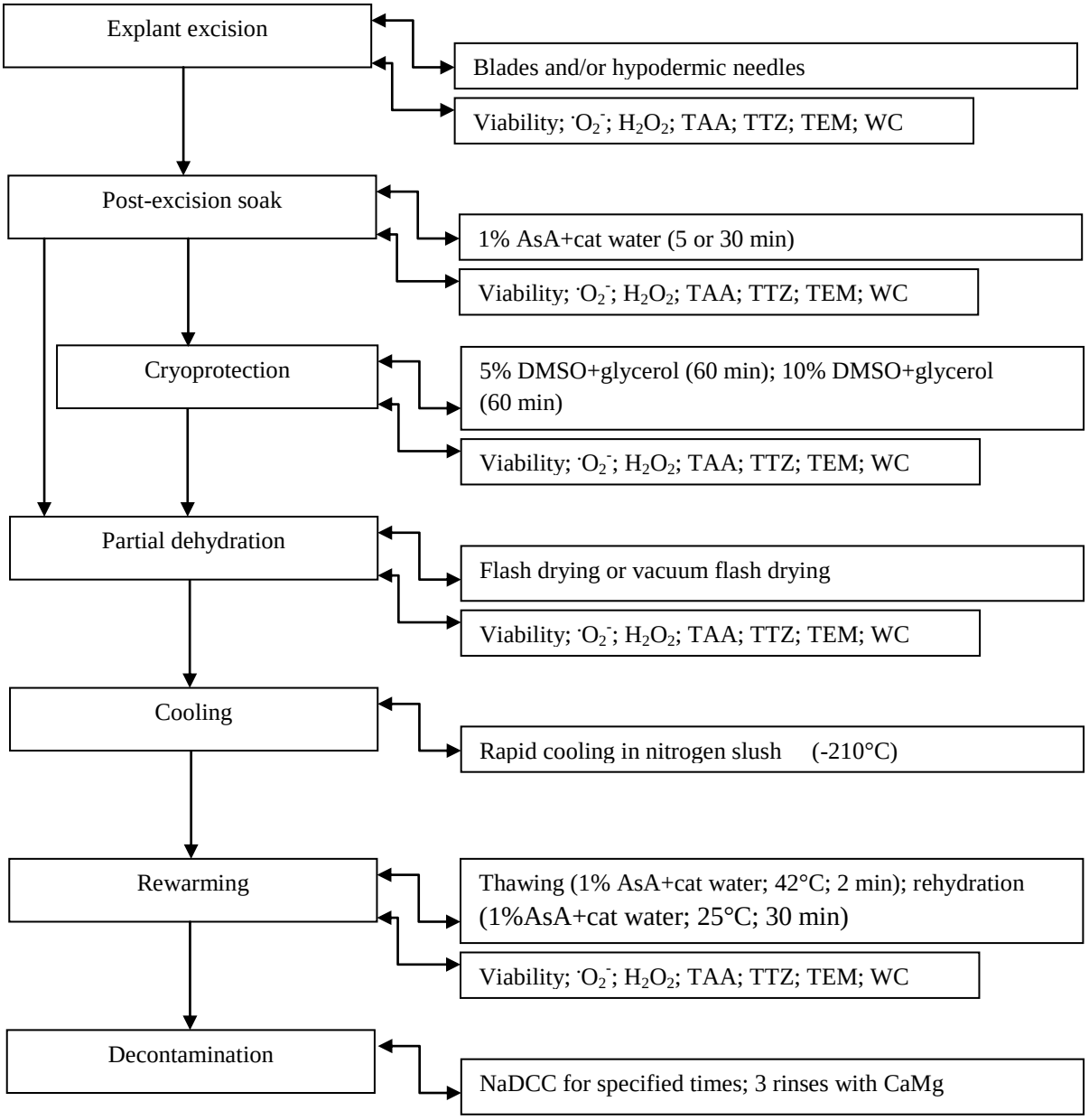

Fig. 2.5: Schematic representation of the experimental design (cryopreservation procedure) and the parameters assessed at each step. These procedures and assessments were carried out on explants of *T. dregeana*, *Q. robur*, *S. gerrardii* and *L. sinensis*.

2.2.1 Water content determination

Water content was determined for fresh (control) explants and after four selected treatments, i.e., post-excision soaking (referred to as soaking henceforth), soaking+flash drying, soaking+cryoprotection and soaking+cryoprotection+drying (described below in sections 2.2.7-2.2.11). Water content was determined gravimetrically and expressed as g H_2O g^{-1} dry mass basis (g g^{-1} dmb). Initial WC of explants was determined periodically during hydrated storage since replicates for various treatments were spread over time using seeds that were in hydrated storage for a few weeks (*L. sinensis*) up to a few months (*T. dregeana*; *Q. robur*; *S. gerrardii*). Using a six-place micro-balance (Mettler MT5; Germany), zygotic explants (n=10) were weighed individually in aluminium foil boats, after which they were dried to constant weight (at 80°C for 48 h). Explants were then brought to ambient temperature in the weighing boats over activated silica gel in closed glass Petri dishes, and reweighed to ascertain the dry mass. The WC was determined using the formula below:

$$WC\ (g\ g^{-1}\ dmb) = FW\ (g) - DW\ (g)/DW\ (g)$$

Where: WC= water content; FW= fresh weight; DW= dry weight

2.2.2 Determination of *in vitro* procedures for explant viability assessment

2.2.2.1 Optimisation of explant decontamination procedure for *in vitro* germination

Explant decontamination was conducted prior to *in vitro* germination in all cases. Trials were conducted with a range of decontaminants for selected time periods (Table 2.1). Viability after decontamination trials was assessed in terms of root production *in vitro* (as described below). The decontaminants tested including NaOCl, calcium hypochlorite [$Ca(OCl)_2$] (active ingredient: hypochlorites) and NaDCC (active ingredient: hypochlorous acid). These decontaminants were selected based on their efficacy as recalcitrant-seeded explant decontaminants during cryopreservation in other studies (Berjak *et al*., 2011; 2014; Naidoo *et al*., 2016; Sershen *et al*., 2016).

The detailed results for these studies are given in Chapter 3 but it is necessary to mention here that the most effective decontamination treatment for explants of all species was a 5 000

ppm (0.5%) solution of NaDCC prepared in sterile distilled water. The duration of exposure to NaDCC ranged from 5 to 20 min pending decontamination requirements for explants of each species.

Table 2.1: Selected decontaminants and duration of exposure for application to explants of *T. dregeana, Q. robur, S. gerrardii* and *L. sinensis*.

Decontaminant (exposure period)	**Species**			
	T. dregeana	***Q. robur***	***S. gerrardii***	***L. sinensis***
0.5% NaDCC (5 min)	-	-	-	+
0.5% NaDCC (10 min)	+	+	+	+
0.5% NaDCC (15 min)	-	-	+	-
0.5% NaDCC (20 min)	-	+	-	-
1% NaOCl (10 min)	+	+	-	-
1% $Ca(OCl)_2$ (10 min)	-	-	+	-

Dashes indicate decontaminants that were not tested for explants of specific species.

2.2.2.2 *In vitro* viability assessment

Viability was assessed for 20 explants (n=20) after each procedural step involved in cryopreservation. Once treated with the most effective decontaminant(s) the explants were rinsed three times with a sterile calcium magnesium solution (CaMg solution; 0.5 mM $CaCl_2.2H_2O$ and 0.5 μM $MgCl_2.6H_2O$ [Mycock, 1999]). Thereafter, the explants were blotted dry using sterile filter paper (Whatman no. 1) and cultured *in vitro* as described below.

Explants were cultured on sterile MS medium, either with or without plant growth regulators see below), in 65 mm sterile plastic Petri dishes (5 explants per dish) under a laminar air flow (Airvolution, Alphalab, Roodeport, South Africa). In the cases of *T. dregeana, S. gerrardii* and *Q. robur* plates were incubated in a growth room at 27°C with a 16 h photoperiod (light intensity approximately 50 μmol m^{-2} s^{1}). For *L. sinensis*, the Petri dishes were maintained in a plant growth chamber (GC-300 TLH, Jeio Tech, South Korea) at 30°C (16 h photoperiod) as this elevated temperature has been reported to improve vegetative growth in certain cultivars (Menzel & Simpson, 1990).

For explants that had been exposed to soaking+flash drying, soaking+cryoprotection+flash drying and subsequent cooling treatments, Petri dishes were initially maintained in the dark for 7 d, or until radicle extension growth was observed, before being transferred to the growth room/growth chamber. This minimises photo-oxidative reactions that may induce further free radical production or accumulation thereof (Benson, 2000; Varghese *et al*., 2009).

Viability was scored in terms of root and shoot production across species, after all procedural steps of cryopreservation. However, in the case of *L. sinensis,* post-cooling viability was assessed in terms of root production and callus development from the shoot meristem.

The culture medium and decontaminants used for explants was optimised accordingly for each species (Table 2.1 & 2.2). The detailed results of these studies are presented in Chapter 3 but for the purposes of this Chapter the most suitable culture medium and decontamination protocols are described below.

Table 2.2: Selected media prepared with/without phytohormones or additives for *in vitro* culture of decontaminated explants of *T. dregeana*, *Q. robur*, *S. gerrardii* and *L. sinensis*.

Media composition	**Species**			
	T. dregeana	***Q. robur***	***S. gerrardii***	***L. sinensis***
Full MS	+	+	+	+
Full MS + 1 mg l^{-1} BAP	+	-	-	-
Full MS + 1 mg l^{-1} BAP+ 2 mg l^{-1} AsA	-	+	-	-
Full MS + 0.4 g l^{-1}AC	-	-	+	-
Full MS + 1 g l^{-1} 2 iP + 2 g l^{-1} trans-zeatin	-	-	-	+
¼ MS + 0.4 g l^{-1} AC	-	-	+	-

Dashes indicate media compostion/s not tested for explants of specified species.

Trichilia dregeana: The *in vitro* germination medium selected for this species was full strength (4.4 g l^{-1}) MS basal medium (Murashige & Skoog, 1962) containing sucrose (30 g l^{-1}) and agar (10 g l^{-1}) at a pH of 5.6-5.8. Media was supplemented with the synthetic cytokinin 6-benzylaminopurine (BAP; 1 mg l^{-1} [Perán *et al*., 2006; Naidoo *et al*., 2011]). Explants were decontaminated with 0.5% NaDCC for 10 min prior to culturing for *in vitro* germination.

Strychnos gerrardii: The *in vitro* germination medium selected for this species was full strength (4.4 g l^{-1}) MS basal medium (Murashige & Skoog, 1962) containing sucrose (30 g l^{-1}) and agar (10 g l^{-1}) at a pH of 5.6-5.8. Activated charcoal (0.4 g l^{-1}; Berjak *et al*., 2011) was suspended in the medium to mitigate phenolic exudations. Explants were decontaminated with 0.5% NaDCC for 15 min prior to culturing for *in vitro* germination.

Quercus robur: The *in vitro* germination medium selected for this species was full strength (4.4 g l^{-1}) MS basal medium (Murashige & Skoog, 1962) containing sucrose (30 g l^{-1}) and agar (10 g l^{-1}) at a pH of 5.6-5.8. Media was supplemented with BAP and AsA (1 and 2 mg l^{-1} respectively). Explants were decontaminated with 0.5% NaDCC for 20 min prior to culturing for *in vitro* germination.

***Lychee sinensis*:** The *in vitro* germination medium selected for this species was full strength (4.4 g l^{-1}) MS basal medium (Murashige & Skoog, 1962) containing sucrose (30 g l^{-1}) and agar (10 g l^{-1}) at a pH of 5.6-5.8. Media was supplemented with the natural cytokinins 6-(γ,γ-Dimethylallylamino) purine (1 mg l^{-1}; Sigma-Aldrich, Germany) and trans-Zeatin (2 mg l^{-1} ; Sigma-Aldrich, Germany). Explants were decontaminated with 0.5% NaDCC for 10 min prior to culturing for *in vitro* germination.

2.2.3 Application of exogenous antioxidants during the various procedural steps of cryopreservation

The various procedural steps involved in the cryopreservation of recalcitrant-seeded germplasm have been shown to result in abnormally high levels of ROS production and reduced antioxidant activity (see Introduction, Section 1.8). The use of exogenous antioxidants to reduce levels of oxidative stress during these various steps has been shown to improve survival in some species (Berjak *et al*., 2011; Naidoo, 2012; Naidoo *et al*., 2016). The present study investigated the potential benefits of two of these antioxidants, viz., cathodic water and AsA, at selected steps of the cryopreservation procedure (Fig. 2.5). Before

application of an exogenously applied antioxidant as part of a cryopreservation protocol, some preliminary experiments were conducted to establish (i) the most effective antioxidant treatment to reduce ROS production and (ii) the duration of antioxidant application.

2.2.4 Generation of cathodic water

Berjak *et al*. (2011) had shown the beneficial effects of using cathodic water to improve viability and reduce $^{\cdot}O_2^-$ production in explants of *S. gerrardii*. Cathodic water, described as electrolysed-reduced water by Shirahata *et al*. (1997) is produced by electrolysis of an electrolyte containing solution. Cathodic water was generated in this study via the electrolysis of CaMg solution (see section 2.3) by the provision of a 60V potential difference, using a BioRad™ Powerpac (BioRad, Hercules, California, USA) to which platinum electrodes were attached (Berjak *et al*., 2011). The setup included two glass beakers, each containing 200 ml autoclaved CaMg solution, with the anode being immersed in one beaker and the cathode in the other (Fig. 2.6). The circuit was completed via a salt bridge which was agar-based and contained saturated potassium chloride. The composition of the salt bridge was prepared by dissolving 30% KCl (w/v) and 3% agar (w/v) in 10 ml of distilled water. The electrolysis of the CaMg solution was conducted over 60 min at room temperature and yielded cathodic (reducing) water with a pH *c*. 11.2 and anodic (acidic) water of pH *c*. 2.4 (Berjak *et al*., 2011).

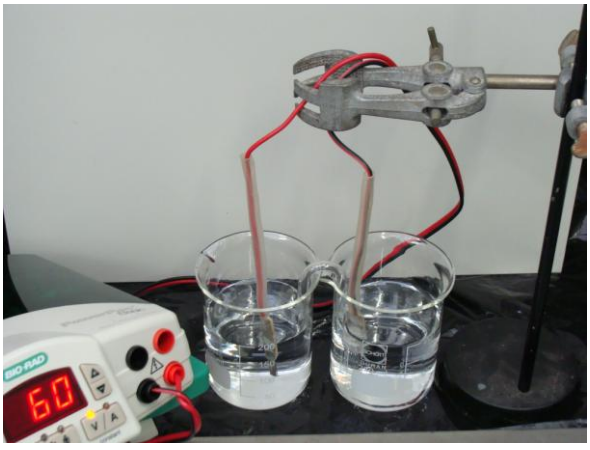

Fig. 2.6: Apparatus used to generate electrolysed-reduced water (cathodic water) via the electrolysis of an electrolyte containing solution.

2.2.5 Assessment of the effect of selected soaking treatments on ROS production using explants of *Q. robur* and *T. dregeana*

Uchendu *et al*. (2010a; b) had shown the benefits of using AsA during cryopreservation procedures to improve viability in *Rubus* shoot tips. Based on the studies of Uchendu *et al*. (2010a; b) and Berjak *et al*. (2011), trials on the most effective exogenous solution to use during cryopreservation were undertaken. Explants of *Q. robur* and *T. dregeana* were selected as representative species for these trials based on the availability of these species at the time. Effectiveness of each treatment was based on the reduction of $^{\cdot}O_2^-$ and H_2O_2 during steps of cryopreservation. The effect of cathodic water (antioxidant properties) was tested against the effect of a CaMg solution (non-antioxidant properties) on ROS reduction in explants of *Q. robur*. Based on those results (reported in Chapter 3), the effect of cathodic water and a 1% solution of AsA (w/v) in cathodic water (w/v) were tested on ROS production in explants of *T. dregeana*. Both these solutions have antioxidant properties. Solutions were applied as a soak (immediately after excision), as a solvent for cryoprotectants (during cryoprotection), as a rehydration solvent (immediately after partial dehydration), and a rewarming solvent (immediately after retrieval of explants from LN). The steps during which assessments were taken are schematically represented in Fig. 2.7.

Results on these trials are presented in Chapter 3, however it is necessary to state here that upon comparison of solutions on the effect of ROS production, a 1% AsA+cathodic water solution was selected as being the most effective in reducing ROS during selected steps of cryopreservation. While the efficacy of this solution in reducing ROS levels was tested only on explants of *T. dregeana*, it was selected to be applied across all species during cryopreservation steps.

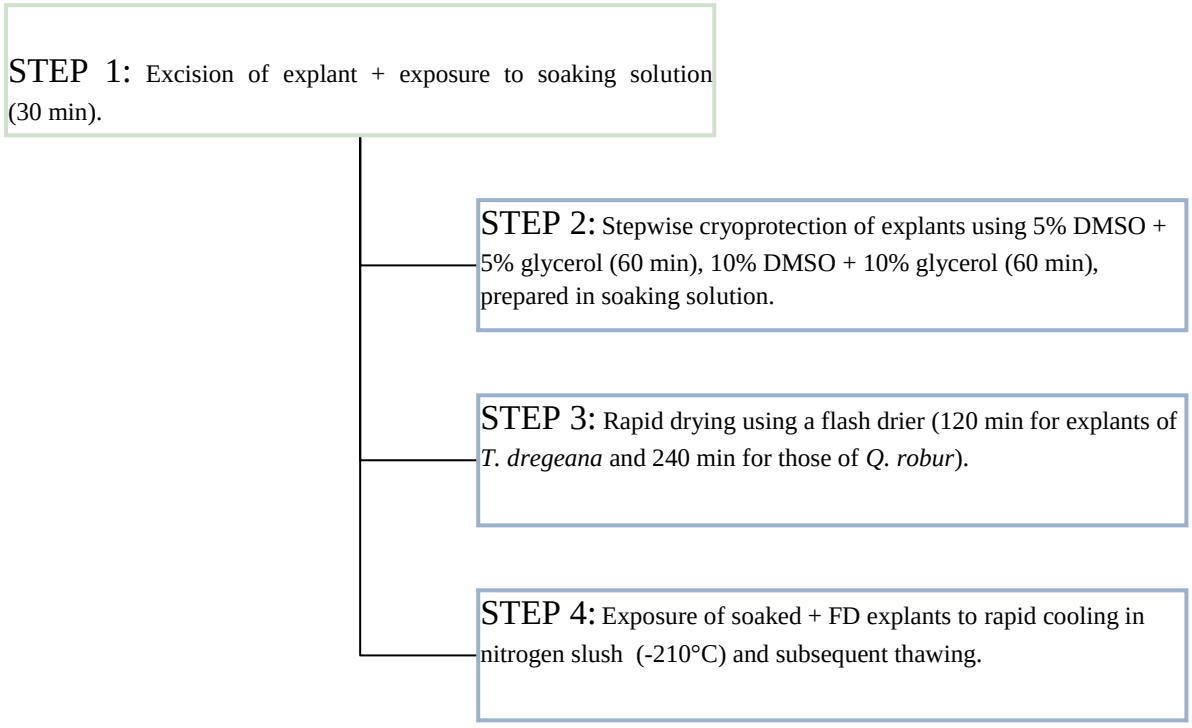

Fig. 2.7: Schematic representation of the experimental design (steps of cryopreservation) used to assess the effects of a soaking/rehydration solution. Assessment of ROS production was carried out on explants of *T. dregeana* and *Q. robur* after exposure to each of these steps. Soaking solution in each step indicate either CaMg or cathodic water for explants of *Q. robur*. For explants of *T. dregeana* soaking solution indicates cathodic water or 1% AsA+cathodic water.

2.2.6 Selection of exposure period to an antioxidant soaking solution for explants of *T. dregeana*, *Q. robur*, *L. sinensis* and *S. gerrardii*

Based on the selection of 1% AsA+cathodic water as the exogenously applied antioxidant treatment, the duration of exposure to this solution for explants of all species had to be determined. This was established after conducting trials that assessed germination capacity and vigour following exposure of explants to 1% AsA+cathodic water for 5, 10, 15, 30 and 60 min. The detailed results of these studies are presented in Chapter 3 but for the purpose of this chapter it should be noted that explants of all species were immersed in a 1% solution of AsA prepared in cathodic water (w/v) after excision (as a soak), prior to partial dehydration (as a soak) and after cooling (as a rewarming medium). Based on those results (Chapter 3), exposure time for soaking was selected as 30 min for explants of *T. dregeana*, *S. gerrardii*

and *Q. robur* and 5 min for explants of *L. sinensis*. However, explants across species were exposed to this solution for 30 min during rehydration and rewarming.

The effects of cathodic water and AsA were assessed in terms of their ability to facilitate seedling development, and their influence on $^{\cdot}O_2^-$, H_2O_2 levels, TAA and respiratory activity during the various procedural steps involved in cryopreservation. Methods used for the cryopreservation procedures referred to, such as cryoprotection (CP), flash drying (FD), cooling and rewarming (C+RW) are described in sections 2.3.2 – 2.3.5. The methods used to measure ROS production, TAA and respiratory activity are described in section 2.4.

2.2.7 Explant excision and soaking

Zygotic explants were excised from seeds using sterile 11 pt (for *T. dregeana* and *L. sinensis*) or 22 pt (*S. gerrardii* and *Q. robur*) surgical blades depending on the species. For explants of *T. dregeana*, hypodermic needles were used to remove excess cotyledon tissue after excision as the use of needles limit damage to the shoot meristem region (Naidoo *et al*., 2011). Explants were accumulated in a Petri dish under 100% relative humidity, as a preventative measure against slow dehydration of the tissue. Excision of explants was conducted in the dark to minimise photo-oxidative reactions. After excision, explants from all species were exposed to an antioxidant soak in 1% AsA+cathodic water (5 min for *L. sinensis* and 30 min for *T. dregeana*, *Q. robur* and *S. gerrardii*). Across all species, control explants (excised only) and those treated with soaking, were surface decontaminated and cultured to assess viability (n=20) as mentioned earlier. ROS production ($^{\cdot}O_2^-$ and H_2O_2), TAA capacity, respiratory activity and WC were assessed in control and soaked explants of all species. Soaked explants were blotted on filter paper prior to WC determination. (WC data reported in Chapter 3, Section 3.3). Transmission electron microscopy was used to assess ultrastructure of shoot meristems from control and soaked explants across all species.

2.2.8 Cryoprotection

Explants were treated with the following penetrating cryoprotectants in a stepwise manner subsequent to soaking: DMSO and glycerol (5 and 10% [v/v] each) individually and in combination, prepared in cathodic water as described in Naidoo *et al*. (2016). Cryoprotectants were applied for 60 min each, i.e., cryoprotection was conducted over 120 min. Naidoo

(2012) reported that this combination of cryoprotectants had ameliorative properties in terms of regulating H_2O_2 and facilitating seedling production after soaking+CP in *T. dregeana* explants which had not previously been achieved. Therefore this was the selected cryoprotectant treatment applied to explants of all species. Explants treated with soaking+CP were surface decontaminated and cultured to assess viability (n=20). Immediately after soaking+CP, ROS production ($^{\cdot}O_2^-$ and H_2O_2), TAA capacity, respiratory activity and WC were assessed in explants of all species. Soaked+CP explants were blotted on filter paper prior to WC determination. Explants across species that were soaked and soaked+CP were also subjected to FD, the methods of which are described below.

2.2.9 Explant partial dehydration: optimisation of drying method and explant target water content

Dehydration is a commonly used technique to remove water from tissues prior to direct immersion in LN (Engelmann, 2011), and is widely used during the cryopreservation of zygotic explants (Engelmann, 1997). Ultra-rapid drying of explants in a stream of compressed air (Berjak *et al*., 1989) usually allows explants to survive freezing at WC between 0.25-0.4 g g^{-1} (dmb) (Vertucci *et al*., 1991; Dereuddre *et al*., 1992; Wesley-Smith *et al*., 1992; Sershen *et al*., 2012a; b). In this study, three drying techniques were employed and compared to establish the technique that facilitated drying as rapidly as possible to WC amenable for cryopreservation (target WC) with minimum loss of viability.

The optimum drying technique was selected using soaked explants. The most efficient drying method selected for explants of each species was then applied to soaked+CP explants from each species to establish a suitable drying period.

Desiccation over silica gel **(after Chmielarz *et al*., 2011):** Fifty grams of activated silica gel was dispensed into 90 mm glass Petri dishes. Filter paper (65 mm) was placed on the silica gel. Explants that were soaked in 1% AsA+cathodic water were blotted dry and placed on the filter paper and were left to desiccate within the closed Petri dish for pre-determined time intervals.

***Flash drying* (after Berjak *et al*., 1990; Pammenter *et al*., 2002):** Explants that were soaked in 1% AsA+cathodic water were blotted dry and placed on a fine-mesh nylon, below which a stream of air was circulated through activated silica gel (250 g) and then over the explants at a rate of approximately 10 *l* min^{-1}. Flash drying occurred within a 500 ml glass bell jar.

***Vacuum flash drying* (after Fu *et al*., 1993):** Explants that were soaked in 1% AsA+cathodic water were flash dried as described above. However, the apparatus used for drying was placed in a desiccator attached to a vacuum, as opposed to a glass bell jar. The activated silica gel used in the desiccator was 500 g.

Independent flash drying curves (indicating WC and viability at different drying intervals) were constructed for each species after soaking+FD and soaking+CP+FD, to optimise duration of drying required to bring the explant WC to an appropriately low level (0.25 – 0.4 g g^{-1} dmb) while still retaining sufficient viability before immersion in a cryogen. Plant organs that usually survive exposure to LN should contain water within this range or less (Vertucci *et al*., 1991; Dereuddre *et al*., 1992; Wesley-Smith *et al*., 1992; Sershen *et al*., 2012a; b) so as to avoid intracellular ice formation. After each drying interval, explants to be assessed for viability were rehydrated in a 1% AsA+cathodic water and decontaminated prior to *in vitro* germination, as described earlier (Section 2.2.2.2). Water content was determined after each drying interval for soaked+FD explants and soaked+CP+FD explants prior to rehydration.

Drying curves are presented in results (Chapter 3, Section 3.3 & 3.4) only for the drying technique selected for each species. Based on those results, flash drying was selected as the desiccation method for explants of *T. dregeana*, *Q. robur* and *S. gerrardii*. Vacuum flash drying was selected as the technique used for explants of *L. sinensis*.

Complete drying profiles for soaked+FD explants and soaked+CP+FD explants across species are presented in Chapter 3 (Section 3.3 and 3.4 respectively). However, it is necessary to note here that after soaking, explants of *S. gerrardii, L. sinensis, T. dregeana* and *Q. robur* were flash dried for 20, 30, 120 and 240 min respectively to reach WC ranging between 0.37-0.42 g g^{-1} (dmb). After soaking+CP, explants of *S. gerrardii, L. sinensis, T. dregeana* and *Q. robur* were flash dried for 25, 40, 150 and 260 min respectively to reach WC ranging

between 0.37-0.42 g g^{-1} (dmb). Biochemical assessments (described below) on explants across species were measured after these selected drying periods only.

Across all species, soaked+FD and soaked+CP+FD explants were rehydrated in 1% AsA+cathodic water for 30 min, surface decontaminated and cultured to assess viability (n=20). Immediately after soaking+FD, ROS production ($O_2^{\cdot-}$ and H_2O_2), TAA capacity and respiratory activity were assessed in explants of all species. Transmission electron microscopy was used to assess ultrastructure of shoot meristems from soaked+FD explants across all species. Biochemical and ultrastructural assessments were not conducted on soaked+CP+FD explants, as this combination of treatments were not suitable for explants of the investigated species (results presented in Chapter 3; Section 3.3) and were not assessed further.

Subsequent to specific pre-desiccation treatments, drying methods and drying intervals were selected for explants of each species. Thereafter, certain drying characteristics were assessed. These characteristics and respective calculations are presented in Table 2.3.

Table 2.3: Description of drying characteristics and the respective calculations used to measure them in soaked and dried explants across species.

Drying characteristic	Calculation
WC after soaking and specified drying interval	(FW – DW)/ DW
Amount of water lost in drying period (g)	*Initial WC – **Final WC
% water loss in drying period	(*Initial WC – **Final WC) /*Initial WC%
Drying rate (g H_2O g^{-1} DW min^{-1})	(*Initial WC – **Final WC)/drying time
Drying rate (% H_2O loss min^{-1})	(*Initial WC – **Final WC)/*Initial WC %/drying time

*Initial WC indicates WC after excision+soaking of explants

**Final WC indicates WC after selected drying period for explants from each species

2.2.10 Cooling

Ultra rapid cooling with nitrogen slush was the technique applied to explants across all species (Echlin, 1992). This was achieved by plunging naked explants directly into nitrogen slush after desiccation. To prepare nitrogen slush [-210°C], 300 ml of liquid nitrogen was dispensed into a polystyrene cup. This was then placed in a desiccator and transformed under vacuum into nitrogen slush (Steponkus & Caldwell, 1993). Explants that were frozen in nitrogen slush were then tumble-mixed in liquid nitrogen (LN) for approximately 1 min; LN slush reverted to the liquid form [-196°C] in minutes. Explants were thereafter transferred under LN into cryovials (Greiner Bio-One®, Frickenhausen, Germany) that were stored for a minimum of 24 h in LN Dewars (Schorn Cryogenics, Boksburg, South Africa) prior to rewarming as described in Section 2.2.11.

Based on viability results obtained during cryo-preparative procedures (Chapter 3; Section 3.1-3.3), the final cryopreservation procedure comprised of soaking and flash drying explants (according to species specific optimisations developed for each) before immediate exposure to ultra-rapid cooling (as described above). Viability, biochemical and ultrastructural assessments were taken after cooled explants were rewarmed and decontaminated.

2.2.11 Rewarming (Perán *et al.*, 2004)

The rate of rewarming has been linked to the rate of cooling (Mazur, 2004; Wesley-Smith *et al*., 2004). Based on the rapid cooling technique selected for explants across species, it was necessary that sufficiently rapid rewarming was employed to prevent recrystallisation in the event of intracellular ice formation during cooling (Walters *et al*., 2008). All cryopreserved explants were rewarmed via direct immersion in a rewarming solution conducted in 65 mm plastic Petri dishes. This entailed rapidly thawing of explants at 42°C for 2 min in 1% AsA+cathodic water (w/v) which was heated in a standard water bath (Juchheim labortechnik, Germany). Explants were subsequently rehydrated at 25°C for 30 min in a solution of the same composition. Explants were then decontaminated (as described in Section 2.2.2.2). All post-cooling procedures were conducted in the dark.

Across all species, explants treated with soaking+FD+C+RW were surface decontaminated and cultured to assess viability (n=20). After these treatments, ROS production ($\cdot O_2^-$ and

H_2O_2), TAA capacity and respiratory activity were assessed in explants of all species. Transmission electron microscopy was used to assess ultrastructure of shoot meristems from soaked+FD+C+RW explants across all species.

2.3 Biochemical assays

For ROS estimations, analysis was conducted on six biological replicates of five explants. For TAA estimations, analysis was conducted on four biological replicates of five explants, after which two technical replicates from each sample was taken. For respiratory activity, analysis was conducted on eight biological replicates of individual explants.

2.3.1 Estimation of extracellular $\cdot O_2^-$ production

To assess extracellular production of $\cdot O_2^-$, the oxidation of epinephrine to adrenochrome was measured spectrophotometrically at a wavelength of 490 nm (Misra & Fridovich, 1972), using the UV-Vis spectrophotometer (Cary 50 Conc UV Vis spectrophotometer, Varian). A 1 mM epinephrine solution (pH 7.0) in 1 M HCl was prepared in the dark prior to the assay. The incubation medium consisted of 0.5 ml epinephrine solution and 1.5 ml distilled water dispensed into six 2 ml Eppendorf® tubes containing five explants per treatment (n=6). These were then placed on an orbital shaker (Labcon, Instrulab CC, Maraisburg, South Africa) at 70 rpm, in the dark, at room temperature, for 15 min (after Roach *et al*., 2008). After incubation, the solutions from each Eppendorf® tube were pipetted into plastic cuvettes (Sarstedt, Sarstedt AG & Company, Nümbrecht, Germany) and the absorbance for each sample was recorded at 490 nm. Explants from each sample were then placed in an oven at 80°C for 48 h to obtain dry mass. The extinction co-efficient of adrenochrome at $A_{490,}$ 4.47 mM^{-1} cm^{-1} was used to calculate $\cdot O_2^-$ production and this was expressed in μmol min^{-1} g^{-1} dmb. The absorbance was also measured for an internal control for each treatment in which zygotic explants were incubated (as described above) in distilled water to account for any leakage from the tissues during incubation (Varghese *et al*., 2011). This absorbance was deducted from all estimations prior to calculations.

Validation of $\cdot O_2^-$ assay: Horseradish SOD was added to the incubation medium described above to give a final concentration of 0.1 μg ml^{-1} before this solution was dispensed into tubes containing explants (both treated and control). In both cases, the addition of SOD to the incubation medium inhibited the oxidation of epinephrine by more than 50%, validating the

use of this assay for extracellular $\cdot O_2^-$ detection (Whitaker *et al*., 2010). This assay was performed using the UV-Vis spectrophotometer (Cary 50 Conc UV Vis spectrophotometer, Varian).

2.3.2 Estimation of extracellular H_2O_2 production

Hydrogen peroxide levels were measured using the xylenol orange assay (Gay & Gebicki, 2000), with a few minor modifications. Six replicates of five explants per treatment (n=6) were incubated in 1.0 ml distilled water on the orbital shaker and rotated at 70 rpm, at room temperature, in the dark for 30 min. Thereafter, 300 µl of the incubation medium (distilled water) was added to 1 500 µl of the working reagent. The working reagent consisted of a ratio mix of Solution A and Solution B (1:100). Solution A contained 25 mM $FeSO_4$; 25 mM $(NH_4)_2SO_4$ and 2.5 M H_2SO_4 prepared in sterile, deionised distilled water. Solution B contained 125 mM xylenol orange (Sigma-Aldrich, Germany) and 100 mM sorbitol prepared in sterile, deionised distilled water. The second incubation was conducted as above for 20 min. Optical densities of the blank and treatment samples were read at 560 nm (Cary 50 Conc UV Vis spectrophotometer, Varian, Palo Alto, CA) and H_2O_2 levels were calculated from a standard curve. All procedures subsequent to primary excision of the explant were conducted under dark conditions to limit ROS generation initiated and perpetuated by photo-oxidation (Touchell & Walters, 2000).

Validation of H_2O_2 assay: Specificity of this assay was confirmed by more than 50% inhibition of H_2O_2 production when 250 µml^{-1} of catalase was added to the assay mixture.

2.3.3 Estimation of total aqueous antioxidant (TAA) capacity

Total aqueous antioxidant capacity (enzymic and non-enzymic) was measured in treated and control explants (n=8) using the ABTS decolourisation assay, which is applicable to lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants (Re *et al*., 1999). The radical monocation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; ABTS•1) is pre-formed (i.e., not continually generated in the presence of an antioxidant), being generated by oxidation of ABTS with potassium persulfate ($K_2S_2O_8$) and is thereafter reduced by hydrogen-donating antioxidants. The protocol for TAA assessment considers both the concentration of the antioxidant and the

duration of the reaction on the inhibition of radical cation absorption. The estimation of TAA is described here in two stages: extraction and spectrophotometric analysis.

Extraction: Four batches of five explants (each weighing approximately 20 mg), were pre-weighed and homogenised in LN and 0.1 g insoluble polyvinylpyrrolidone (PVP), using pre-cooled pestles and mortars. Explants that were treated with antioxidant solutions were briefly rinsed 3 times with distilled water (to remove traces of treatment substances, e.g., AsA or cathodic water) and blotted with filter paper prior to homogenisation in LN. Extraction buffer (50 mM KH_2PO_4 buffer; pH 7.0, containing 1mM $CaCl_2$, 1mM KCl and 1mM EDTA), was prepared in advance and kept in a refrigerator at 4°C. Using a micropipette, 1 ml of extraction buffer was added to the homogenised explants and mixed well. Each extract was dispensed into pre-chilled 2 ml Eppendorf® tubes held on ice. Each mortar was then rinsed twice with 0.5 ml of extraction buffer (0.5 ml per rinse), bringing the total volume of extract to 2 ml.

The Eppendorf® tubes containing the extracts were maintained on ice for 15 min and vortexed (Snijders, Holland) at 5 min intervals; these tubes were then centrifuged at 4ºC for 10 min at 14 000 rpm (Hermle Labortechnik, Germany). After centrifugation, the supernatant (1 ml) was dispensed into pre-chilled Eppendorf® tubes placed on ice and centrifuged for a further 5 min under similar conditions. Thereafter, 600 µl of the antioxidant extract was dispensed into another set of pre-chilled Eppendorf® tubes and kept on ice for the ABTS assay.

Spectrophotometry: The spectrophotometer was zeroed with 1 ml phosphate buffered saline (PBS). Prior to assessment of TAA in the extracts, a working solution of ABTS was prepared. This involved the dilution of the ABTS radical solution (7 mM ABTS and 2.45 mM $K_2S_2O_8$ in 1 ml of distilled water and incubated in the dark 12-16 h before use) with phosphate buffer saline (PBS; 5 mM Na_2HPO_4 and 37.5 mM NaCl, pH 7.4) until an absorbance of 0.70 ± 0.02 at a wavelength of 732 nm was attained (Johnston *et al*., 2006). Once the working solution was optimised, 1 ml of working solution was pipetted into a plastic cuvette and absorbance read at 0 min before the reaction was initiated. Immediately thereafter, 100 µl (*T. dregeana*); 80 µl (*Q. robur*); 60 µl (*S. gerrardii*) and 50 µl (*L. sinensis*) of extract from each sample was added to the cuvette which was inverted three times using a piece of Parafilm® to initiate reaction of the contents. The decline in absorbance was subsequently recorded after 0.5, 1

and 2 min. The assay was performed twice for each replicate and hence the values presented represent the mean of eight estimations. Phosphate buffer saline was used as the blank during the assay.

Standard curve for ABTS: For each set of experiments a standard curve for the ABTS assay was generated for the calculation of TAA. For this purpose, a 5 mM Trolox® (6-hydroxy-2.5.7.8-tetramethylchromeane-2-carboxylic acid, 97%; Sigma-Aldrich, Germany) stock was prepared using TAA extraction buffer. From the stock solution, 1 ml of the following standard concentrations was prepared: 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1 mM, using the extraction buffer. To generate a standard curve, the assay for Trolox® was conducted exactly as the ABTS assay for extracts from tissues (described above), except that the volume of each standard solution changed according to the extract volume specific to each species. The standard solutions were added to the ABTS working solution in place of the extract from each treatment. Absorbance readings were taken from each of the standard solutions three times and averaged.

The ABTS radical decolourisation assay was conducted on explants after each step as outlined in Fig. 2.5. All calculations for TAA capacity were expressed on a fresh mass basis (fmb).

2.3.4 Estimation of extracellular respiratory activity

Respiratory activity was measured using a protocol modified after Harding and Benson (1995). Individual explants from each species (n=8) were incubated in 1.5 ml of aqueous 2,3,5-Triphenyl tetrazolium chloride (TTC) solution, consisting of 300 µl of 2% TTC [w/v] in Tris-HCl buffer [0.05 M at pH 7.5]) + 1.2 ml Tris-HCl buffer [0.05 M, pH 7.5]. This was conducted after every step of cryopreservation (outlined in Fig. 2.5). The explants were incubated in the dark at room temperature for 12 h after which the TTC solution was decanted from the Eppendorf® tubes, and immediately replaced with 1 ml ethanol (95 % v/v) for the purpose of formazan extraction. Explants were maintained in ethanol in the dark for 24 h. The absorbance of the extract was read at 500 nm using a UV-Vis Spectrophotometer (Cary 50 Conc UV Vis spectrophotometer, Varian). Explants were dried individually for 48 h in an oven at 80°C after which the dry weight was recorded using a 6 place balance (Mettler

MT5; Germany). Respiratory activity was calculated and expressed as μmol formazan g^{-1} per 12 h dmb.

All spectrophotometric estimations, were carried out using an ultra-violet - visible (UV-Vis) spectrophotometer (Cary 50 Conc UV Vis spectrophotometer, Varian, Palo Alto, CA), at a constant temperature of 25°C, in the dark.

2.4 Ultrastructural studies

Transmission electron analysis was assessed on five individual explants per treatment. Assessment of ultrastructure was done for all species on shoot meristems excised from explants that were (i) untreated, (ii) soaked, (iii) soaked+FD and (iii) soaked+FD+C+RW. These treatments formed the procedural steps that were carried out to cryopreserve explants across species.

Immediately after the selected treatments, explants were incubated on MS medium for 48 h prior to fixation. Thereafter, specimens were fixed in 2.5% phosphate-buffered glutaraldehyde (0.1 M, pH 7.2) for 24 h at 4°C (Sabatini *et al*., 1964) after which they were rinsed several times with phosphate buffer before being post-fixed in 0.5% osmium tetraoxide for 60 min at room temperature. Dehydration of specimens was achieved using a graded acetone series (30%, 50%, 75% acetone each for 5 min and 100% acetone for 10 min). This was followed by infiltration and embedding of the specimens in low-viscosity Spurr's resin (Spurr, 1969). Polymerization of the specimens was achieved in 8 h at 70°C. Ultra-thin sections of the shoot meristem region of the explants (60-100 nm) were obtained using a Reichert-Jung Ultracut E Microtome and collected on copper grids. Post-staining was done sequentially with saturated (2.5%) uranyl acetate and lead citrate (Reynolds, 1963) for 10 min each. Viewing of sections was done on a Jeol 1010 (Japan) transmission electron microscope.

2.5 Imaging

Digital images of experimental apparatuses, whole seeds, fruit and explants were captured using a Nikon Coolpix® digital camera.

2.6 Data interpretation and analysis

A One Way Analysis of Variance (ANOVA) was used to assess (i) differences in WC across species (Chapter 3, Section 3.1.1, 3.2.1 & 3.3.1) and (ii) differences between biomarkers across procedural steps within species (Chapter 3, Section 3.7.1-3.7.4). Means were contrasted using a Tukey post-hoc test. Alphabetical values were assigned to mean values recorded for each treatment. Mean values assigned with different alphabets were recognised as being significantly different. A Mann-Whitney U test was performed in instances where data did not satisfy parametric assumptions.

For each step of cryopreservation (Chapter 3, Section 3.1-3.5), total levels of $^{\cdot}O_2^-$, H_2O_2, TAA, respiratory activity were compared against the respective control within each species to assess how these parameters changed relative to basal levels. Differences between control and treatment mean values for each species at each step of cryopreservation were tested for using an Independent Samples T-Test, where data was normally distributed or a Mann-Whitney-U test in instances where the assumptions of normality or equal variance were not met. Differences between soaking treatments in explants of *Q. robur* and *T. dregeana* between treatments, within steps of cryopreservation (Chapter 3, Section 3.1.3) were analysed with an Independent Samples T-test. Differences between WC in explants of *L. sinensis* during each time interval using a flash drying or vacuum flash drying method (Chapter 3, Section 3.3.1) was analysed with an Independent Samples T-test. Alphabetical values were assigned to mean values recorded for each treatment within independent variables. Mean values assigned with different alphabets were recognised as being significantly different.

Correlations between post-cryo viability with explant physical characteristics during after excision and drying characteristics during desiccation (Chapter 3, Section 3.3.1) were assessed with a Spearman correlation. Correlations between various biomarkers (individual and pooled) and viability (Chapter 3, Section 3.7) were analysed using a Spearman correlation (correlations within individual species) and a Pearson correlation (correlations across species). All viability percentage data were arcsine transformed to conform data to parametric test assumptions. All statistical analyses were performed at the 0.05 level of significance using SPSS statistical package (Version 24; SPSS Inc. Chicago, Illinois, USA).

CHAPTER 3: Results

Aspects of this chapter are published in Sershen *et al*. (2016) and Naidoo *et al*. (2016).

The results of this study are presented in this chapter in sub-sections based on the procedural steps of cryopreservation investigated. Four species were investigated and where possible results for all four are compared; however, in cases where some aspects were studied for selected species only, this is indicated. For each procedural step, parameters regarding oxidative metabolism and respiratory activity are related to viability data. Qualitative observations of cellular ultrastructure following the various procedural steps and the treatments applied during them are also described.

3.1 Explant selection

3.1.1 Physiological and morphological characteristics of explants across selected recalcitrant-seeded species

The physiology and anatomy of explants selected for cryopreservation of recalcitrant-seeded species can vary greatly, and it has been reported that the variability in characteristics such as germinability and explant topography, morphology and tissue architecture can influence physiological responses of explants to cryopreservation (Pammenter *et al*., 2011). Explant characteristics also influence how cryogenic steps are manipulated. Anatomy can also be used to explain the variability in physiological responses of explants across recalcitrant-seeded species to cryopreservation procedures. The following results constitute a comparison of selected observable and measurable explant morphological characteristics across the four recalcitrant-seeded species investigated here, viz., *Trichilia dregeana*, *Quercus robur*, *Strychnos gerrardii* and *Lychee sinensis*.

Table 3.1 shows the physiological and morphological characteristics of zygotic explants excised to the smallest size possible while keeping meristematic regions intact. Explants from all species except *T. dregeana* produced seedlings on the optimised *in vitro* growth medium (see Table 3.2 for a description of these results). This was the only species in which excision damage to the explants was flush with the shoot meristem (Fig. 3.1a). In *T. dregeana* the primary explant excision (P) involved separating the zygotic embryo from the non-endospermic seed. This primary explant was excised with a blade such that it remained attached to remnants of the cotyledons. Thereafter the remnant pieces of cotyledon were

trimmed down as close as possible to the embryo (indicated by dashed red lines - Fig. 3.1a; F) with hypodermic needles. This method of excision was less injurious but does not completely inhibit physical injury to the shoot meristem which was in close proximity to the points of excision. This may explain why explants of *T. dregeana* showed 100% root production but only 40% shoot production. The final explant (Fig. 3.1a; F) for *T. dregeana* consisted of the embryo and tiny segments of cotyledon attached. These explants exhibited a significantly higher shedding WC than those of the other species with a fresh mass significantly lower than explants of *L. sinensis* and *Q. robur* but significantly higher than explants of *S. gerrardii* (Table 3.1). The length of *T. dregeana* explants was comparable to those of *Q. robur*, both of these explants exhibiting lengths greater than those of *L. sinensis* and *S. gerrardii* (Table 3.1).

Seeds of *S. gerrardii* are endospermic and possess papery thin cotyledons attached to the embryo. Shedding WC was similar to that measured in *L. sinensis* explants (Table 3.1); WC in explants of both these species was significantly lower than that of *T. dregeana* explants (Table 3.1). *Strychnos gerrardii* embryos could be easily removed from the seed, forming the primary explant (Fig. 3.1b; P). To reduce the size of the explant, the bottom half of the cotyledons were cut off with a horizontal incision (Fig. 3.1b) to form the final explant (Fig. 3.1b; F). Direct incisions (dashed red lines, Fig. 3.1b) were made away from the shoot meristematic region in this species. Full seedling development was recorded in explants, confirming no physical damage being incurred during excision. This is likely due to *S. gerrardii* seeds being endospermic, therefore the nutrient and reserve material was not absorbed by the embryo, which is the case with non-endospermic seeds such as *T. dregeana* and *Q. robur*. The fresh mass of explants of *S. gerrardii* was recorded to be significantly lower than explants of the other three species (Table 3.1). This is in spite of the length of explants being only slightly smaller than that of *T. dregeana* and *Q. robur* (Table 3.1).

Seeds of *L. sinensis* are endospermic with tiny (1-3 mm) zygotic embryos that are completely embedded within endosperm tissue (Fig. 3.1c). The primary explant consisted of the embryo within the endospermic tissue (Fig. 3.1c; P). The surrounding endosperm tissue, which was firmly attached to the embryo was then partially cut away (incision points indicated by dashed red lines) to obtain an embryo that was surrounded by a thin layer of endosperm; this represented the final explant (Fig. 3.1c; F). Despite having the smallest length, *L. sinensis*

explants exhibited the significantly highest fresh mass (Table 3.1), possibly due to the endosperm tissue surrounding the embryo. As a consequence of their anatomy and the excision strategy used, the final *L. sinensis* explant (Fig. 3.1c; F) was subjected to little to no physical injury to the embryo, which may explain why 100% of the explants produced seedlings (Table 3.1).

The primary explants of *Q. robur* were embryos that were loosely embedded within the dicotyledonous, non-endospermic seed (Fig. 3.1d; P), superficially attached to the cotyledons at two points (indicated by dashed red lines in Fig. 3.1d). These embryos were easily lifted away from the cotyledons and formed the final explant (Fig. 3.1d; F). These explants exhibited the second highest fresh mass and lowest shedding WC, with a length comparable to that of *T. dregeana* explants (Table 3.1). Due to the ease with which the explants are detached from the cotyledons (no incisions needed), no physical injury was inflicted on the embryo with 100% of the explants producing seedlings (Table 3.1).

After the selection of explants from each species, trials were undertaken to implement the most suitable *in vitro* procedures to facilitate contaminant-free seedling production. Control explants from each species were used for these trials.

Table 3.1: Morphological characteristics of zygotic explants from the four species studied.

Species	Explant fresh mass(mg)	Explant sheddingWC (g H_2O g^{-1}DM)	Explant length (mm)	% Viability and type of growth displayed	Physical excision damage to root or shoot
T. dregeana	0.007±0.0005^{c}	2.07±0.02^{a}	4-5	40% shoot production, 100% root production	Yes, shoot
S. gerrardii	0.001±0.0001^{d}	1.24±0.15^{b}	3-4	100% seedling production	No
L. sinensis	0.010±0.0003^{a}	1.24±0.02^{b}	1-3	100% seedling production	No
Q. robur	0.009±0.0002^{b}	1.09±0.03^{c}	4-6	100% seedling production	No

Viability data (n=20) for excised, untreated explants were recorded after 3 weeks in culture and damage to meristematic regions, if incurred, are indicated. Fresh mass and shedding WC values represent mean±SD (n=10). $p<0.05$ when explant fresh mass and shedding WC were tested for significant differences across species (ANOVA).

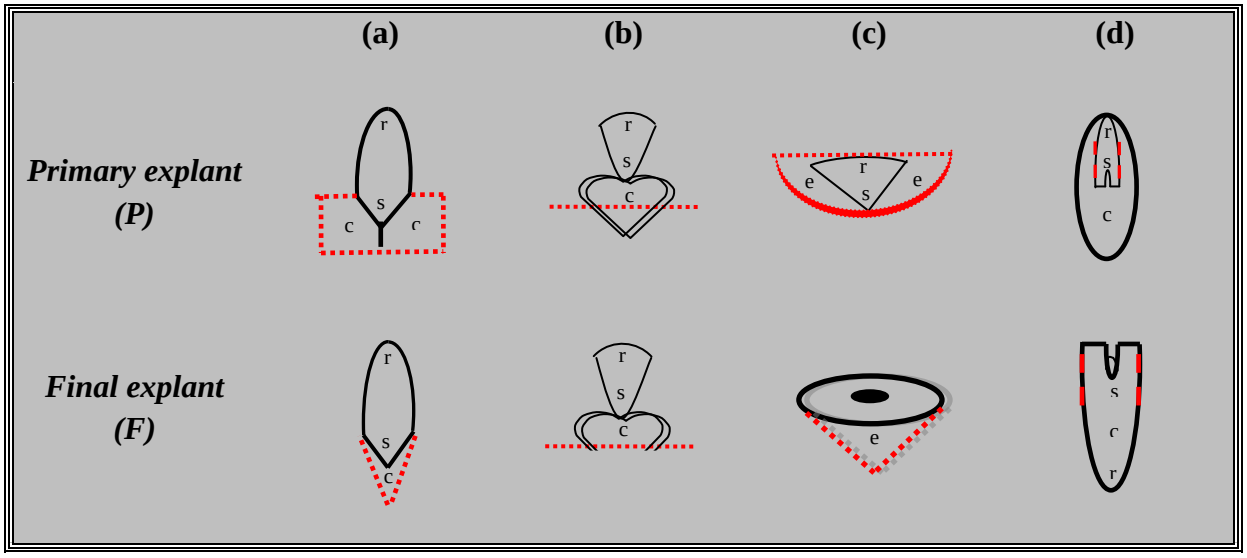

Fig. 3.1a-d: Anatomical illustrations of explants selected for *T. dregeana* (a), *S. gerrardii* (b), *L. sinensis* (c) and *Q. robur* (d). Explants across species undergo a primary (P) and final (F) excision to remove other parts of the surrounding seed tissues. The location of the root (r) and shoot (s) meristems and cotyledonary (c) and endosperm (e) tissue is given in each case. Explants of *L. sinensis* (c) had root and shoot meristems completely embedded in endosperm and those of *Q. robur* (d) had shoot meristems enclosed by cotyledon. Dashed red lines represent the position at which excisions were made when removing the explant.

3.1.2 Optimisation of *in vitro* procedures

3.1.2.1 Selection of a decontamination protocol for *in vitro* procedures

Decontaminants for *in vitro* culture of *T. dregeana* (Kioko, 2002, Goveia, 2007, Varghese *et al*., 2011), *S. gerrardii* (Berjak *et al*., 2011; Sershen *et al*., 2016) and *Q. robur* (Chmielarz, 1997; Berjak *et al*., 1999b) zygotic explants have been reported in previous studies. The present study compared these previously tested decontaminants against a 0.5% sodium dichloroisocyanurate (NaDCC) solution, for various periods of exposure. For zygotic explants of *L. sinensis*, decontaminants were assessed for the first time as no published data on decontamination of zygotic explants of this species were available at the time of this study. The decontamination protocol was eventually selected for each species based on its efficacy to combat bacterial and fungal contamination whilst still facilitating high viability retention. Viability was recorded as root production in all cases, since shoot development was compromised as a consequence of excision injury in *T. dregeana* (Table 3.1).

Untreated explants of *T. dregeana* showed no contamination *in vitro* when decontaminated with 1% sodium hypochlorite (NaOCl) or 0.5% NaDCC (Table 3.2). There was better root production observed when 0.5% NaDCC was used as a decontaminant as compared with 1% NaOCl; therefore 0.5% NaDCC applied for 10 min was selected as the decontamination protocol for this species.

Untreated explants of *Q. robur* decontaminated with 1% NaOCl for 10 min showed no contamination but only 80% root production (Table 3.2). However, decontamination of explants with 0.5% NaDCC for 10 min, facilitated 100% root production but 10% of these explants exhibited contamination. Extending the exposure period of 0.5% NaDCC by a further 10 min resulted in contamination-free explants and 100% root production. Hence, 0.5% NaDCC for 20 min was selected as the decontamination protocol for *Q. robur* explants.

Untreated explants of *S. gerrardii* decontaminated with 1% calcium hypochlorite [$Ca(OCl)_2$] showed no contamination *in vitro* but exhibited only 80% root production (Table 3.2). NaDCC (0.5%) applied for 10 and 15 min facilitated 100% root production with the latter exposure time being more effective in combating contamination. Therefore, 0.5% NaDCC for 15 min was selected as the decontamination protocol for *S. gerrardii* explants.

Untreated explants of *L. sinensis* were decontaminated with 0.5% NaDCC for 5 and 10 min (Table 3.2). The latter exposure time was effective in facilitating contamination-free explants showing 100% root production. Exposure of explants to 0.5% NaDCC for 10 min was selected as the decontamination protocol for *L. sinensis*.

Upon selection of 0.5% NaDCC as the common decontaminant across species, the most efficacious germination medium for *in vitro* seedling development could be developed.

Table 3.2: Effect of the concentration of, and exposure time to, different decontaminants on zygotic explant (n=20) contamination and viability in *T. dregeana*, *Q. robur*, *S. gerrardii* and *L. sinensis*.

Decontaminant (exposure period)	**Species**							
	T. dregeana		***Q. robur***		***S. gerrardii***		***L. sinensis***	
	% Cont.	**% Roots**	**% Cont.**	**% Roots**	**% Cont.**	**% Roots**	**% Cont.**	**% Roots**
0.5% NaDCC (5 min)	-	-	-	-	-	-	15	100
0.5% NaDCC (10 min)	**0**	**100**	10	100	5	100	**0**	**100**
0.5% NaDCC (15 min)	-	-	-	-	**0**	**100**	-	-
0.5% NaDCC (20 min)	-	-	**0**	**100**	-	-	-	-
1% NaOCl (10 min)	0	80	0	80	-	-	-	-
1% $Ca(OCl)_2$ (10 min)	-	-	-	-	0	80	-	-

Percentage contamination (Cont.) reflects the number of explants exhibiting fungal and/or bacterial contamination. Percentage contamination and root production were recorded after 3 weeks of *in vitro* culture. The decontaminant and period of exposure associated with values indicated in bold were regarded as optimum. Dashes indicate decontaminants that were not tested on explants.

3.1.2.2 Selection of media for *in vitro* germination

Media selected for *in vitro* germination was based on their efficacy to facilitate seedling development, i.e., root and shoot production. Previous studies have optimised the appropriate medium to achieve this outcome for some of the species investigated here and in these cases the suitability of the growth medium was simply confirmed. Specific concentrations of additives to germination medium for each species are detailed in Chapter 2. Previous studies on *T. dregeana* (Goveia, 2007; Naidoo *et al*., 2011), *S. gerrardii* (Goveia, 2007; Berjak *et al*., 2011) and *Q. robur* (Chmielarz, 1997) have not indicated a problem with root production in

zygotic explants of these species. Therefore, only growth regulators and antioxidant additives known to promote shoot development were investigated here.

Untreated explants of *T. dregeana* showed better shoot production on full strength MS medium supplemented with benzylaminopurine (BAP) than on full strength MS medium alone (Table 3.3). Therefore, the BAP supplemented medium was selected for *in vitro* culture of *T. dregeana* explants.

Untreated explants of *Q. robur* showed better shoot production on full strength MS medium supplemented with cytokinins and ascorbic acid (AsA) than on full strength medium alone (Table 3.3). MS medium supplemented with BAP and AsA was the *in vitro* culture medium selected for explants of this species.

Untreated explants of *S. gerrardii* showed better shoot production on full strength MS medium supplemented with activated charcoal (AC) compared with ¼ MS supplemented with the same concentration of AC, or full strength MS medium alone (Table 3.3). Full strength MS medium supplemented with AC was therefore selected as the *in vitro* culture medium for *S. gerrardii* explants.

Untreated explants of *L. sinensis* showed better shoot production on full strength MS medium supplemented with 2 iP and trans-zeatin than on full strength MS medium supplemented with BAP, or full strength MS medium alone (Table 3.3). Benzylaminopurine, a synthetic phytohormone, promoted shoot development in explants of *T. dregeana* and *Q. robur*, while explants of *L. sinensis* produced 100% seedlings only when cultured on medium supplemented with natural phytohormones. Full strength MS medium supplemented with 2 iP and trans-zeatin was therefore the *in vitro* germination medium selected for explants of *L. sinensis*.

After the selection of explants and optimisation of *in vitro* culture protocols, experiments designed to select the post-excision soaking (henceforth referred to as soaking) treatment that was most effective at reducing excision-induced oxidative stress were initiated.

Table 3.3: Percentage root and shoot production in zygotic explants of *T. dregeana*, *Q. robur*, *S. gerrardii* and *L. sinensis* on media supplemented with growth regulators, and/or antioxidant additives and/or activated charcoal.

Media composition	Species							
	T. dregeana		***Q. robur***		***S. gerrardii***		***L. sinensis***	
	%Root	**%Shoot**	**%Root**	**%Shoot**	**%Root**	**%Shoot**	**%Root**	**%Shoot**
Full MS	100	0	100	60	100	50	100	75
Full MS + 1 mg l^{-1} BAP	**100**	**40**	-	-	-	-	100	80
Full MS + 1 mg l^{-1} BAP+ 2 mg l^{-1} AsA	-	-	**100**	**100**	-	-	-	-
Full MS + 0.4 g l^{-1}AC	-	-	-	-	**100**	**100**	-	-
Full MS + 1 g l^{-1} 2iP + 2 g l^{-1} trans-zeatin	-	-	-	-	-	-	**100**	**100**
¼ MS + 0.4 g l^{-1} AC	-	-	-	-	100	80	-	-

Percentage root and shoot production were recorded after 3 weeks *in vitro* culture. The decontaminant and period of exposure associated with values indicated in bold were regarded as optimum. Dashes indicated media not tested for explants of specified species.

3.1.3 Effect of soaking in solutions with and without antioxidants on ROS production in zygotic explants of *T. dregeana* and *Q. robur* during the procedural steps of cryopreservation

Berjak *et al*. (2011) showed that the cathodic fraction of an electrolysed solution of calcium magnesium (CaMg), known as cathodic water and henceforth, abbreviated to CW, decreases $^{\cdot}O_2^-$ production in recalcitrant-seeded zygotic explants during cryopreservation. For the purpose of selecting an antioxidant soaking solution to ameliorate oxidative stress in the explants during the various potentially injurious cryopreservation steps, this section compared the effects of CW and a solution of CaMg on $^{\cdot}O_2^-$ and H_2O_2 production. Calcium magnesium is a common rehydration (R) and rewarming (RW) medium in recalcitrant seed cryopreservation protocols (Mycock, 1999; Hajari *et al*., 2011; Kistnasamy *et al*., 2011; Varghese *et al*., 2011; Sershen *et al*., 2012a; c). These studies were conducted on the explants of *Q. robur* and focused on the effects of these antioxidant (soaking) solutions during the various procedural steps involved in cryopreservation: excision, cryoprotection, partial

drying, cooling and rewarming (the specific methods for which are described in detail in Chapter 2). In each case, the production of $^{\cdot}O_2^-$ and H_2O_2 is related to explant viability.

The use of an antioxidant soak (CW) during various steps of cryopreservation significantly reduced both ROS measured in explants of *Q. robur* compared with the antioxidant-free solution (CaMg) (Figs 3.2a & b). The influence on ROS production in explants effected by CW treatment was consistent across all steps of cryopreservation. These trials confirmed the action of CW as a treatment which reduces ROS in explants of a representative recalcitrant-seeded species, *Q. robur*.

Based on studies that have reported the efficacy of AsA in improving germination and reducing ROS during cryopreservation of recalcitrant-seeded germplasm (Uchendu *et al*., 2010a; b; Reed *et al*., 2012), trials to comparatively assess the effects of CW alone and AsA prepared in CW on ROS production was undertaken. The concentration of AsA was selected as 1% (w/v) based on results reported for *T. dregeana* in Naidoo (2012). Explants of *T. dregeana* were used to establish the most effective exogenous antioxidant soaking solution to apply during the various cryopreservation steps.

Treatment of *T. dregeana* explants with 1%AsA+CW significantly reduced ($p<0.05$) both ROS measured compared with treatment of explants with CW alone (Figs 3.3a & b). The effect of exogenously applied AsA+CW on ROS levels in explants was consistent across all treatments. This formed the premise for selecting a 1% AsA+CW solution as the antioxidant soaking solution to be used during the various steps of cryopreservation for explants of all species investigated in the present study.

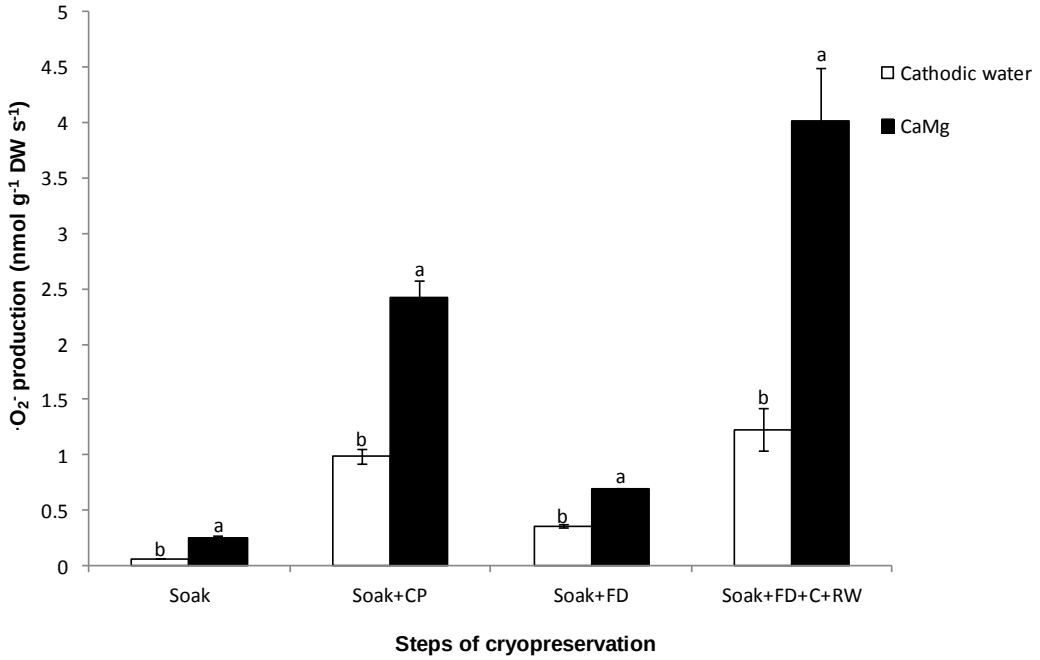

Fig. 3.2a: Extracellular $\cdot O_2^-$ production in zygotic explants of *Q. robur* exposed to CaMg or CW during soaking, during DMSO+glycerol cryoprotection (CP), prior to flash drying (FD) and after cooling (C) during subsequent rewarming (RW). Bars represent mean±SD (n=6) and when labelled with different letters are significantly different when compared between treatments within a procedural step ($p<0.05$, Independent sample t-test).

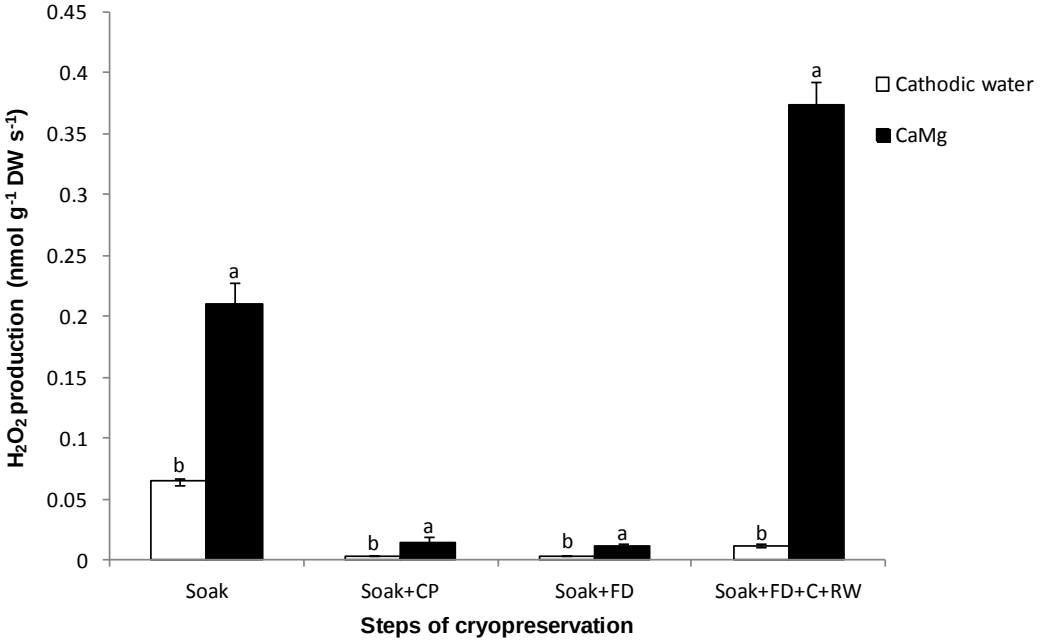

Fig. 3.2b: Extracellular H_2O_2 production in zygotic explants of *Q. robur* exposed to CaMg or CW during soaking, during DMSO+glycerol cryoprotection (CP), prior to flash drying (FD) and after cooling (C) during subsequent rewarming (RW). Bars represent mean±SD (n=6) and when labelled with different letters are significantly different when compared between treatments within a procedural step ($p<0.05$, Independent sample t-test).

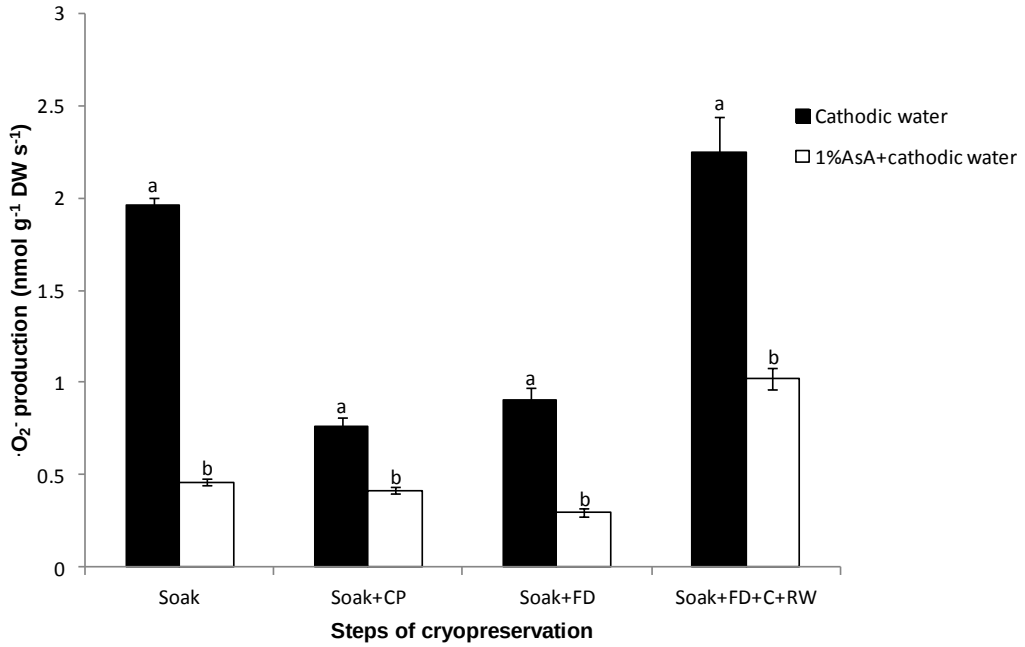

Fig. 3.3a: Extracellular $^{\cdot}O_2^-$ production in zygotic explants of *T. dregeana* exposed to CW or 1% AsA+CW during soaking, during DMSO+glycerol cryoprotection (CP), prior to flash drying (FD) and after cooling (C) during subsequent rewarming (RW). Bars represent mean±SD (n=6) and when labelled with different letters are significantly different when compared between treatments within a procedural step ($p<0.05$, Independent sample t-test).

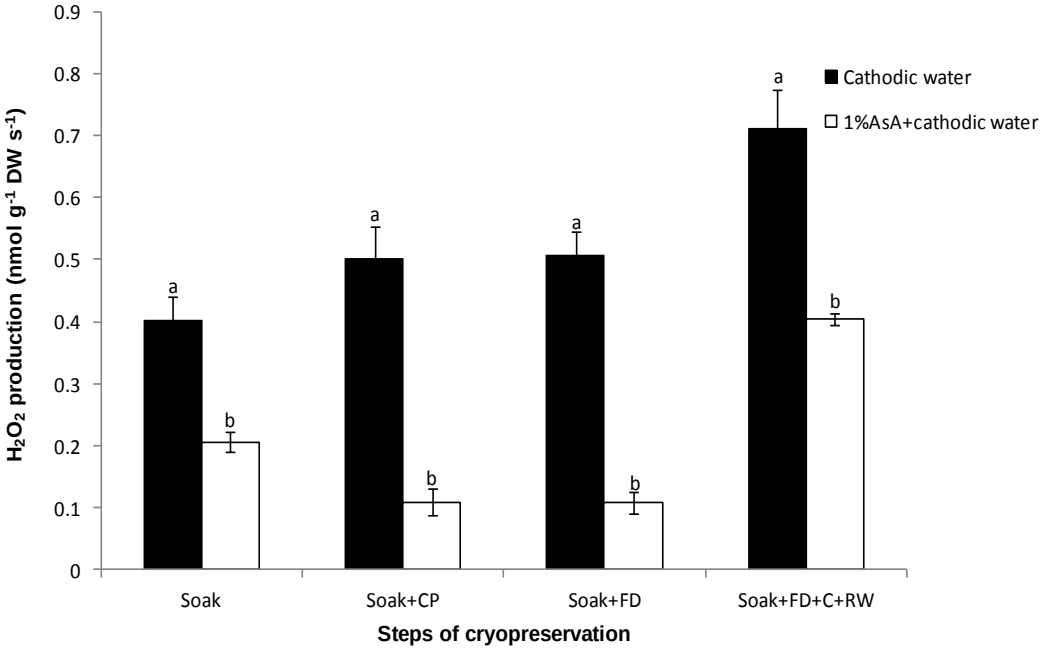

Fig. 3.3b: Extracellular H_2O_2 production in zygotic explants of *T. dregeana* exposed to CW or 1% AsA+CW during soaking, during DMSO+glycerol cryoprotection (CP), prior to flash drying (FD) and after cooling (C) during subsequent rewarming (RW). Bars represent mean±SD (n=6) and when labelled with different letters are significantly different when compared between treatments within a procedural step ($p<0.05$, Independent sample t-test).

3.1.4 Selection of a soaking period for the application of 1% AsA prepared in CW to zygotic explants

A 30 min soak in 1% AsA+CW was previously shown to be efficacious in promoting seedling production and reducing ROS production in excised zygotic *T. dregeana* explants (Naidoo, 2012) after various procedural steps of cryopreservation. Based on results from that study and trials conducted presently (section 3.1.3), the effects of various exposure times to 1% AsA+CW were tested for excised zygotic explants of all four species investigated in this study (Table 3.4). Exposure times in soaking treatments were eventually selected based on *in vitro* seedling production and vigour (measured in terms of 50% root emergence by explants).

When explants were exposed to 1% AsA+CW for various periods (those of *Q. robur*, *S gerrardii* and *L. sinensis* showed 100% seedling production after treatment with 1% AsA for 5 min (Table 3.4). This was expected since explants from these species showed full viability retention after excision without any application of any exogenous antioxidants (Table 3.4). Nevertheless, soaking was applied to all species in an attempt to reduce excision-induced ROS levels. Explants of *T. dregeana* produced only 40% seedlings, after a 5 min exposure. Explants from all species were exposed to longer durations in the antioxidant solution. Especially in *T. dregeana*, this was undertaken to establish if a longer period of exposure would facilitate higher % shoot development; this was observed to be the case after a 30 min exposure period (Table 3.4). After 30 min of soaking, explants of *T. dregeana* produced 80% shoot production which did not increase after a further 30 min exposure. For explants of the remaining species, the effect of an extended time period was assessed to determine if the antioxidant solution may have harmful effects with prolonged exposure. None of the other species showed signs of growth retardation after extended periods (15 & 30 min) of exposure.

Based on high seedling production (80%) and improved vigour (50% root emergence after 10 days), 30 min exposure to 1% AsA+CW was selected as the exposure period for soaking explants of *T. dregeana* (Table 3.4). As full seedling production was observed in explants of the remaining species for all exposure times tested, optimisation of a soaking period was based on vigour. Explants of both *S. gerrardii* and *Q. robur* showed 50% root emergence after 7 days without exposure to a soak and when exposed to 1% AsA+CW for 5 and 15 min. However, 50% root emergence occurred in these species after 4 and 5 days respectively, after a 30 min exposure. This was therefore selected as the exposure time for explants of both

these species. Explants of *L. sinensis* were the only to show an improvement in vigour (50% root emergence after 2 days) relative to unsoaked (control) explants, which exhibited 50% root emergence after 4 days (Table 3.4). Root emergence (50%) was observed after 2 days in explants of *L. sinensis* after a 5 min exposure period to soaking, and vigour did not improve with longer exposure periods. Therefore a 5 min period of exposure to 1% AsA+CW was selected for explants of *L. sinensis* after excision. Seedling production and vigour results obtained for 30 min (*T. dregeana*, *Q. robur* and *S. gerrardii*) and 5 min *(L. sinensis*) exposure to the soaking treatment were acceptable across all species. An exposure period longer than 30 min was tested only in *T. dregeana* as development of shoots after excision is a documented problem in this species (Goveia, 2007; Naidoo *et al*., 2011; Naidoo, 2012).

The duration for the application of 1% AsA+CW after drying (as a rehydration medium) and after cooling (as a rewarming solution) prior to *in vitro* germination, was based on the duration for these procedures used in other cryopreservation studies on zygotic explants from recalcitrant seeds (Berjak *et al*., 1999b; Kioko, 2002; Goveia, 2007; Berjak *et al*., 2011).

Table 3.4: Effect of exposure time in a soaking solution (1% AsA+CW) on seedling production and vigour (determined by time taken for 50% root emergence) in *T. dregeana*, *Q. robur*, *S. gerrardii* and *L. sinensis* zygotic explants (n=20).

	Species			
Period of exposure (min)	*T. dregeana*	*Q. robur*	*S. gerrardii*	*L. sinensis*
	%seedling production			
0	40	100	100	100
5	40	100	100	100
15	40	100	100	100
30	80	100	100	100
60	80	-	-	-
	Time taken for 50% root emergence (days)			
0	14	7	7	4
5	14	7	7	2
15	14	7	7	2
30	10	5	4	2
60	10	-	-	-

Dashes indicate exposure times for which 1% AsA+CW were not applied to explants.

The subsequent sections in this chapter features results on viability and selected stress biomarkers assessed in explants of all four species during procedural steps of cryopreservation.

3.1.5 Extracellular ROS production and viability in zygotic explants with and without soaking

Explants were measured for extracellular $\cdot O_2^-$ and H_2O_2 production, total aqueous antioxidant capacity (TAA), extracellular respiratory activity and viability in control explants and in those exposed to exogenous antioxidants. Results on ultrastructural responses to the various procedural steps are presented for explants of all species but only for selected procedural steps.

Treatment with antioxidants involved immersion in 1% AsA+CW (labelled 'soaked', henceforth) for 5 min in *L. sinensis* and 30 min for the remaining species, based on results presented in Table 3.4, which showed that soaking for longer periods, did not improve explant viability or vigour.

Superoxide production was significantly lower in soaked explants compared with the control (unsoaked) in all species (Fig. 3.4). More specifically, $\cdot O_2^-$ levels declined by more than 90% relative to the control in *T. dregeana* and *Q. robur*, and by more than 80% in *L. sinensis* and more than 60% in *S. gerrardii* (Table 3.5). Except for *T. dregeana*, where viability increased by 40% following soaking, 100% of explants were viable across all species, with or without soaking (Figs 3.4-3.7).

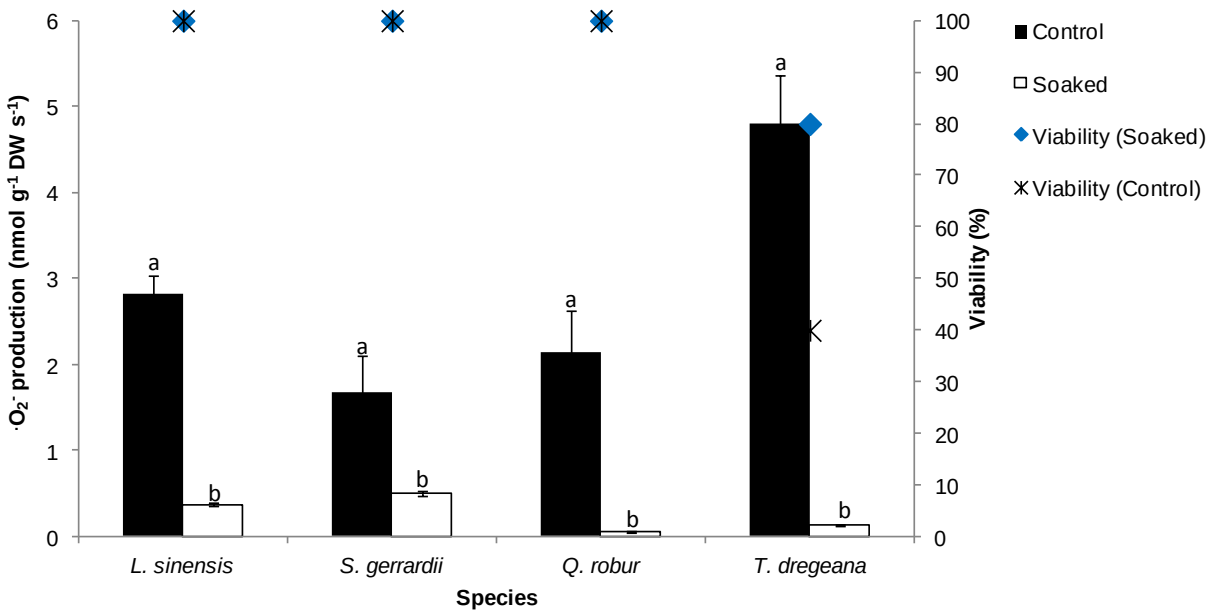

Fig. 3.4: Extracellular $\cdot O_2^-$ production and viability for excised explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to exogenous antioxidants. Values represent mean±SD (n=6 for $\cdot O_2^-$ and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.5: $\cdot O_2^-$ production in treated (soaked) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative $\cdot O_2^-$ production (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	-86	-69	-97	-97

Hydrogen peroxide production in soaked explants was significantly higher in comparison with those of the control in *L. sinensis* and *S. gerrardii* (Fig. 3.5), increasing by 550 and 500% relative to their respective controls (Table 3.6). Soaked explants of *Q. robur* and *T. dregeana* showed a less marked but significant reduction in H_2O_2 production (Fig. 3.5) of 61% and 17% respectively, relative to their respective controls (Table 3.6)

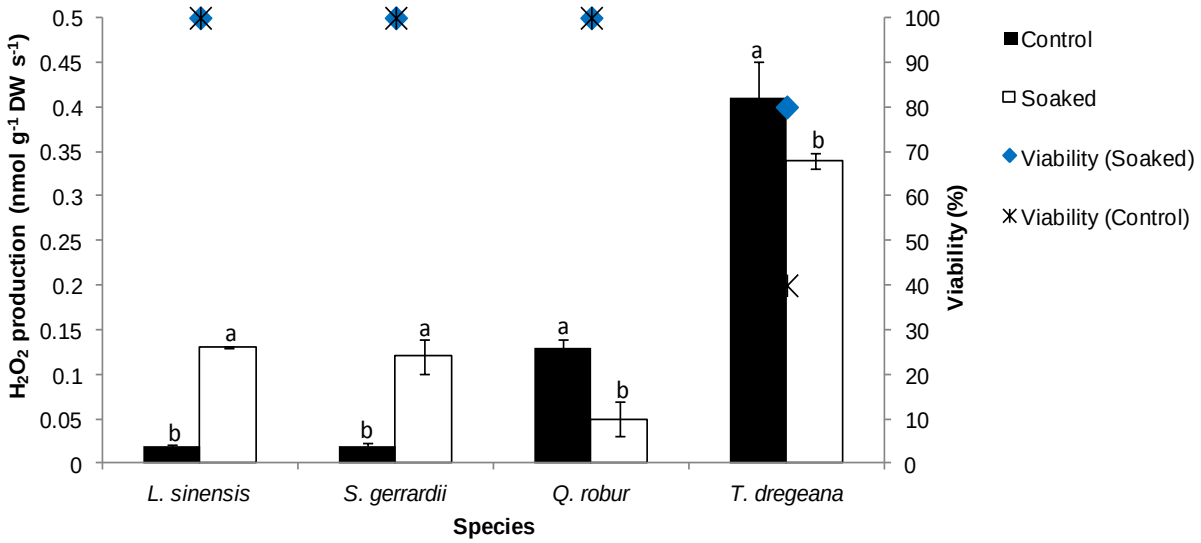

Fig. 3.5: Extracellular H_2O_2 production and viability for excised explants of *L. sinensis, S. gerrardii, Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to exogenous antioxidants. Values represent mean±SD (n=6 for H_2O_2 and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.6: H_2O_2 production in treated (soaked) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative H_2O_2 production (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	550	500	-61	-17

3.1.6 Total aqueous antioxidant capacity and viability in zygotic explants with and without soaking

Total aqueous antioxidant capacity was significantly lower in explants of all species after soaking compared with the control (Fig. 3.6). TAA decreased by 8, 14, 78 and 81% relative to the control in explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana*, respectively (Table 3.7). Untreated explants of *L. sinensis*, *S. gerrardii* and *Q. robur* exhibited antioxidant levels almost three times that present in untreated explants of *T. dregeana.*

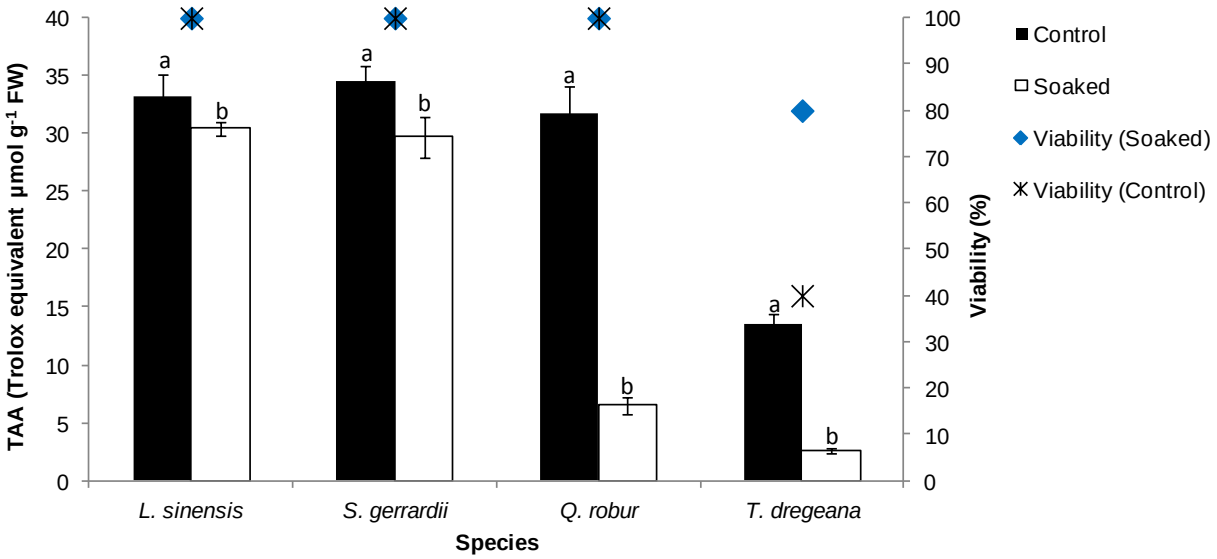

Fig. 3.6: Total aqueous antioxidant capacity and viability for excised explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to exogenous antioxidants. Values represent mean±SD (n=8 for TAA and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.7: TAA capacity in treated (soaked) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative TAA capacity (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	-8	-14	-78	-81

3.1.7 Extracellular respiratory activity and viability in zygotic explants with and without soaking

Respiratory activity was significantly lower in soaked *Q. robur* (35%) and *S. gerrardii* (61%) explants relative to the control (Fig. 3.7; Table. 3.8). In contrast, respiratory activity in soaked *T. dregeana* explants was significantly higher than the control. Only explants of *T. dregeana* exhibited an increase in respiration relative to the control (by 25%; Table 3.8) after soaking. A slight reduction in respiration was observed in explants of *L. sinensis* relative to the control.

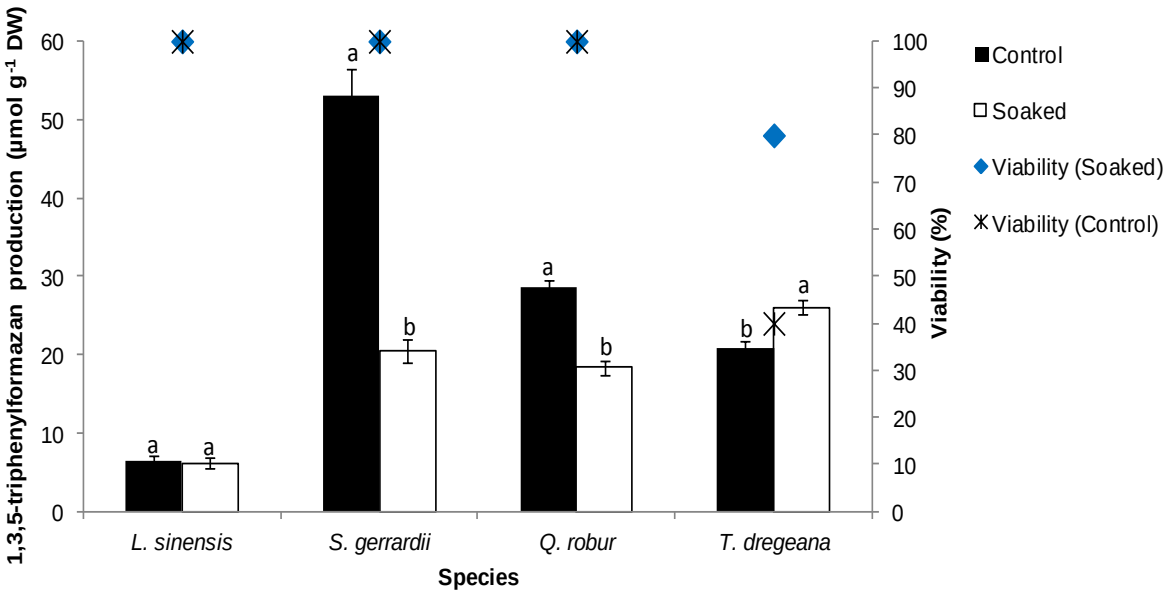

Fig. 3.7: Extracellular respiratory activity and viability for excised explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to exogenous antioxidants. Values represent mean±SD (n=8 for TTZ and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.8: Respiratory activity in treated (soaked) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative respiratory activity (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	-6	-61	-35	25

3.1.8 Effects of exogenous application of antioxidants on shoot meristem ultrastructure of zygotic explants

Micrographs shown in Figs 3.8-3.11 illustrate the shoot meristematic region (tunica layer) in untreated explants (Figs 3.8a & b, 3.9a & b, 3.10a and 3.11a & b) and in explants treated with soaking in 1% AsA+CW (Figs 3.8c &d, 3.9c & d, 3.10b &c and 3.11c & d) from *T. dregeana* (Fig. 3.8), *Q. robur* (Fig. 3.9), *S. gerrardii* (Fig. 3.10) and *L. sinensis* (Fig. 3.11). Shoot meristems were selected as the explants for ultrastructural assessment based on the sensitivity of this region of recalcitrant zygotic embryos to cryopreservation procedures (Berjak & Pammenter, 2013a; Wesley-Smith *et al*., 2015).

Before describing the ultrastructure of these shoot meristems it is worth noting the following: (i) Explants showed increased hydration upon exposure to a soaking treatment (by 0.11 g g^{-1}, 0.04 g g^{-1}, 0.08 g g^{-1} , 0.12 g g^{-1} in *T. dregeana*, *Q. robur*, *S. gerrardii* and *L. sinensis* respectively; (ii) soaked explants of *T. dregeana* (WC: 2.18±0.30 g g^{-1}; Fig. 3.8c & d) showed increased seedling production (80%) compared with untreated explants (40%), while % seedling production in *Q. robur* (WC: 1.13±0.02 g g^{-1}; Fig. 3.9c & d) *S. gerrardii* (WC: 1.32±0.30 g g^{1}; Fig. 3.10b & c) and *L. sinensis* (WC: 1.36±0.02 g g^{-1}; Fig. 3.11c & d) was unaffected by soaking (see Section 3.3.1 for full description of WC and viability profiles associated with these treatments).

Ultrastructure of the shoot meristems of both untreated and soaked explants showed a high level of subcellular organisation. In untreated (control) explants, mitochondria (M) in shoot meristematic cells of all species were frequent with well developed cristae (Figs 3.8b, 3.9a, 3.10a, 3.11a & b).

Mitochondria exhibited particularly dense matrices in untreated (Fig. 3.8b) and soaked *T. dregeana* explants (Fig. 3.8d). Many well developed mitochondria within the granulated cytoplasm were observed in soaked explants of *T. dregeana* (Fig. 3.8c), consistent with the highest respiratory activity compared with the control in this species (Table 3.8). The nucleus (N) was observed to be spherical and dense with heterochromatin in shoot meristems of untreated explants of *T. dregeana* (Fig. 3.8a) and the integrity of the nucleus was maintained after soaking (Fig. 3.8d). Shoot meristems of soaked *T. dregeana* explants showed plastoglobuli (pg; Fig. 3.8d) and numerous large vacuoles with distinct boundaries

(Fig. 3.8c). Vacuolar inclusions were also noted here which may be suggestive of active metabolism and protein and organelle turnover (Marty, 1999). Plasmamembrane associated vesicles were also noted in soaked explants (indicated by arrows, Fig. 3.8d).

In untreated (Fig. 3.9a) and soaked explants (Fig. 3.9d) of *Q. robur* many mitochondria (M) with visible cristae were observed in the cytomatrix, indicative of active respiration. In shoot meristems of *Q. robur*, short profiles of endoplasmic reticulum (er) were observed in the cytoplasm after soaking (Fig. 3.9d) suggestive of ongoing protein synthesis. Vesicles (indicated by arrows) indicate ongoing membrane synthesis in soaked *Q. robur* explants (Fig. 3.9c & d).

Numereous lipid deposits (L) were visible in the cytomatrix in shoot meristematic cells of untreated *S. gerrardii* explants (Fig. 3.10a), implying reserve uptake during active metabolism. This was shown to be consistent in shoot meristems of soaked explants of this species (Fig. 3.10b) where plastoglobuli (pg) were also present (Fig. 3.10b). A few, large vacuoles (V) were observed within the cytomatrix in untreated (Fig. 3.10a) and soaked explants (Fig. 3.10b & c), in addition to spherical nuclei (N) in soaked explants (Fig. 3.10c). Black arrows in Fig. 3.10a indicate plasmamembrane associated vesicles in meristems of untreated explants and suggestive of exocytosis or endocytosis (Battey *et al.*, 1999). As tunica layers of the shoot meristem are not associated with vascular tissue (Esau, 1960), the incidence of phagocytotic activity may be considered as normal cellular events.

In untreated (Fig. 3.11b) and soaked explants of *L. sinensis* (Fig. 3.11d) many mitochondria (M) with visible cristae were observed in the cytomatrix, indicative of active respiration. Starch bodies (S) were observed in untreated explants (Fig. 3.11a). Large vacuoles (V) were noted in both untreated (Fig. 3.11b) and soaked explants (Fig. 3.11c). A spherical nucleus (N) with dense heterochromatin (indicated by arrows) was present in soaked explants (Fig. 3.11c).

Shoot meristematic cells in all species exhibited an ultrastructure typical of actively respiring, highly metabolic cells, consistent with full viability retention. As no signs of ultrastructural stress/loss of integrity were observed in untreated *T. dregeana* axes, it is unlikely that the cause of inhibited shoot production in this species was linked to ultrastructural abnormalities.

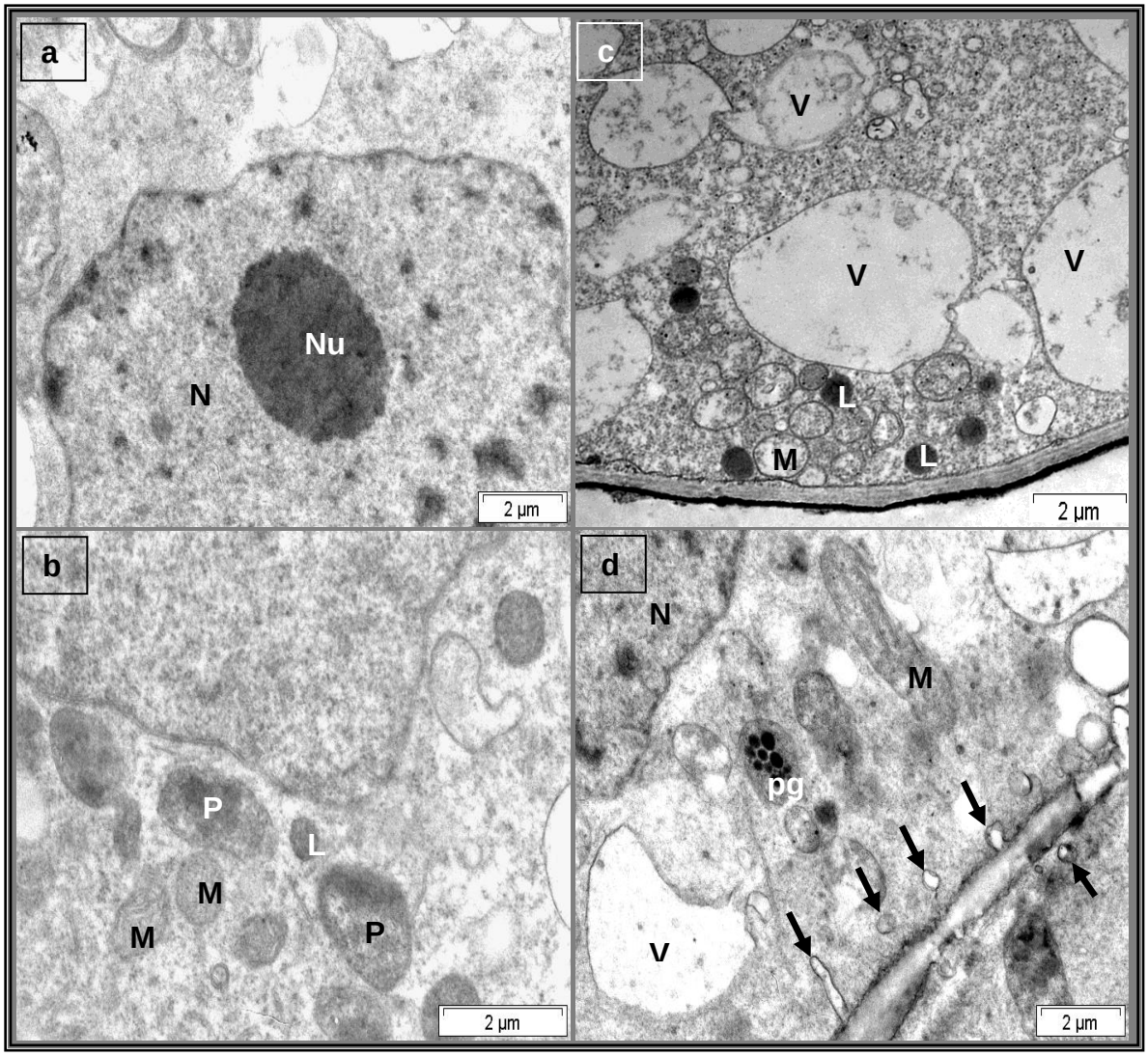

Fig. 3.8a-d: The ultrastructure of shoot meristems excised from untreated (Control; a & b) and soaked (1% AsA+CW solution for 30 min; c & d) zygotic *T. dregeana* explants. The shoot meristematic region in both untreated (a & b) and soaked (c & d) explants showed intact cellular ultrastructure with well developed organelles. Figure 3.8a shows a well developed nucleus and nucleolus. Figure 3.8b shows well defined lipid (L), mitochondria (M) and plastids (P). Numerous mitochondria (M) with well developed cristae were observed throughout the cytomatrix in shoot meristems of soaked explants (Fig. 3.8c & d). Numerous dense lipid bodies (L) accumulated in the cytomatrix (Fig. 3.8c). Dense plastoglobuli (pg) were found within plastids (Fig. 3.8d). Vacuoles (V) were large and frequently occurring (Fig. 3.8c & d). Numerous vesicles were associated with the plasma membrane (indicated by arrows Fig. 3.8d).

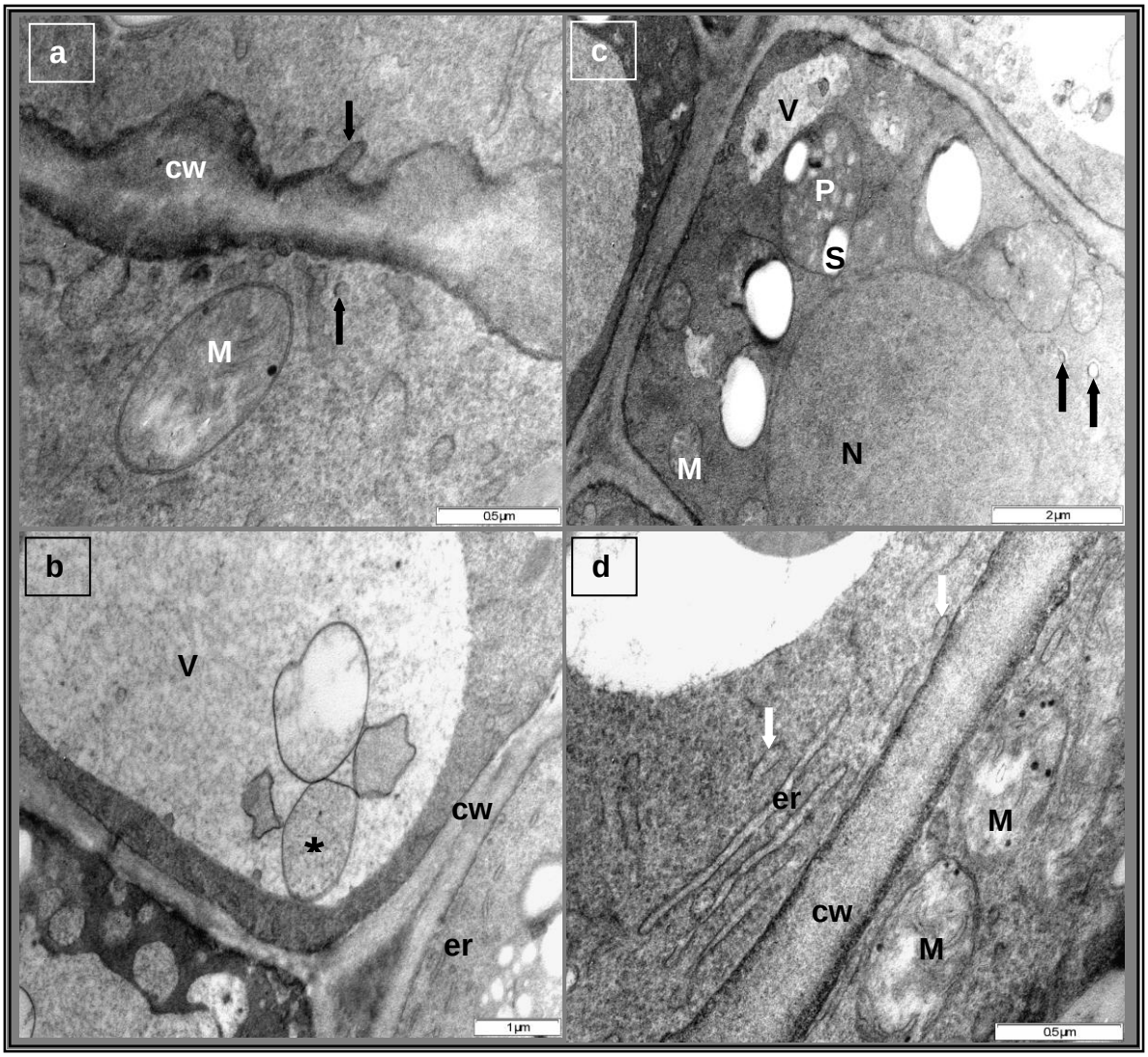

Fig. 3.9a-d: The ultrastructure of shoot meristems excised from untreated (Control; a & b) and soaked (1% AsA+CW solution for 30 min; c & d) zygotic *Q. robur* explants. The shoot meristematic region in both untreated (a & b) and soaked (c & d) explants showed intact cellular ultrastructure with well developed organelles. Figure 3.9a shows a well developed mitochondrion (M) with defined cristae as well as vesicle activity (indicated by arrows). Figure 3.9b shows a large vacuole (V) with vacuolar inclusions (indicated by an asterisk) and numerous short profiles of endoplasmic reticulum (er). Well developed mitochondria (M) with defined cristae were also present in the cytoplasm of shoot meristems in soaked explants (Fig. 3.9c & d). Shoot meristems of soaked explants (Fig. 3.9c & d) also showed numerous plasmamembrane associated vesicles (indicated by arrows) present in the cytomatrix with elongated endoplasmic reticulum (er) (Fig. 3.9d). Plastids (P) with starch granules (S) were present in the cytoplasm (Fig. 3.9c) and nuclei with evenly dispersed granular chromatin (N) were also observed (Fig. 3.9c) in shoot meristems of soaked explants.

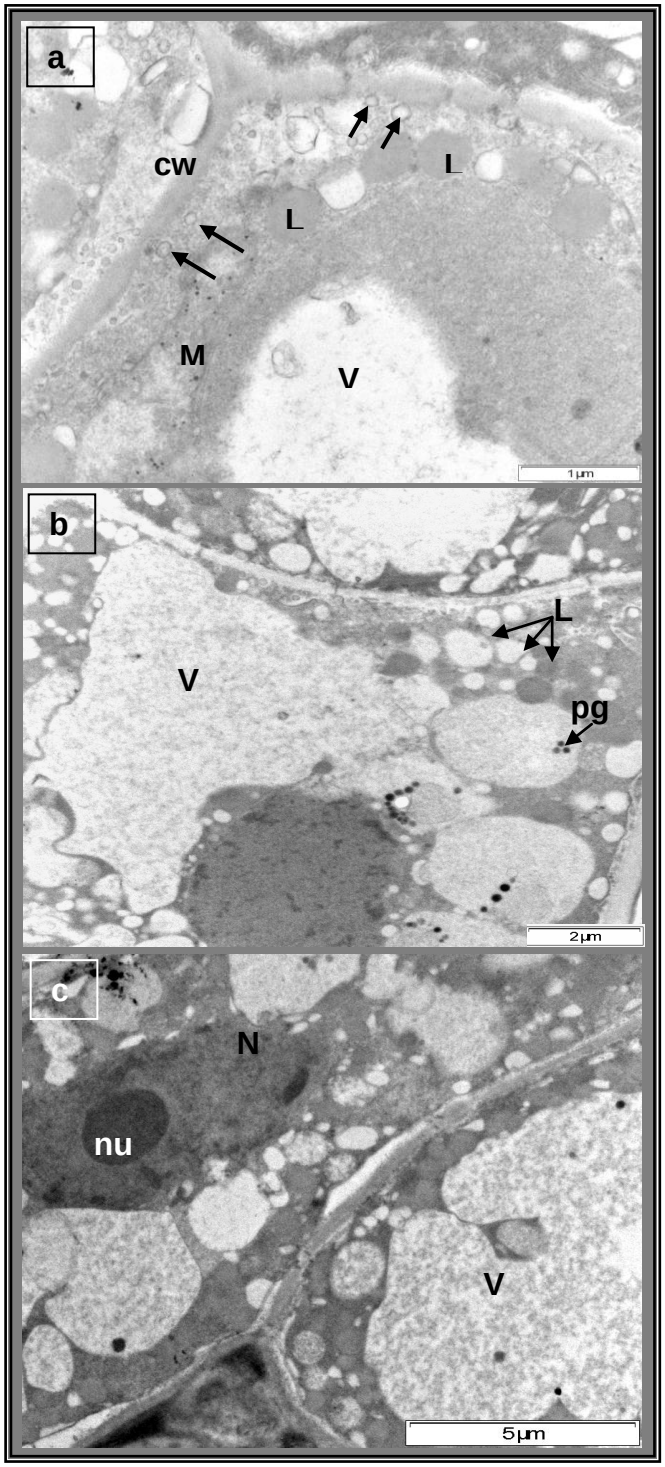

Fig. 3.10a-c: The ultrastructure of shoot meristems excised from untreated (Control; a) and soaked (1% AsA+CW solution for 30 min; b & c) zygotic *S. gerrardii* explants. The shoot meristematic region of untreated and soaked explants showed intact cellular ultrastructure with well developed organelles (Fig. 3.10a). The ultrastructure displayed frequently dispersed dense lipid bodies (L) in untreated (Fig. 3.10a) and soaked explants (Fig. 3.10b). Numerous plasmamembrane associated vesicles (indicated by arrows) were noted in untreated explants (Fig. 3.10a). The mitochondria (M) showed defined cristae (Fig. 3.10a). A few, large vacuoles (V) were evident in the cytoplasm of shoot meristems of soaked explants (Fig. 3.10b & c). Well developed nuclei (N) and nucleoli (Nu) were also noted (Fig. 3.10c).

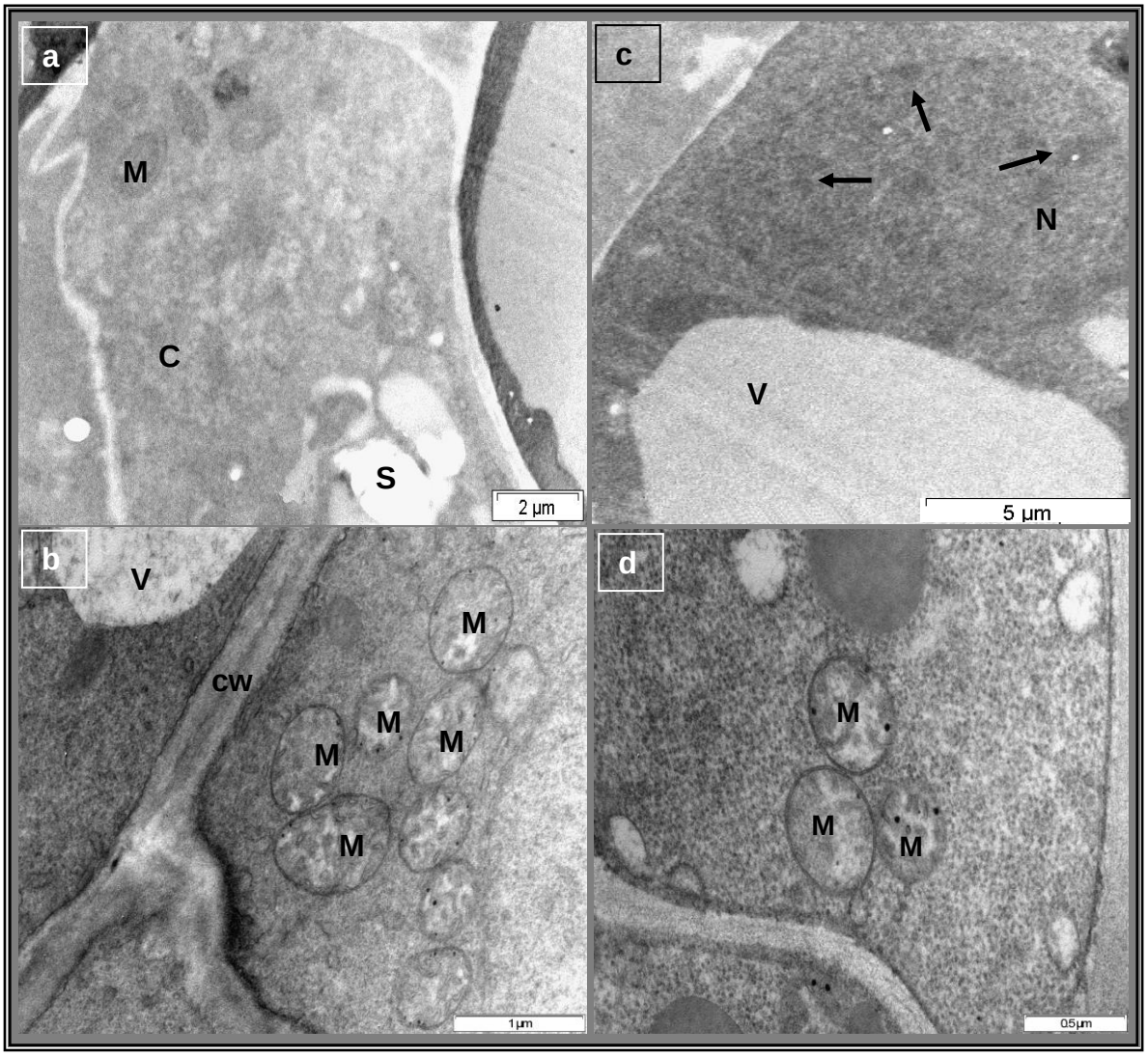

Fig. 3.11a-d: The ultrastructure of shoot meristems excised from untreated (Control; a & b) and soaked (1% AsA+CW solution for 5 min; c & d) zygotic *L. sinensis* explants. Frequently dispersed, developed mitochondria (M) were present in the cytomatrix of shoot meristems in untreated (Fig. 3.11a & b) and soaked explants (Fig. 3.11d). Starch grains (S) were evident in the cytoplasm of shoot meristems in untreated explants (Fig. 3.11a). In shoot meristems of soaked explants, single, large vacuoles (V) were noted in the cytoplasm (Fig. 3.11c) and the nucleus (N) was well developed with numerous patches of heterochromatin (indicated by arrows) (Fig. 3.11c).

3.2 Explant cryoprotection

3.2.1 Effects of soaking and the combination of soaking and cryoprotection on explant water content and viability

Explants across species were exposed to soaking and cryoprotection with 10% DMSO+glycerol (henceforth, referred to as soaking+CP). Cryoprotectants were prepared in CW as a measure of potentially regulating ROS production in explants during cryoprotection. Exposure to penetrating cryoprotectants variably affects the WC and viability of recalcitrant-seeded explants (Kioko, 2002; Varghese *et al*., 2009; Hajari *et al*., 2011; Sershen *et al*., 2012a; b). The effects of these cryo-preparative treatments (prior to partial dehydration) on explant WC and viability data are described below for all species investigated here.

Soaked explants exhibited a significant increase in WC only in *S. gerrardii* and *L. sinensis* relative to the control. Explant viability remained unchanged (*Q. robur*, *S. gerrardii* & *L. sinensis*) or improved (*T. dregeana*) after soaking relative to the control. After soaking+CP, explants across species exhibited a significant increase in WC relative to control and significant increases in WC were also observed relative to soaked explants of *T. dregeana* and *Q. robur* (Table 3.9). Explant viability after soaking+CP decreased in *Q. robur*, *S. gerrardii* and *L. sinensis* and remained unchanged in *T. dregeana* relative to the control, but explant viability across all species declined relative to soaked explants. Explants exposed to soaking and soaking+CP were subjected to drying (section 3.3.2). The implications of the physiological responses of these explants to cryoprotection on post drying viability are discussed in the section on explant partial dehydration (Section 3.3).

Table 3.9: Viability and water content of explants exposed to soaking or soaking and cryoprotection across species.

	T. dregeana		***Q. robur***		***S. gerrardii***		***L. sinensis***	
Treatment	**WC ($g\ g^{-1}$)**	**V (%)**	**WC ($g\ g^{-1}$)**	**V (%)**	**WC ($g\ g^{-1}$)**	**V (%)**	**WC ($g\ g^{-1}$)**	**V (%)**
Control	2.07 ± 0.02^{b}	40	1.09 ± 0.03^{b}	100	1.24 ± 0.15^{b}	100	1.24 ± 0.02^{b}	100
Soak	2.18 ± 0.30^{b}	80	1.13 ± 0.02^{b}	100	1.32 ± 0.30^{ab}	100	1.36 ± 0.02^{a}	100
Soak+CP	2.29 ± 0.08^{a}	40	1.19 ± 0.04^{a}	70	1.51 ± 0.06^{a}	70	1.38 ± 0.01^{a}	90

Viability (V) data (n=20) for control, soaked and soaked+CP explants are shown after 3 weeks in culture. Water content (WC) values represent the mean±SD (n=10). $p<0.05$ when explant WC levels were tested for significant differences across treatments within species (ANOVA).

3.2.2 Extracellular ROS production and viability in zygotic explants with and without the combination of soaking and cryoprotection

Explants were measured for extracellular $\cdot O_2^-$ and H_2O_2 production, total aqueous antioxidant capacity, respiratory activity and viability in control explants and in those soaked+cryoprotected. Treatment with antioxidants involved immersion in 1% AsA+CW for 5 min in *L. sinensis* and 30 min for the remaining species, and subsequent stepwise cryoprotection in 5% and 10% DMSO+glycerol prepared in CW over 120 min.

Superoxide production decreased significantly (by 59, 58 and 44% in *L. sinensis*, *Q. robur* and *T. dregeana*, respectively [Table 3.10]) in explants of three species treated with soaking+CP (Fig. 3.12), compared with their controls. There was a slight increase in $\cdot O_2^-$ production observed in *S. gerrardii* compared with the control. After soaking+CP, explant viability declined by 10, 30 and 30% in *L. sinensis*, *S. gerrardii*, and *Q. robur* respectively, relative to the control (Figs 3.12-3.15). Soaked+CP explants of *T. dregeana* showed no change in viability relative to the control (Figs 3.12-3.15).

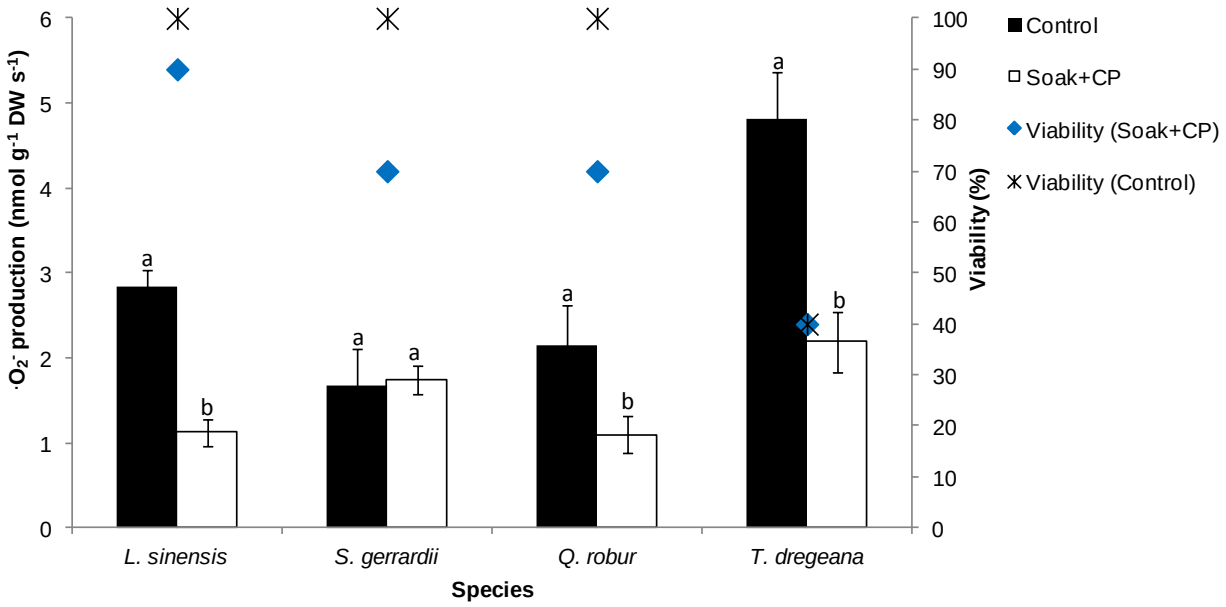

Fig. 3.12: Extracellular $\cdot O_2^-$ production and viability for excised explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to the combination of exogenous antioxidant soaking and cryoprotection with 5% & 10% DMSO+glycerol. Values represent mean±SD (n=6 for $\cdot O_2^-$ and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.10: $\cdot O_2^-$ production in treated (soaked+cryoprotected) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative $\cdot O_2^-$ production (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	-59	4	-58	-44

Hydrogen peroxide production significantly decreased by 30, 97 and 97% relative to the control in soaked+CP explants that were excised from *L. sinensis, Q. robur* and *T. dregeana,* respectively (Fig. 3.13; Table 3.11). In contrast, there was a significant (175%) increase in H_2O_2 production in *S. gerrardii* explants relative to the control (Fig. 3.13; Table 3.11).

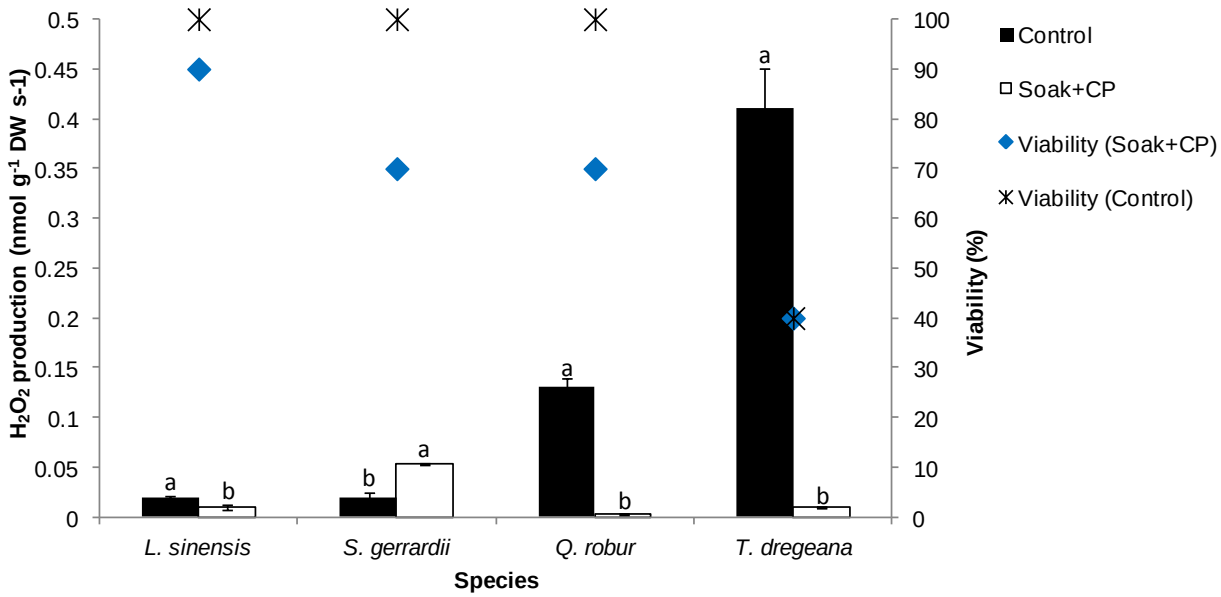

Fig. 3.13: Extracellular H_2O_2 production and viability for excised explants of *L. sinensis, S. gerrardii, Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to the combination of exogenous antioxidant soaking and cryoprotection with 5% & 10% DMSO+glycerol. Values represent mean±SD (n=6 for H_2O_2 and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.11: H_2O_2 production in treated (soaked+cryoprotected) zygotic explants of the four species investigated, expressed as a % of the control.

	H_2O_2 production (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	-30	175	-97	-97

3.2.3 Total aqueous antioxidant capacity and viability in zygotic explants with and without the combination of soaking and cryoprotection

There was no notable change in antioxidant capacity in explants of *L. sinensis* and *S. gerrardii* relative to the control. Antioxidant capacity significantly decreased relative to the control in soaked+CP *Q. robur* and *T. dregeana* explants; by 84 and 75%, respectively (Fig. 3.14; Table 3.12).

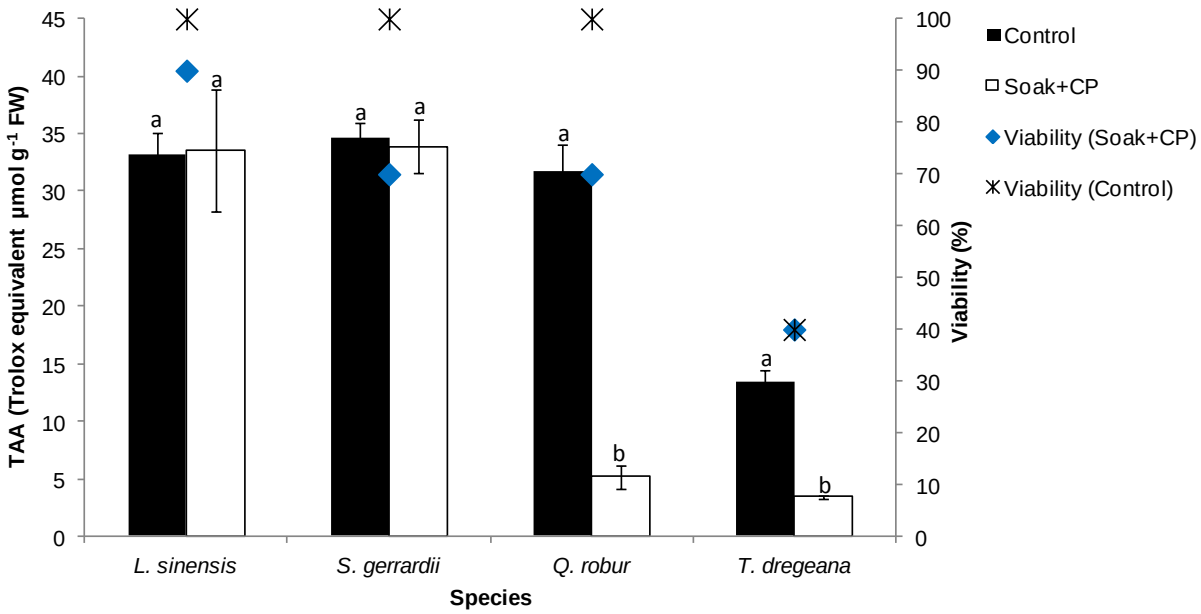

Fig. 3.14: Total aqueous antioxidant capacity and viability for excised explants of *L. sinensis, S. gerrardii, Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to the combination of exogenous antioxidant soaking and cryoprotection with 5% & 10% DMSO+glycerol. Values represent mean±SD (n=8 for TAA and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.12: TAA capacity in treated (soaked+cryoprotected) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative TAA capacity (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	1	-2	-84	-75

3.2.4 Extracellular respiratory activity and viability in zygotic explants with and without the combination of soaking and cryoprotection

Respiratory activity increased significantly (70%) in soaked+CP explants of *L. sinensis* compared with the control (Fig. 3.15; Table 3.13). In soaked+CP explants of *S. gerrardii*, *Q. robur* and *T. dregeana*, there was a significant decrease (77, 10 and 52%, respectively) in respiratory activity in comparison with the control (Fig. 3.15; Table 3.13).

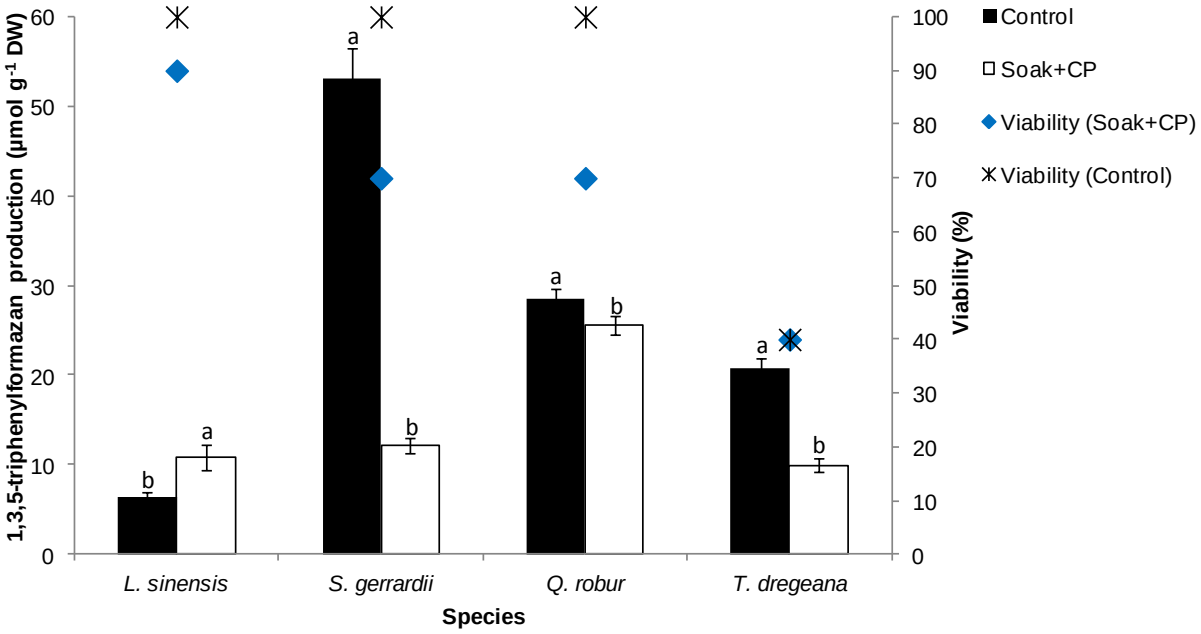

Fig. 3.15: Extracellular respiratory activity and viability for excised explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to the combination of exogenous antioxidant soaking and cryoprotection with 5% & 10% DMSO+glycerol. Values represent mean±SD (n=8 for TTZ and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.13: Respiratory activity in treated (soaked+cryoprotected) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative respiratory activity (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	70	-77	-10	-52

3.3 Explant partial dehydration

This section presents physiological and biochemical results obtained after partial dehydration of explants. As reported in sections 3.1 and 3.2, explants underwent two procedural steps prior to partial dehydration: soaking and soaking+cryoprotection. Both steps were applied to explants (represented as undried; WC and viability indicated at 0 on the x-axis) prior to partial dehydration to establish the ideal combination of cryo-preparative treatments before cooling. The following results represent data for explants that were exposed to (i) soaking+flash drying (soak+FD); and (ii) soaking+cryoprotection+flash drying (soak+CP+FD).

3.3.1 Selection of drying technique

Multiple desiccation techniques have been used to slowly or rapidly dry recalcitrant-seeded germplasm based on explant type (Fu *et al*., 1993; Chandel *et al*., 1995; Chmielarz, 1997; Berjak *et al*., 1999b; Chaudhury & Malik, 1999; Chmielarz *et al*., 2011). In this study, three rapid drying techniques were investigated: desiccation over silica gel (Chmielarz *et al*., 2011), flash drying (FD) (Berjak *et al*., 1990; Pammenter *et al*., 2002) and vacuum flash drying (henceforth referred to as vacuum FD; Fu *et al*., 1993). These techniques were compared in terms of their effects on explant drying rate and viability. Desiccation over silica gel resulted in extremely slow drying rates (data not shown) not ideal for zygotic explants from recalcitrant seeds (Pammenter *et al*., 1998; Varghese *et al*., 2011) and was therefore not included in further investigations. Vacuum FD of explants improved the drying rate for *L. sinensis* explants only, when compared with conventional flash drying. Hence, the drying profile of explants that were vacuum and conventionally flash dried are presented for comparative purposes only for this species. Drying profiles are presented for explants of *T.*

dregeana, *Q. robur*, *S. gerrardii* and *L. sinensis* after the employment of the flash drying technique.

3.3.2 Optimisation of explant WC and drying time for cryopreservation

(i) Soaked explants

The flash drying curves presented in Figs 3.16 to 3.19 illustrate WC and viability (seedling production) at selected drying intervals for each species. Using these data, drying rates and % water loss were calculated for the optimum drying period/WC.

Figures 3.16-3.19 were constructed to select an optimum drying period/WC for desiccation prior to cooling. Based on the improvement of vigour (Table 3.4) and the significant reduction in $\cdot O_2^-$ production compared with the control immediately after excision in all species (Fig. 3.4 & Table 3.5), a soaking treatment was deemed necessary after excision, prior to dehydration. As discussed in Chapter 1, the drying period for partial dehydration of recalcitrant-seeded germplasm should result in sufficiently low WC within minimum drying time and with minimal viability loss, in order to be deemed suitable. Viability in this section is based on seedling production (root and shoot development) as henceforth, the study focused on optimising cryo-preparative procedures that would facilitate maximum seedling growth. In cases where only roots developed (usually after drying periods longer than that selected as optimum), viability was recorded as zero.

Explants of *T. dregeana* lost approximately 50% of its shedding WC within the first 30 min of drying (Fig. 3.16). Thereafter, a further 90 min of drying was required to sufficiently desiccate explants to a WC amenable for cooling (0.41 g g^{-1}). After this desiccation period (120 min FD), 50% seedling production was recorded. Further desiccation did not permit seedling growth. Therefore, soaked explants of this species were flash dried for 120 min prior to cooling.

Explants of *Q. robur* lost approximately 50% of its shedding WC within 120 min of drying (Fig. 3.17). Thereafter, a further 120 min of drying was required to sufficiently desiccate explants to a WC amenable for cooling (0.37 g g^{-1}). After this desiccation period (240 min FD), high viability retention, represented by 80% seedling production, was recorded. Further

desiccation permitted 5% seedling growth. Therefore, soaked explants of this species were flash dried for 240 min prior to cooling.

Explants of *S. gerrardii* lost approximately 50% of its shedding WC within 15 min of drying (Fig. 3.18). Thereafter, a further 5 min of drying was required to sufficiently desiccate explants to a WC amenable for cooling (0.38 g g^{-1}). After this desiccation period (20 min FD), high viability retention, represented by 75% seedling production, was recorded. Further desiccation permitted no seedling growth. Therefore, soaked explants of this species were flash dried for 20 min prior to cooling.

Explants of *L. sinensis* that were flash dried lost approximately 50% of its shedding WC within 30 min of drying (Fig. 3.19). Explants flash dried under vacuum lost approximately 60% of water within 15 min of drying (Fig. 3.19). Explants flash dried under vacuum required a further 15 min of drying to sufficiently desiccate explants to a WC amenable for cooling (0.36 g g^{-1}). Explants flash dried in the absence of a vacuum also required a further 15 min of drying to sufficiently desiccate explants to a WC amenable for cooling (0.35 g g^{-1}). While 100% seedling production by explants was observed under both drying methods, flash drying under vacuum reduced drying time by 15 min to reach the target WC range. There was a significant reduction in water loss ($p<0.05$; Independent Samples T-Test) at each time interval in excised zygotic explants desiccated under a vacuum flash drier compared with those dried for the same period in a conventional flash drier (Fig. 3.19). Therefore, flash drying under vacuum was the method applied to desiccate of *L. sinensis* explants for 30 min prior to cooling. Further desiccation permitted only 5% seedling growth.

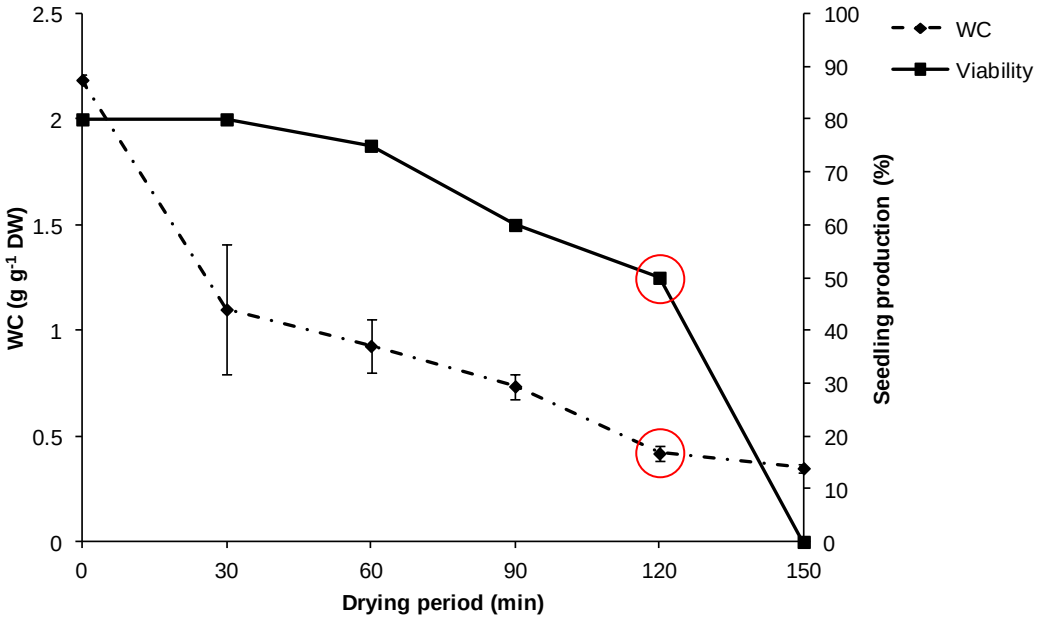

Fig. 3.16: Water content (WC) and seedling production (n=20) in undried and in soaked and flash dried zygotic explants of *T. dregeana* during selected drying intervals. WC values represent mean±SD (n=10). Red circles indicate explant WC, drying time (0.42 g g^{-1}; 120 min) and associated viability (considered to be suitable for partial dehydration of this species prior to cooling).

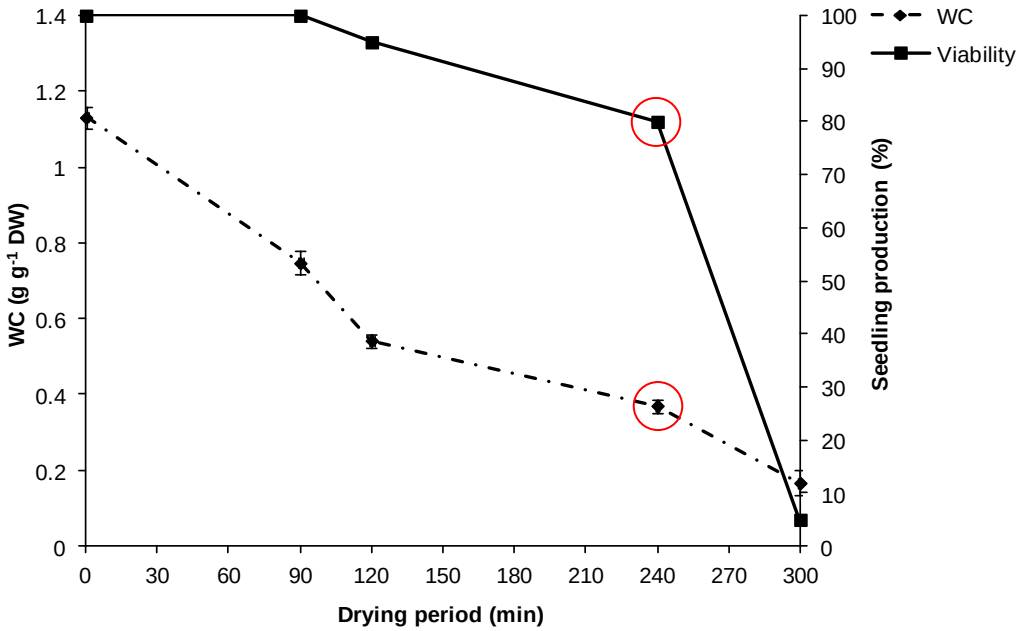

Fig. 3.17: Water content (WC) and seedling production (n=20) in undried and in soaked and flash dried zygotic explants of *Q. robur* during selected drying intervals. WC values represent mean±SD (n=10). Red circles indicate explant WC, drying time (0.37 g g^{-1}; 240 min) and associated viability (considered to be suitable for partial dehydration of this species prior to cooling).

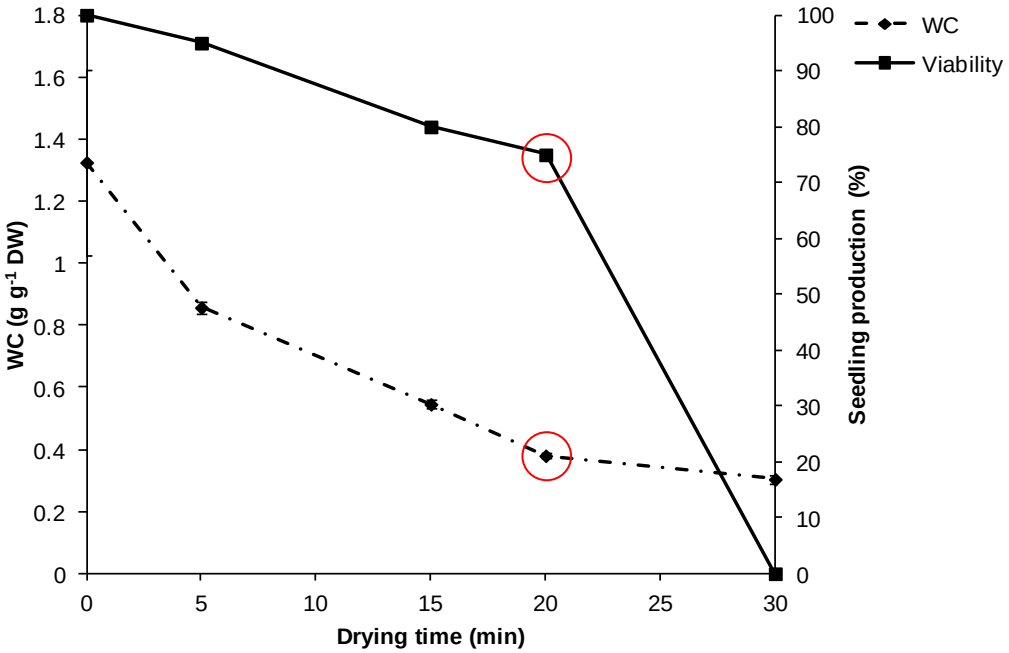

Fig. 3.18: Water content (WC) and seedling production (n=20) in undried and in soaked and flash dried zygotic explants of *S. gerrardii* during selected drying intervals. WC values represent mean±SD (n=10). Red circles indicate explant WC, drying time (0.38 g g^{-1}; 20 min) and associated viability (considered to be suitable for partial dehydration of this species prior to cooling).

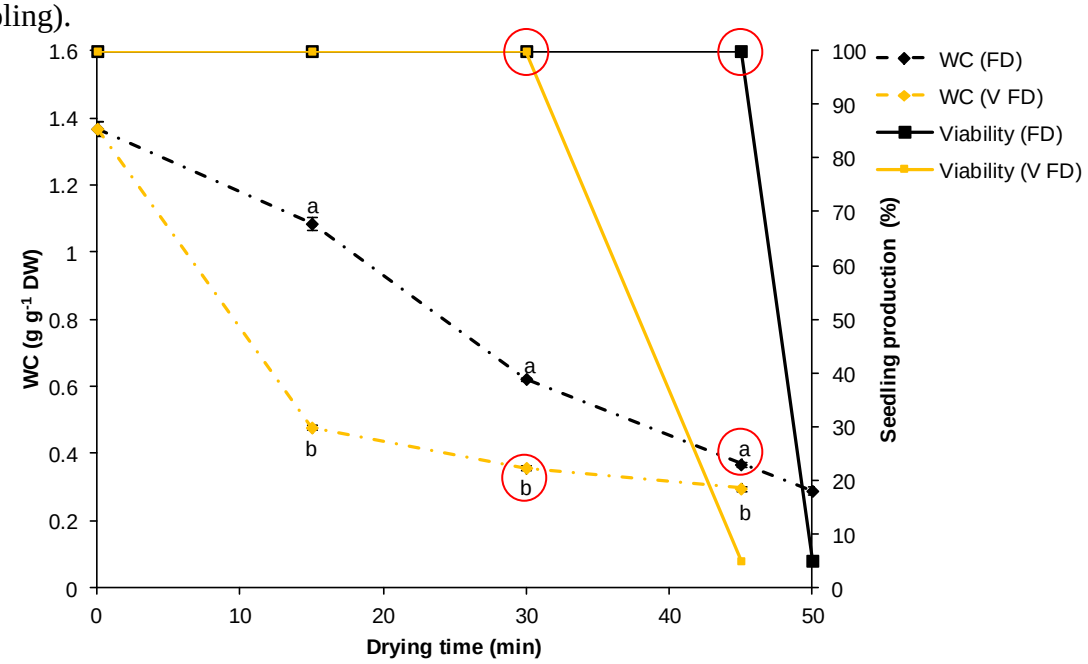

Fig. 3.19: Water content (WC) and seedling production (n=20) in undried and in soaked and flash dried zygotic explants of *L. sinensis* during selected drying intervals. WC values represent mean±SD (n=10). Red circles indicate explant WC, drying time (0.36 g g^{-1}; 45 min and 0.35 g g^{-1}; 30 min for flash dried [FD] and vacuum flash dried [V FD] explants respectively) and associated viability (considered to be suitable for partial dehydration of this species prior to cooling). Data points that indicate WC and are labelled with different letters are significantly different when compared between flash drying methods, within drying intervals ($p<0.05$, Independent sample t-test).

(ii) Soaked and cryoprotected explants

After selecting the drying technique for partial dehydration of soaked explants, the selected techniques were applied to soaked+CP explants (FD for explants of *T. dregeana, Q. robur* and *S. gerrardii* and vacuum FD for explants of *L. sinensis*). From the flash drying curves illustrated below, optimum drying intervals were selected for soaked+CP explants of each species based on the viability retained at WC that were amenable for cooling.

Figures 3.20-3.23 exhibit data that was generated to select an optimum drying period for partial dehydration of soaked+CP explants of each species. The drying interval was selected (as for soaked explants) based on the period that resulted in sufficiently low WC within minimum time and with minimal viability loss.

Explants of *T. dregeana* lost approximately 50% of its shedding WC within 60 min of drying (Fig. 3.20). Thereafter, a further 90 min of drying was required to desiccate explants to a WC amenable for cooling (0.42 g g^{-1}). After this desiccation period (150 min FD), 40% seedling production was recorded. Further desiccation did not permit seedling growth.

Explants of *Q. robur* lost approximately 50% of its shedding WC within 240 min of drying (Fig. 3.21). Thereafter, a further 20 min of drying was required to desiccate explants to a WC amenable for cooling (0.37 g g^{-1}). After this desiccation period (260 min FD), viability retention represented by 65% seedling production by explants was recorded. Further desiccation permitted a mere 5% seedling growth.

Explants of *S. gerrardii* lost approximately 50% of its shedding WC within 15 min of drying (Fig. 3.22). Thereafter, a further 10 min of drying was required to desiccate explants to a WC amenable for cooling (0.38 g g^{-1}). After this desiccation period (25 min FD), viability retention represented by 65% seedling production was recorded. Further desiccation permitted no seedling growth.

Explants of *L. sinensis* lost approximately 50% of its shedding WC within 15 min of drying (Fig. 3.23). Thereafter, a further 25 min of drying was required to desiccate explants to a WC amenable for cooling (0.37 g g^{-1}). After this desiccation period (40 min FD), viability retention represented by 60% seedling production was recorded. Further desiccation permitted no seedling growth.

It was observed that cryoprotected (soak+CP) and non-cryoprotected (soaked) explants across species exhibited the same pattern in terms of drying period necessary for achieving WC amenable for cooling, with drying periods increasing in the following order in both cryoprotected and non-cryoprotected material: *S. gerrardii*>*L. sinensis*> *T. dregeana*> *Q. robur*. Based on the viability obtained after soaking+FD (Figs 3.16-3.19) and soaking+CP+FD (Figs 3.20-3.23), the latter combination of cryo-preparative treatments were not selected for the cryopreservation protocols of the four species. This was based on explant viability after soaking+FD (Figs 3.16-3.19) being higher than explant viability after soaking+CP+FD (Figs 3.20-3.23). It was also observed that the selected drying period required to reach target WC was shorter in soaked+FD explants (Figs 3.16-3.19) than in soaked+CP+FD explants (Figs 3.20-3.23). Explants prepared for cryopreservation across all species were therefore treated with soaking and flash drying (soak+FD) prior to cryogen exposure.

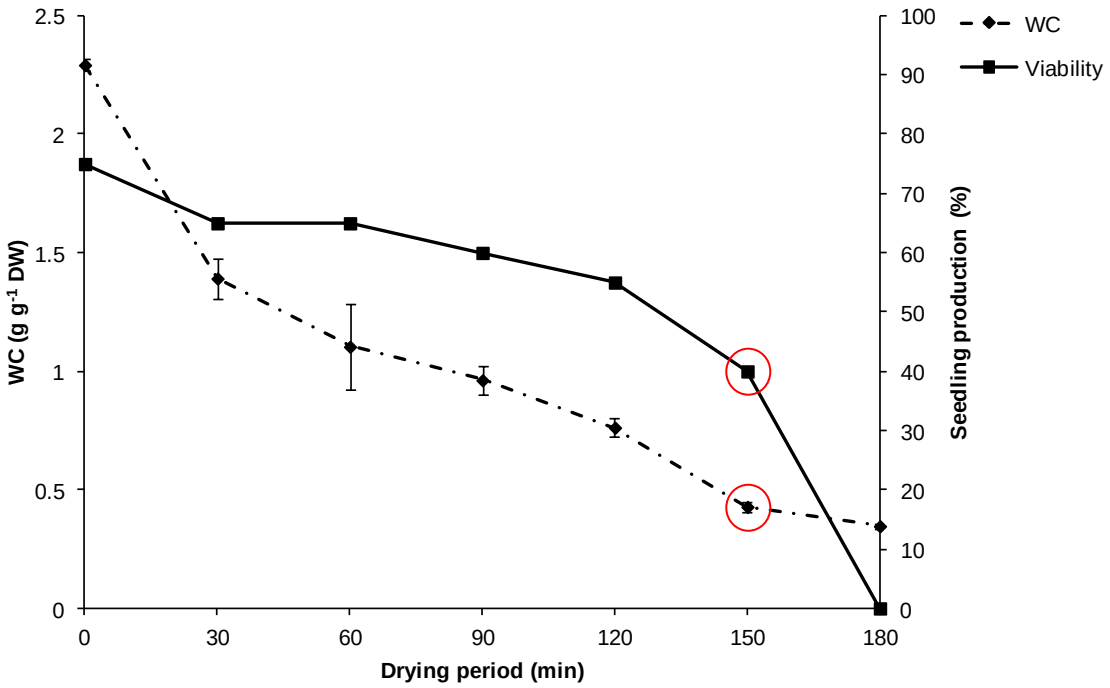

Fig. 3.20: Water content (WC) and seedling production (n=20) in undried and in soaked, cryoprotected and flash dried zygotic explants of *T. dregeana* during selected drying intervals. WC values represent mean±SD (n=10). Red circles indicate explant WC, drying time (0.42 g g^{-1}; 150 min) and associated viability (considered to be suitable for partial dehydration of this species).

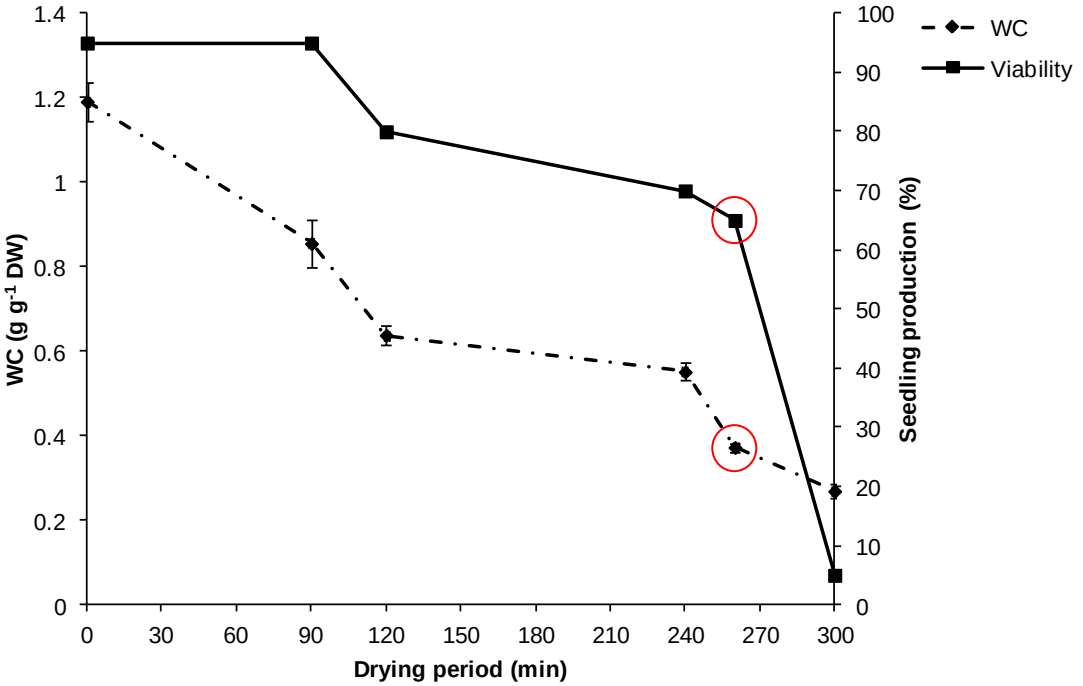

Fig. 3.21: Water content (WC) and seedling production (n=20) in undried and in soaked, cryoprotected and flash dried zygotic explants of *Q. robur* during selected drying intervals. WC values represent mean±SD (n=10). Red circles indicate explant WC, drying time (0.37 g g^{-1}; 260 min) and associated viability (considered to be suitable for partial dehydration of this species).

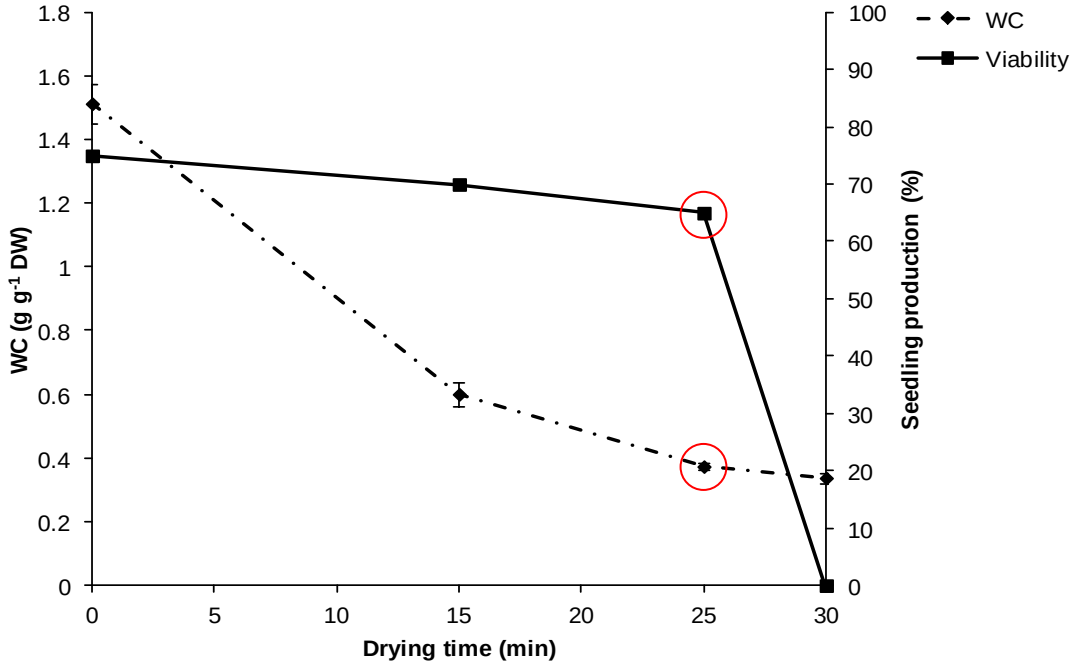

Fig. 3.22: Water content (WC) and seedling production (n=20) in undried and in soaked, cryoprotected and flash dried zygotic explants of *S. gerrardii* during selected drying intervals. WC values represent mean±SD (n=10). Red circles indicate explant WC, drying time (0.38 g g^{-1}; 25 min) and associated viability (considered to be suitable for partial dehydration of this species).

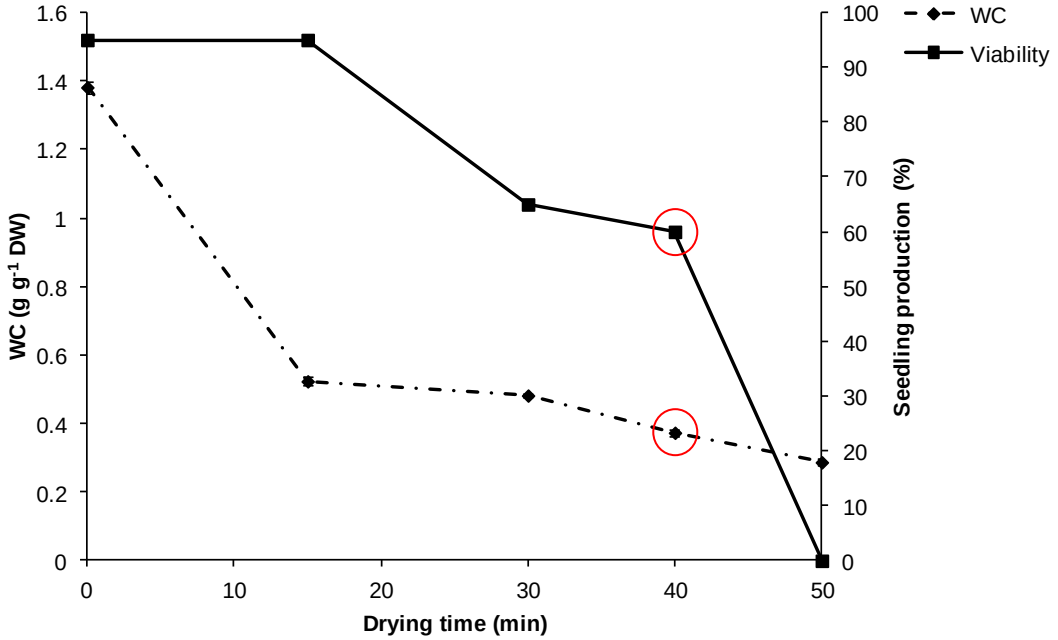

Fig. 3.23: Water content (WC) and seedling production (n=20) in undried and in soaked, cryoprotected and vacuum flash dried zygotic explants of *L. sinensis* during selected drying intervals. WC values represent mean±SD (n=10). Red circles indicate explant WC, drying time (0.37 g g^{-1}; 40 min) and associated viability (considered to be suitable for partial dehydration of this species).

3.3.3 Extracellular ROS production and viability in zygotic explants with and without the combination of soaking and rapid dehydration

Explants of all four species were measured for extracellular $^{\cdot}O_2^-$ and H_2O_2 production, total aqueous antioxidant capacity, respiratory activity and viability in control explants and in those treated with exposure to a combination of antioxidant soaking and flash drying (soaking+FD). Treated explants were immersed in 1% AsA+CW for 5 min in *L. sinensis* and 30 min for the remaining species, before flash drying for selected drying intervals (species-specific; see Section 3.3.2).

Superoxide production in soaked+FD explants of *L. sinensis*, *Q. robur* and *T. dregeana* decreased significantly, by 89, 82 64% respectively, compared with their controls (Fig. 3.24; Table 3.14). However, there was a significant increase (44%) in this ROS in soaked+FD explants of *S. gerrardii* compared with the control (Fig. 3.24; Table 3.14). After soaking+FD, seedling production declined by 25 and 20% in *S. gerrardii* and *Q. robur* explants respectively relative to the control (Figs 3.24-3.27). A 10% increase in seedling production was observed in soaked+FD explants of *T. dregeana* relative to the control (Figs 3.24-3.27).

No decline in viability was observed in soaked+FD explants of *L. sinensis* relative to the control (Figs 3.24-3.27).

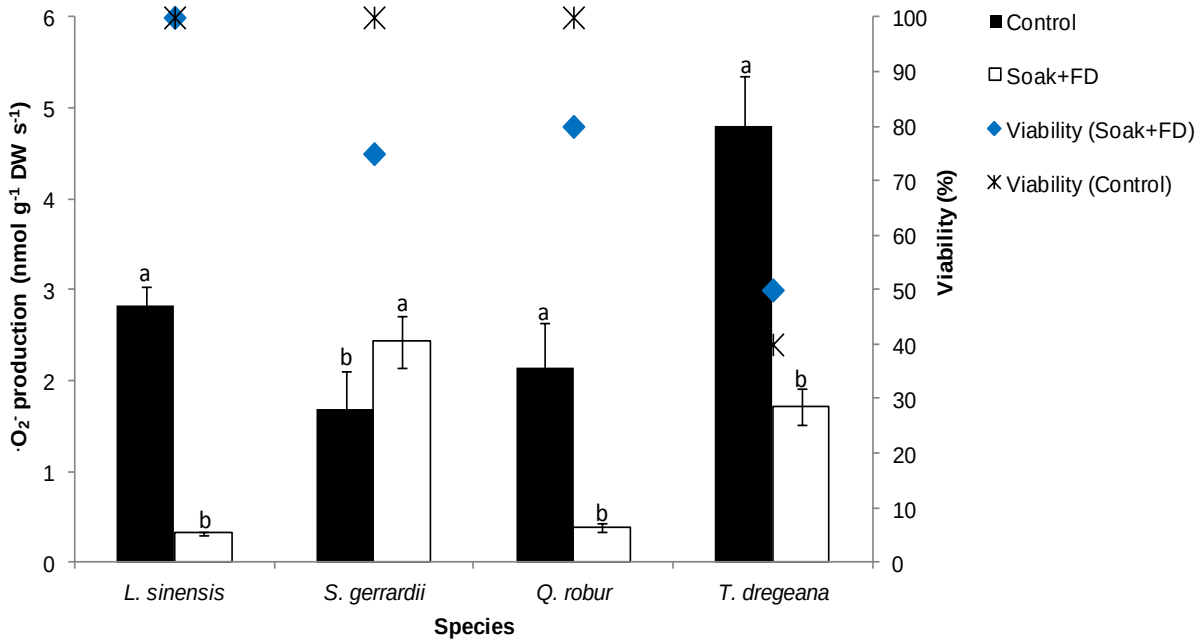

Fig. 3.24: Extracellular $\cdot O_2^-$ production and viability for excised explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to the combination of exogenous antioxidant soaking and flash drying. Explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* were flash dried for 30, 20, 240 and 120 min, respectively. Values represent mean±SD (n=6 for $\cdot O_2^-$ and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.14: $\cdot O_2^-$ production in treated (soaked+flash dried) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative $\cdot O_2^-$ production (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	-89	44	-82	-64

Hydrogen peroxide production increased significantly in soaked+FD explants of *L. sinensis* and *S. gerrardii* (by 250 and 1176% respectively) compared with the control (Fig. 3.25; Table 3.15). This ROS species decreased significantly in soaked+FD explants of *Q. robur* and *T. dregeana* (by 97 and 95% respectively) in comparison with the control (Fig. 3.25; Table 3.15)

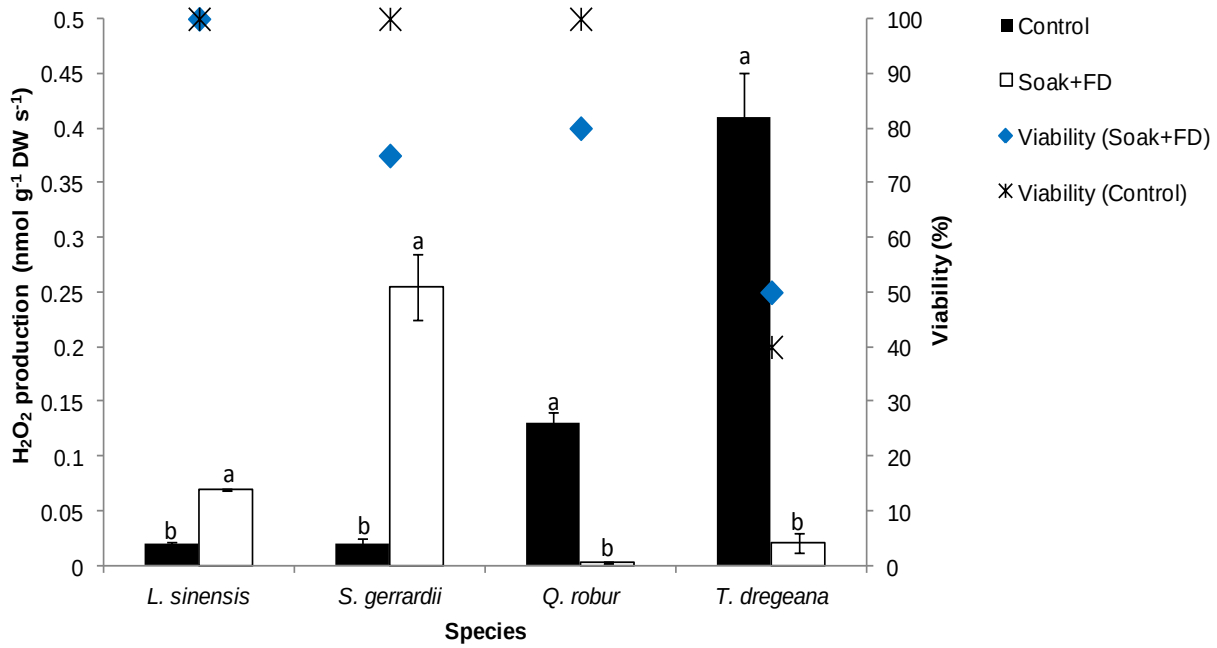

Fig. 3.25: Extracellular H_2O_2 production and viability for excised explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to the combination of exogenous antioxidant soaking and flash drying. Explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* were flash dried for 30, 20, 240 and 120 min, respectively. Values represent mean±SD (n=6 for H_2O_2 and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.15: H_2O_2 production in treated (soaked+flash dried) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative H_2O_2 production (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	250	1176	-97	-95

3.3.4 Total aqueous antioxidant capacity in zygotic explants with and without the combination of soaking and rapid dehydration

Total aqueous antioxidant capacity increased significantly in soaked+FD explants of *S. gerrardii* (by 27%) compared with the control (Fig. 3.26; Table 3.16). Antioxidant capacity

decreased significantly in soaked+FD explants of *L. sinensis*, *Q. robur* and *T. dregeana* (by 23, 70 and 75%, respectively) compared with the control (Fig. 3.26; Table 3.16).

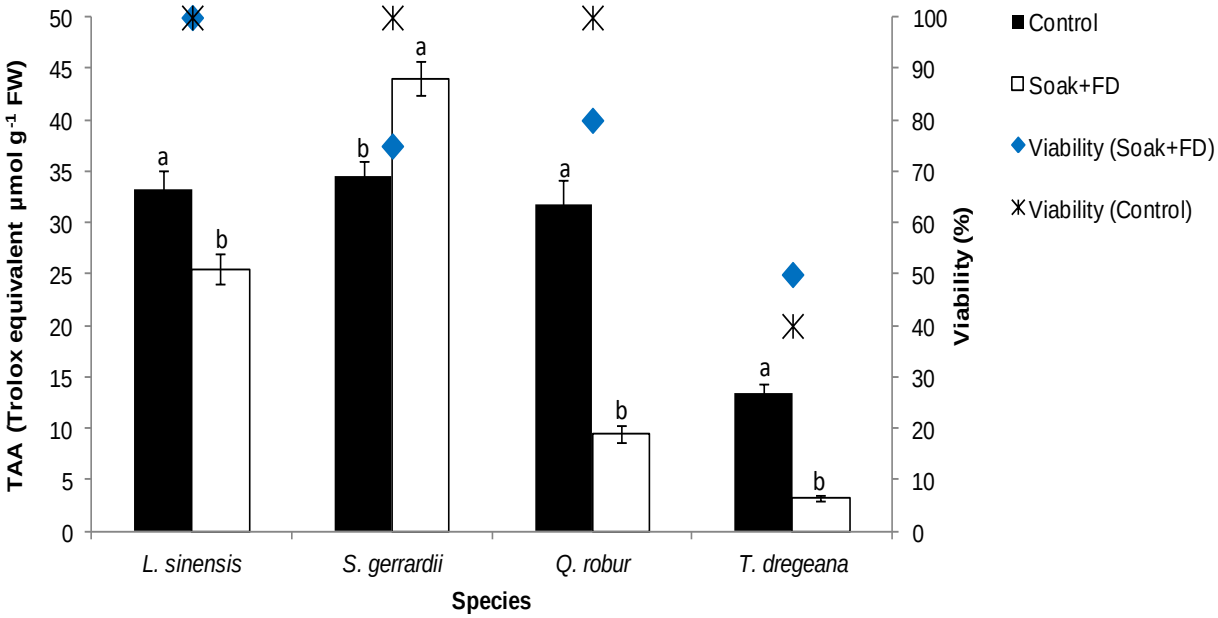

Fig. 3.26: Total aqueous antioxidant capacity and viability for excised explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to the combination of exogenous antioxidant soaking and flash drying. Explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* were flash dried for 30, 20, 240 and 120 min, respectively. Values represent mean±SD (n=8 for TAA and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.16: TAA capacity in treated (soaked+flash dried) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative TAA capacity (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	-23	27	-70	-75

3.3.5 Extracellular respiratory activity in zygotic explants with and without the combination of soaking and rapid dehydration

Respiratory activity decreased significantly in soaked+FD explants of all species in comparison with their respective controls (Fig. 3.27; Table 3.17). This decrease was measured as a 50, 74, 15 and 25% decline in soaked+FD explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana*, respectively (Fig. 3.27; Table 3.17).

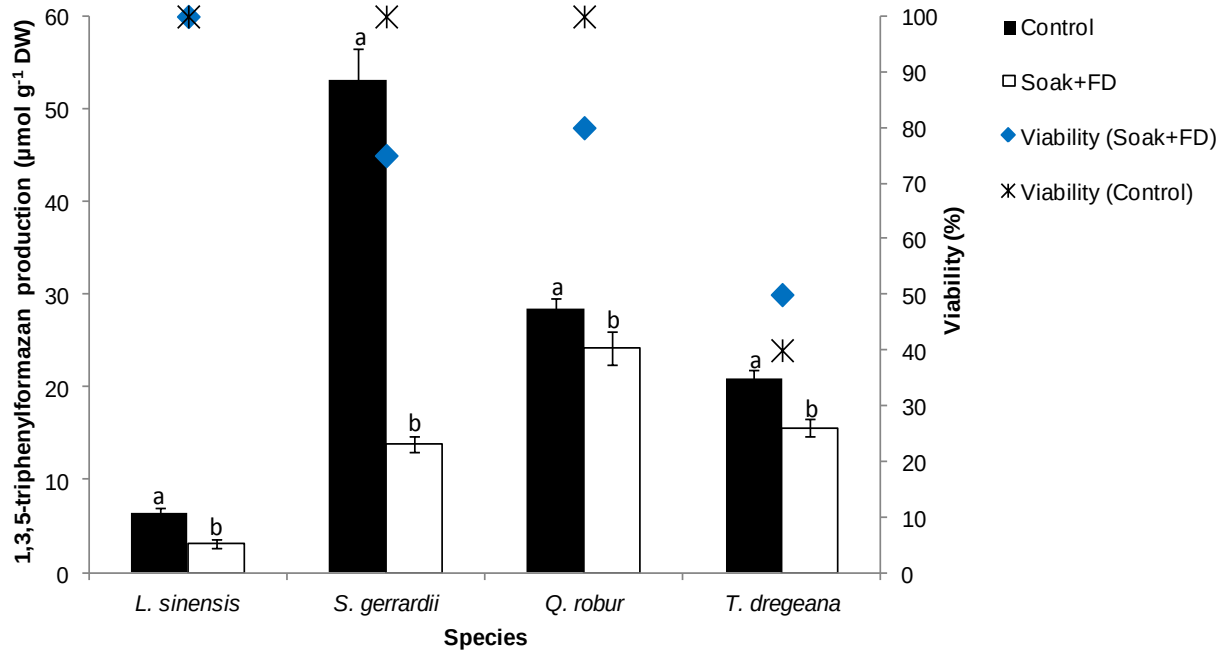

Fig. 3.27: Extracellular respiratory activity and viability for excised explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to the combination of exogenous antioxidant soaking and flash drying. Explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* were flash dried for 30, 20, 240 and 120 min, respectively. Values represent mean±SD (n=8 for TTZ and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.17: Respiratory activity in treated (soaked+flash dried) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative respiratory activity (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	-50	-74	-15	-25

3.3.6 Effects of the combination of exogenous application of antioxidants and rapid dehydration on shoot meristem ultrastructure of zygotic explants

Ultrastructural studies were conducted only on non-cryoprotected explants given that cryoprotection was shown to be unsuitable for zygotic explants of these species. The following micrographs (Figs 3.28-3.31) illustrate the shoot meristematic ultrastructure in soaked and flash dried (soaked+FD) zygotic explants from *T. dregeana*, *Q. robur*, *S. gerrardii* and *L. sinensis*, respectively.

Figures 3.28-3.31 (a & b) depict shoot meristem ultrastructure of soaked+FD explants. Viability of explants of *L. sinensis* remained unchanged at 100% in untreated (Fig.3.11a & b), soaked (Fig. 3.11c & d) and soaked+FD (Fig. 3.31a & b) explants. Soaked explants of *Q. robur* dried for 240 min (WC: 0.37±0.01 g g^{-1}; Fig. 3.29a & b), *S. gerrardii* dried for 20 min (WC: 0.38±0.01 g g^{-1}; Fig. 3.30) and *L. sinensis* dried for 30 min (WC: 0.35±0.005 g g^{-1}; Fig. 3.31a & b), retained 80, 75 and 100% viability, respectively (see Section 3.3 for complete WC and viability profiles). While viability after partial dehydration decreased relative to soaked explants (Fig. 3.8c & d, 3.9c & d & 3.10b & c) in three of these species (*T. dregeana*, *Q. robur* & *S. gerrardii*), all the micrographs reflect ultrastructure representative of viable explants.

The lowest post-drying viability (50%) across species was exhibited by soaked explants of *T. dregeana*, flash dried for 120 min (WC: 0.42±0.01 g g^{-1}; Fig. 3.28a & b). However, the ultrastructure of shoot meristems of this species (Fig. 3.28a & b) features attributes of viable tissue. The presence of plastids (P) and numerous mitochondria (M) indicate active respiration (Fig. 3.28a). Lipid bodies (L), spherical nuclei (N; Fig.3.28a) and endoplasmic reticulum (er; Fig. 3.33b) were observed. There were no obvious ultrastructural abnormalities observed in the nucleus which showed intact nuclear membranes, patches of heterochromatin (indicated by arrows) in the nucleoplasm and dense nucleoli (Fig. 3.28a). Mitochondrial development, however, was not as advanced as in untreated material (Fig. 3.8a & b) after desiccation and subsequent rehydration (Fig. 3.28a & b).

Q. robur explants retained 80% viability after being soaked and dried for 240 min (WC: 0.37±0.01 g g^{-1}; Fig. 3.29a & b). Ultrastructural integrity was well preserved in these shoot

meristems where relatively long profiles of endoplasmic reticulum (er), starch bodies (S) and mitochondria (M) with well developed cristae were also observed (Fig. 3.29a & b). Mitochondrial development was advanced in terms of cristae compared with soaked explants (Fig. 3.9d). The presence of these organelles were indicative of actively respiring cells and ongoing membrane development.

Explants of *S. gerrardii* soaked and dried for 20 min (WC: 0.38±0.01 g g^{-1}; Fig. 3.30), retained 75% viability. Storage reserves in the form of lipid were evident in the ultrastructure of *S. gerrardii* in soaked+FD explants (Fig. 3.30). Generally, the ultrastructure was indicative of germinable tissue. The higher degree of vacuolation (V) visible in shoot meristems of soaked+FD explants (Fig. 3.30) compared with those of untreated (Fig. 3.10a) or soaked ones (Fig. 3.10b & c) are indicative of a typical response of recovery from drying stress in recalcitrant-seeded zygotic explants (Berjak *et al*., 1989). The prevention of intracellular collapse in this species can be inferred by the numerous lipid bodies (L).

Explants of *L. sinensis* that were soaked and vacuum flash dried for 30 min (WC: 0.35±0.005 g g^{-1}; Fig. 3.31a & b), retained 100% viability. Numerous, well developed mitochondria with clearly visible cristae were distributed throughout the cytomatrix (M; Fig. 3.31a & b), as observed in untreated (Fig. 3.11c) and soaked explants (Fig. 3.11d). Well developed nuclei with patches of heterochromatin and dense nucleoli were evident throughout the meristem (Nu; Fig. 3.31a), similar to the situation in soaked explants (Fig. 3.11c) indicating ongoing mitotic activity (Berjak & Pammenter, 2000). Water loss from the tissue did not show a marked effect on ultrastructural integrity in this species (Fig. 3.31a & b), which was consistent with full viability retention after desiccation.

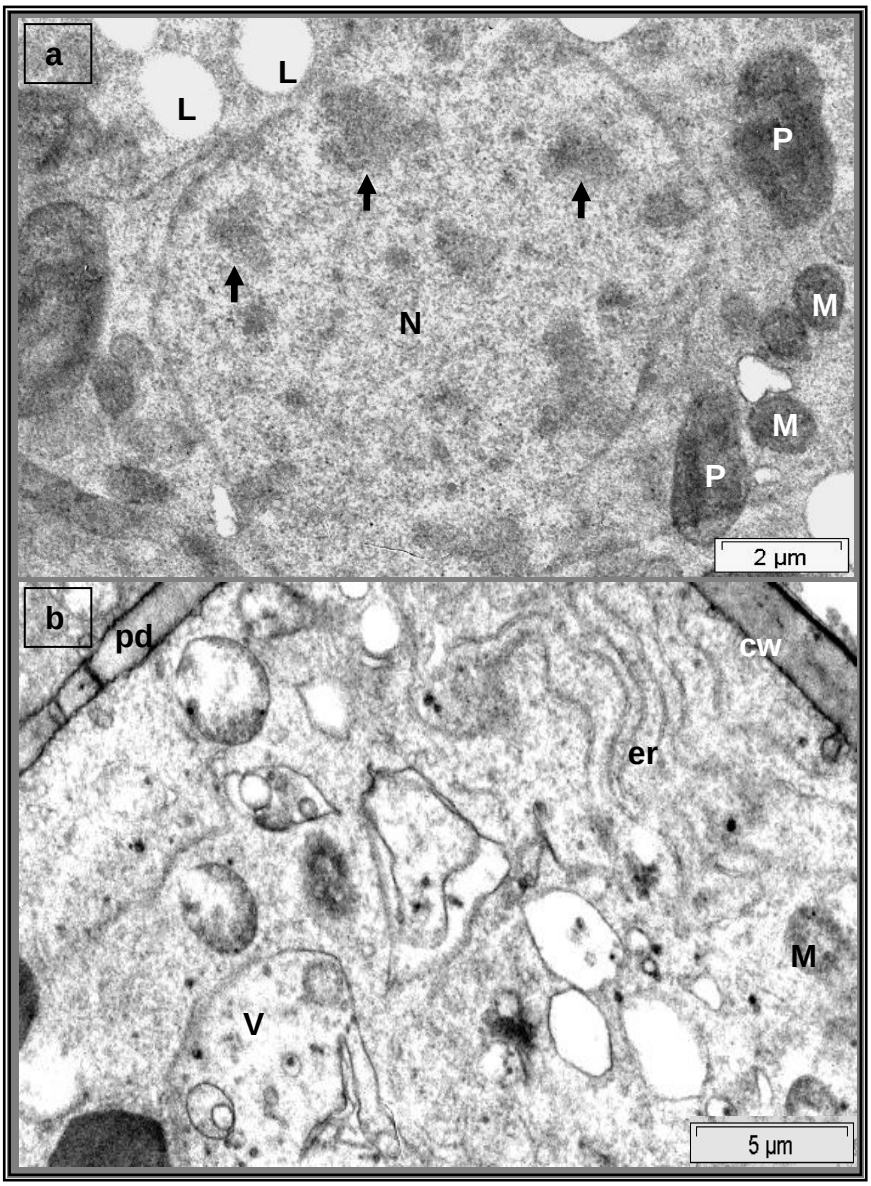

Fig. 3.28a & b: The ultrastructure of shoot meristems excised from soaked (1% AsA+CW solution for 30 min) and flash dried (120 min) zygotic *T. dregeana* explants. Well developed nuclei (N) (Fig. 3.28a) with numerous patches of heterochromatin (indicated by arrows) were evident in the ultrastructure. Numerous electron dense mitochondria (M) (Figs 3.28a & b) were present throughout the cytomatrix. Large, well developed plastids (P) (Fig. 3.28a) were noted in the cytomatrix. Long profiles of endoplasmic reticulum (er), irregular shaped vacuoles (V), plasmodesmata (pd) and vesicle activity (indicated by arrows) was observed (Fig. 3.28b).

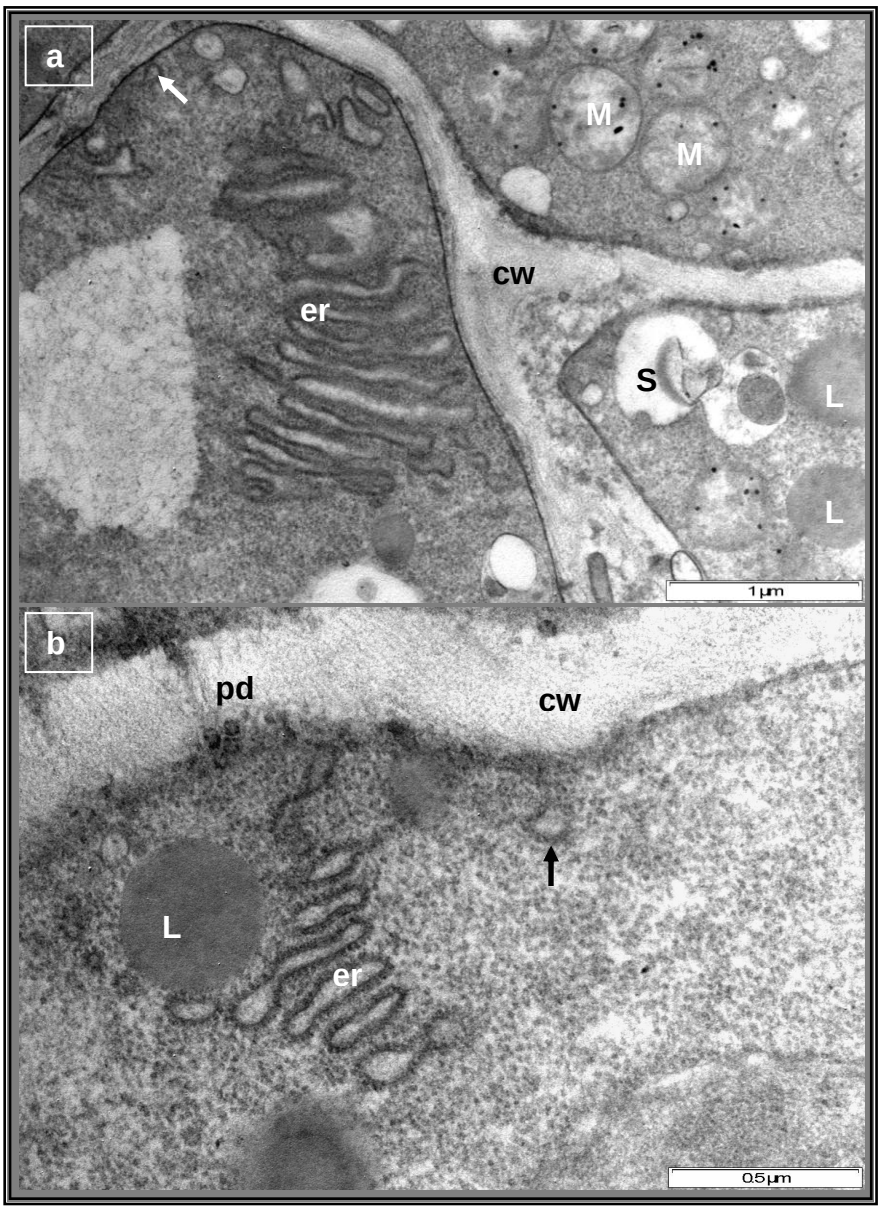

Fig. 3.29a & b: The ultrastructure of shoot meristems excised from soaked (1% AsA+CW solution for 30 min) and flash dried (240 min) zygotic *Q. robur* explants. Figures 3.29a & b show long profile endoplasmic reticulum (er) and dense lipid bodies (L) as well as numerous vesicles (indicated by arrows). Mitochondria (M) appear well developed with defined cristae (Fig. 3.29a).

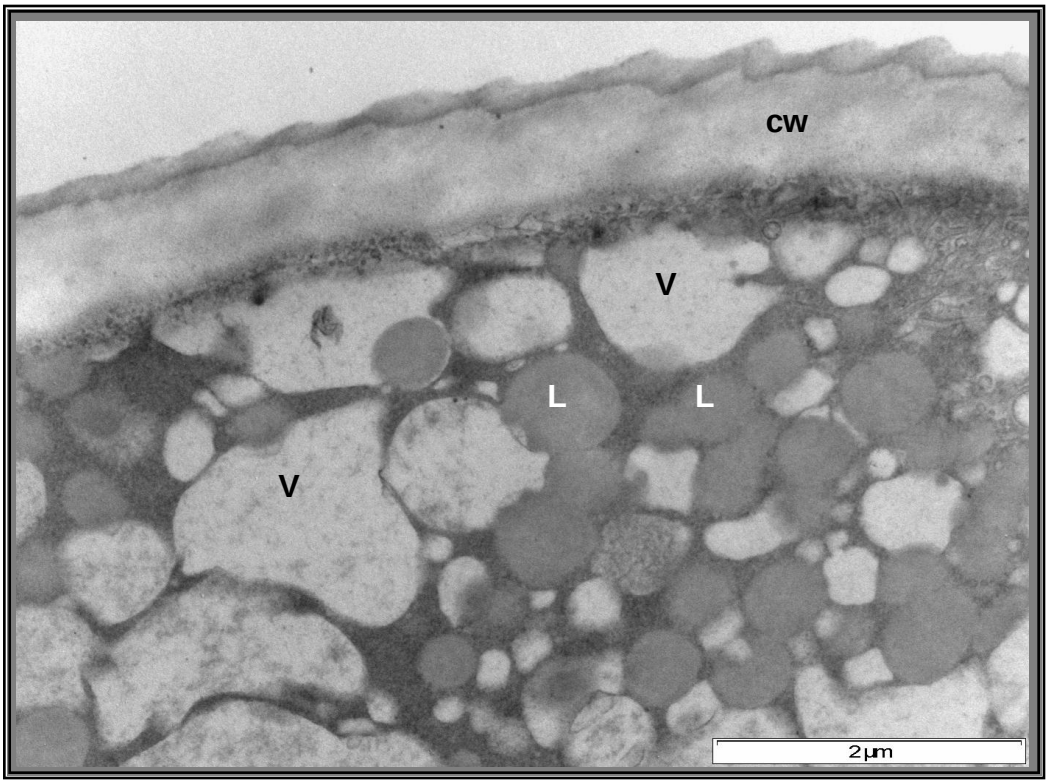

Fig. 3.30: The ultrastructure of shoot meristems excised from soaked (1% AsA+CW solution for 30 min) and flash dried (20 min) zygotic *S. gerrardii* explants. Numerous lipid bodies (L) and large, compacted vacuoles (V) were visible throughout the cytoplasm.

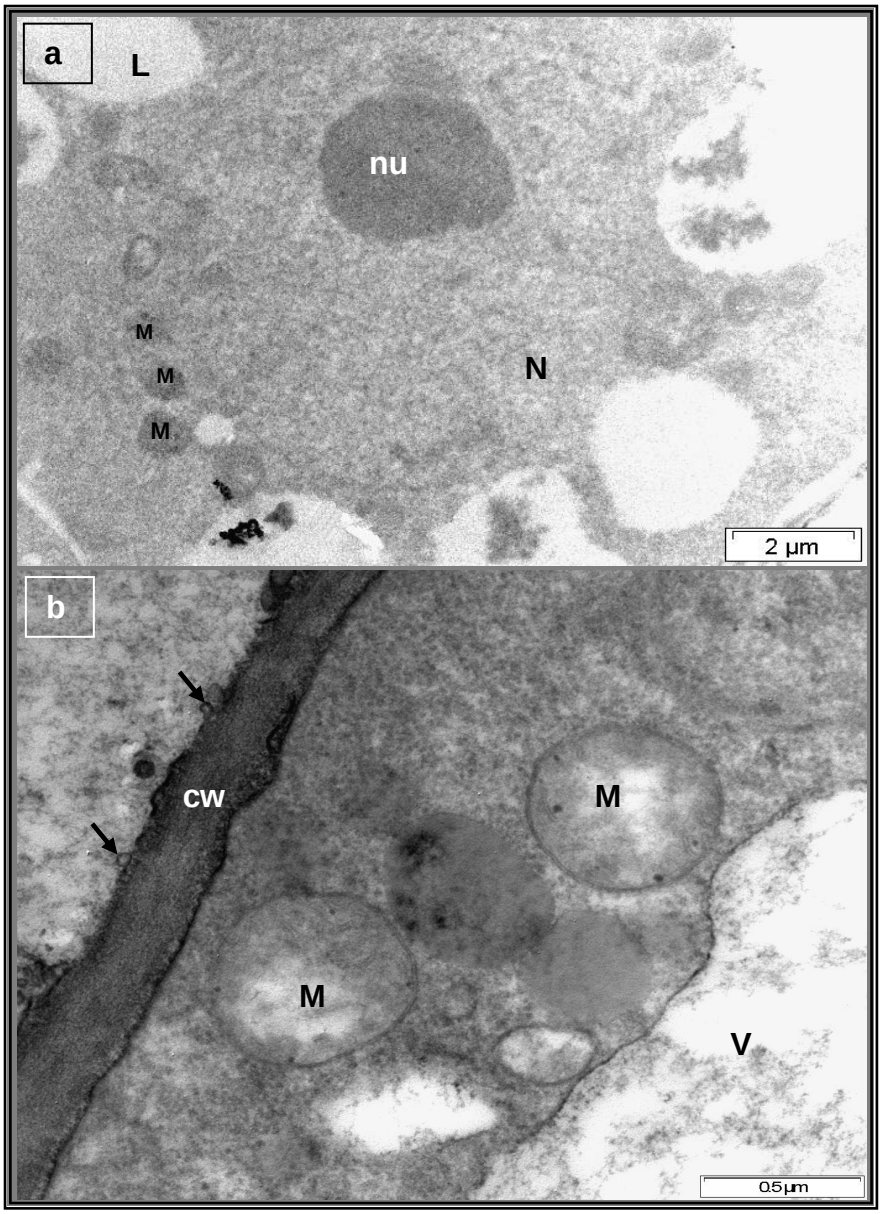

Fig. 3.31a & b: The ultrastructure of shoot meristems excised from soaked (1% AsA+CW solution for 5 min) and flash dried (30 min) zygotic *L. sinensis* explants. The nucleus (N) and nucleolus (nu) (Fig. 3.31a) were spherical and well developed. Numerous, developed mitochondria (M) (Figs 3.31a & b) were present throughout the cytomatrix. Lipid bodies (L) (Fig.3.31a) appeared less dense in the cytoplasm. Plasmamembrane associated vesicles (indicated by arrows) were visible (Fig. 3.31b).

3.4 Explant cooling (exposure to cryogen) and rewarming

Cooling of explants to cryogenic temperatures can be achieved using a variety of methods, as described in Chapter 1. In the present study explants were cooled using nitrogen slush (-210°C), given that this method has been found to be the best in terms of promoting seedling survival in recalcitrant-seeded zygotic explants after rewarming (Berjak & Pammenter, 2013b) and has been used to successfully cryopreserve explants from a number of recalcitrant-seeded species (Berjak *et al*., 1999b; Wesley-Smith *et al*., 2001; Berjak *et al*., 2011; Hajari *et al*., 2011; Sershen *et al*., 2012a; b).

Explants were measured for extracellular $^{\cdot}O_2^-$ and H_2O_2 production, total aqueous antioxidant capacity, respiratory activity and viability in control explants and in those treated with exposure to a combination of antioxidant soaking, partial dehydration, cooling and rewarming (soaking+FD+C+RW). Treated explants were exposed to immersion in 1% AsA+CW for 5 min in *L. sinensis* and 30 min for the remaining species, flash drying for selected drying intervals (species specific), ultra-rapid cooling in nitrogen slush and rewarming in 1%AsA+CW held at 42°C for 2 min and rehydrated in the same solution at 25°C for 30 min.

3.4.1 Extracellular ROS production in zygotic explants with and without the combination of soaking, rapid dehydration, cooling and rewarming

Superoxide production increased significantly in soaked+FD+C+RW explants of *L. sinensis* (by 32%) but decreased significantly (by 49%) in similarly treated explants of *T. dregeana* compared with their controls (Fig. 3.37; Table 3.22). Superoxide production was observed to marginally increase in soaked+FD+C+RW explants of *S. gerrardii* (by 18%) and decrease in similarly treated explants of *Q. robur* (by 15%). Viability declined by 60, 30 and 40% in soaked+FD+C+RW explants of *L. sinensis*, *S. gerrardii* and *Q. robur* relative to untreated explants. No viability was recorded in similarly cryopreserved *T. dregeana* explants.

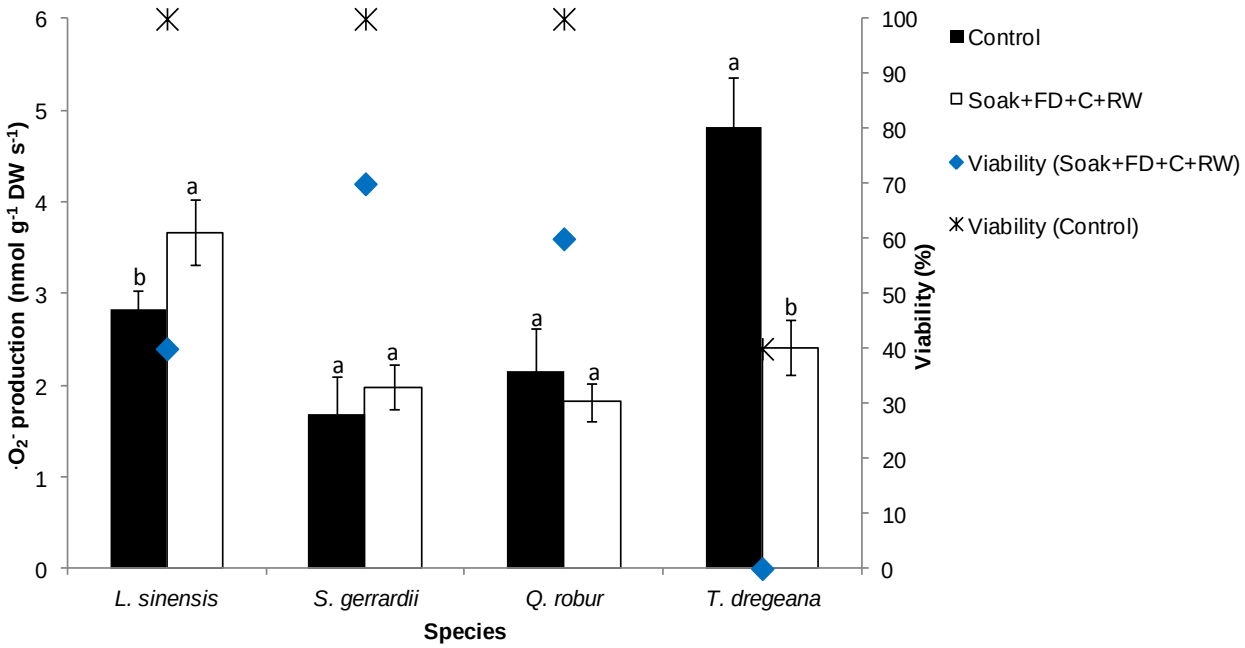

Fig. 3.32: Extracellular $\cdot O_2^-$ production and viability for excised explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to the combination of exogenous antioxidant soaking, flash drying, cooling and rewarming. Explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* were flash dried for 30, 20, 240 and 120 min respectively, prior to cryogen exposure. Viability indicates seedling production for *S. gerrardii* and *Q. robur* explants, and root production with callus development at the shoot apex for *L. sinensis*. Values represent mean±SD (n=6 for $\cdot O_2^-$ and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.18: $\cdot O_2^-$ production in treated (soaked+flash dried+cooled+rewarmed) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative $\cdot O_2^-$ production (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	32	18	-15	-49

Hydrogen peroxide production increased significantly in soaked+FD+C+RW explants of *L. sinensis* and *S. gerrardii* by 234 and 74% respectively, in comparison with their controls (Fig. 3.33; Table 3.19). There was a significant decrease in this ROS in similarly treated explants of *Q. robur* and *T. dregeana* (by 43 and 93%, respectively) in comparison with their controls (Fig. 3.33; Table 3.19).

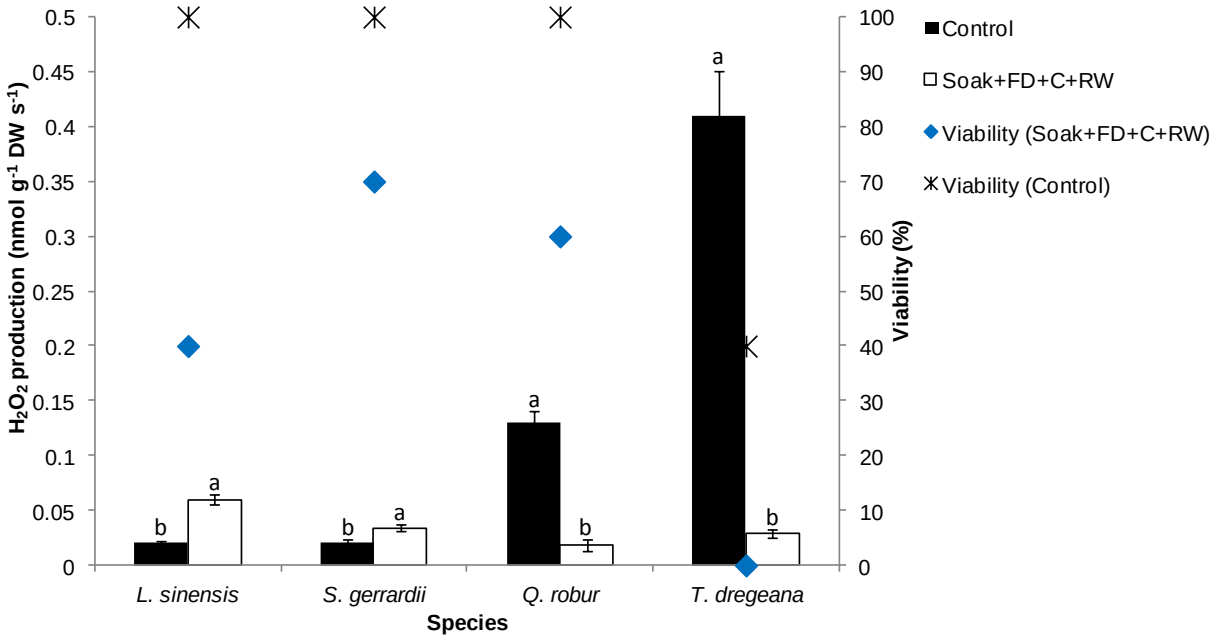

Fig. 3.33: Extracellular H_2O_2 production and viability for excised explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to the combination of exogenous antioxidant soaking, flash drying, cooling and rewarming. Explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* were flash dried for 30, 20, 240 and 120 min respectively, prior to cryogen exposure. Viability indicates seedling production for *S. gerrardii* and *Q. robur* explants, and root production with callus development at the shoot apex for *L. sinensis*. Values represent mean±SD (n=6 for H_2O_2 and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.19: H_2O_2 production in treated (soaked+flash dried+cooled+rewarmed) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative H_2O_2 production (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	234	74	-43	-93

3.4.2 Total aqueous antioxidant capacity and viability in zygotic explants with and out the combination of soaking, rapid dehydration, cooling and rewarming

There was a significant decrease in antioxidant capacity in soaked+FD+C+RW explants in all species in comparison with their respective controls (Fig. 3.34). This decrease was measured

as a 44, 13, 58 and 83% decline in antioxidant capacity compared with their controls in *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana*, respectively (Table 3.20).

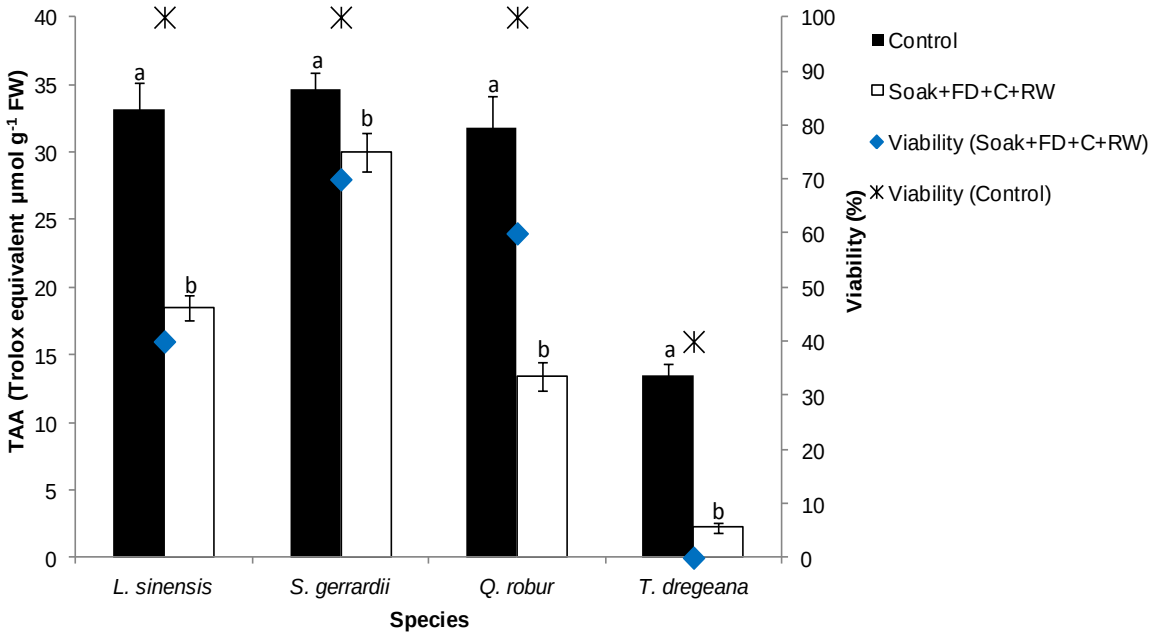

Fig. 3.34: Total aqueous antioxidant capacity and viability for excised explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to the combination of exogenous antioxidant soaking, flash drying, cooling and rewarming. Explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* were flash dried for 30, 20, 240 and 120 min respectively, prior to cryogen exposure. Viability indicates seedling production for *S. gerrardii* and *Q. robur* explants, and root production with callus development at the shoot apex for *L. sinensis*. Values represent mean±SD (n=8 for TAA and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.20: TAA capacity in treated (soaked+flash dried+cooled+rewarmed) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative TAA capacity (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	-44	-13	-58	-83

3.4.3 Extracellular respiratory activity and viability in zygotic explants with and without the combination of soaking, rapid dehydration, cooling and rewarming

Respiratory activity decreased significantly in soaked+FD+C+RW explants in all species in comparison with the controls (Fig. 3.35). This decrease was measured as a 26, 60, 56 and 89% decline in respiratory activity in soaked+FD+C+RW explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana*, respectively (Table 3.21).

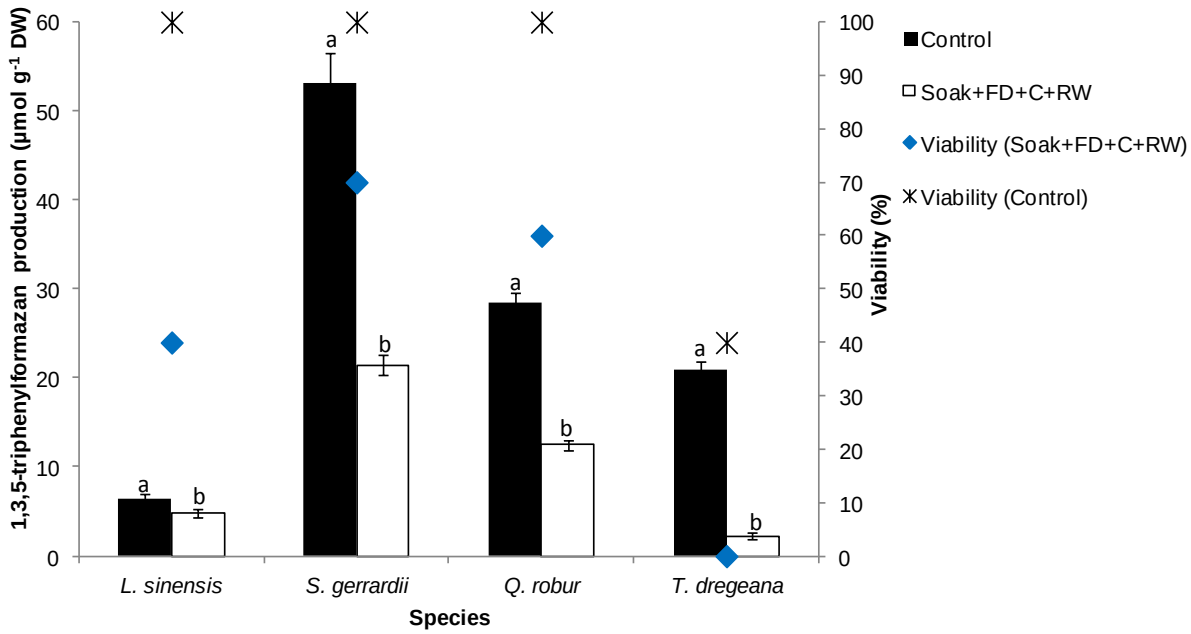

Fig. 3.35: Extracellular respiratory activity and viability for excised explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* with (Treated) and without (Control) exposure to the combination of exogenous antioxidant soaking, flash drying, cooling and rewarming. Explants of *L. sinensis*, *S. gerrardii*, *Q. robur* and *T. dregeana* were flash dried for 30, 20, 240 and 120 min respectively, prior to cryogen exposure. Viability indicates seedling production for *S. gerrardii* and *Q. robur* explants, and root production with callus development at the shoot apex for *L. sinensis*. Values represent mean±SD (n=8 for TTZ and n=20 for viability). Bars labelled with different letters are significantly different when compared between treatments within species ($p<0.05$, Independent sample t-test). Viabilities for control (crosses) and treated (diamonds) explants are also shown for each species.

Table 3.21: Respiratory activity in treated (soaked+flash dried+cooled+rewarmed) zygotic explants of the four species investigated, expressed as a % of the control.

	Relative respiratory activity (%)			
	L. sinensis	***S. gerrardii***	***Q. robur***	***T. dregeana***
	-26	-60	-56	-89

3.4.4 Effects of the combination of exogenous application of antioxidants, rapid dehydration and subsequent cooling and rewarming on shoot meristem ultrastructure of zygotic explants

The following micrographs (Figs 3.36-3.39) illustrate the shoot meristematic region of *T. dregeana, Q. robur, S. gerrardii and L. sinensis* explants exposed to the combination of soaking, flash drying, cooling and rewarming (referred to a cryopreserved explants henceforth).

Cryopreserved explants of all four species showed a decline in viability relative to untreated, soaked and soaked+FD explants. Cryopreserved *T. dregeana* explants, that were soaked and flash dried for 120 min (WC: 0.42±0.01 g g^{-1}; Fig. 3.28a & b), and cooled and rewarmed (Fig. 3.36a & b) thereafter, showed no viability retention. Ultrastructure in shoot meristems of these explants was consistent with complete derangement of cellular architecture (Fig. 3.36a & b). Complete detachment of the plasmamembrane (pm) from the cell wall was visible (Fig. 3.36b). No visible organelles were noted (Fig. 3.36a & b) except for the presence of a few vacuoles (V) within the cytoplasm (C) (Fig. 3.36b). The deteriorated ultrastructure visible in cryopreserved explants (Fig. 3.36a & b) are in complete contrast to the ultrastructural integrity observed in shoot meristems of soaked+FD (Fig. 3.28a & b) and untreated and soaked explants (Fig. 3.8a-d), where numerous organelles typical of active metabolism were present.

Q. robur explants that were soaked and flash dried for 240 min (WC: 0.37±0.01 g g^{-1}; Fig. 3.29a & b), and subsequently cooled and rewarmed (Fig. 3.37a & b) showed 60% viability retention (seedling production). Starch bodies (S) and numerous mitochondria (M) with well defined cristae were visible in cryopreserved explants (Fig. 3.37a & b); these were also noted in explants exposed to soaking+FD (Fig. 3.29a & b). Vacuoles (V) often displayed inclusions (Fig. 3.37a & b), indicative of autophagic activity. Starch bodies (S) (Fig. 3.37a), vesicles (indicated by arrows; Fig. 3.37b) and numerous, dense mitochondria were also observed in cryopreserved explants (Fig. 3.37a & b). The structured organisation observed in flash dried explants of this species (Fig. 3.29a & b) was maintained in cryopreserved explants (Fig. 3.37a & b).

Explants of *S. gerrardii* that were soaked and flash dried for 20 min (WC: 0.38±0.01 g g^{-1}; Fig. 3.30) and subsequently cooled and rewarmed (Fig. 3.38), showed 70% viability retention (seedling production). A high degree of vacuolation (V) with vacuolar inclusions was observed amongst the frequently dispersed lipid bodies (L) in cryopreserved explants (Fig. 3.38). This was similar to vacuolation observed in flash dried explants (Fig. 3.30). The ultrastructure of the shoot meristematic region showed structured organisation in flash dried explants (Fig. 3.30), which was maintained after cryopreservation (Fig. 3.38).

Explants of *L. sinensis* that were soaked and vacuum flash dried for 30 min (WC: 0.35±0.005 g g^{-1}; Fig. 3.31 a & b) and subsequently cooled and rewarmed (Fig. 3.39a & b) showed 40% root development and organised shoot growth, which can be attributed to the retention of cellular organisation of the shoot meristematic tissue. Despite retarded shoot development, shoot meristems of cryopreserved explants exhibited numerous mitochondria (M) (Fig. 3.39b) with dense matrices, lipid bodies (L) (Fig. 3.39b) and well developed nuclei (N) (Fig. 3.39a), indicative of metabolically active tissue. However, some cells exhibited plasmolysis (indicated by arrows; Fig. 3.39b) and electron translucent patches within mitochondria (indicated by asterisk; Fig 3.39b) suggesting that some damage/stress was incurred during cryopreservation of these explants. This was also supported by poorly developed mitochondria (Fig. 3.39a & b) compared with untreated (Fig. 3.11a & b), soaked (Fig. 3.11d) and flash dried explants (Fig. 3.31a & b) which may have contributed to the post-cooling decline in viability (40%).

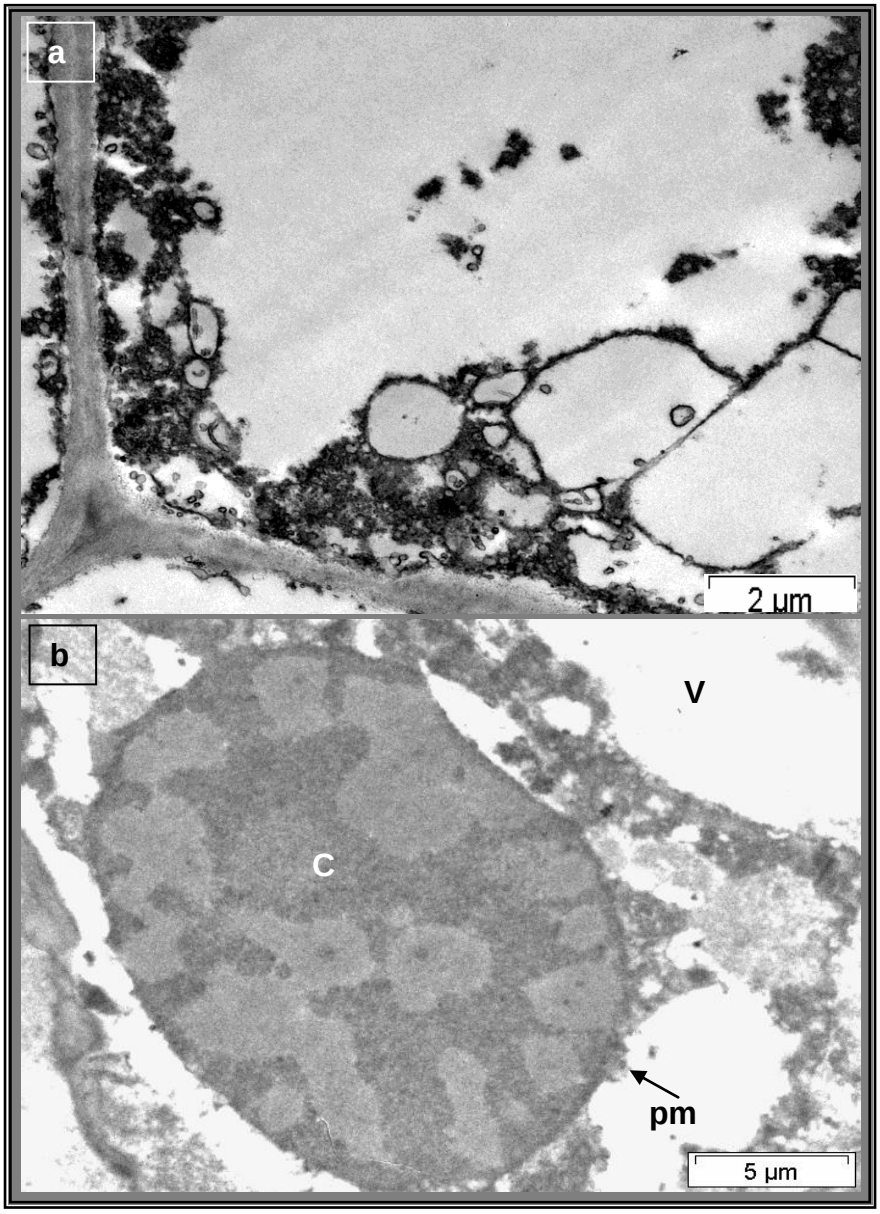

Fig. 3.36a & b: The ultrastructure of shoot meristems excised from soaked (1% AsA+CW solution for 30 min), flash dried (120 min), cooled (in nitrogen slush) and rewarmed zygotic *T. dregeana* explants. There were no discernible organelles in the cytoplasm (Figs 3.36a & b). Plasmolysis is evident in Fig. 3.36b (indicated by an arrow).

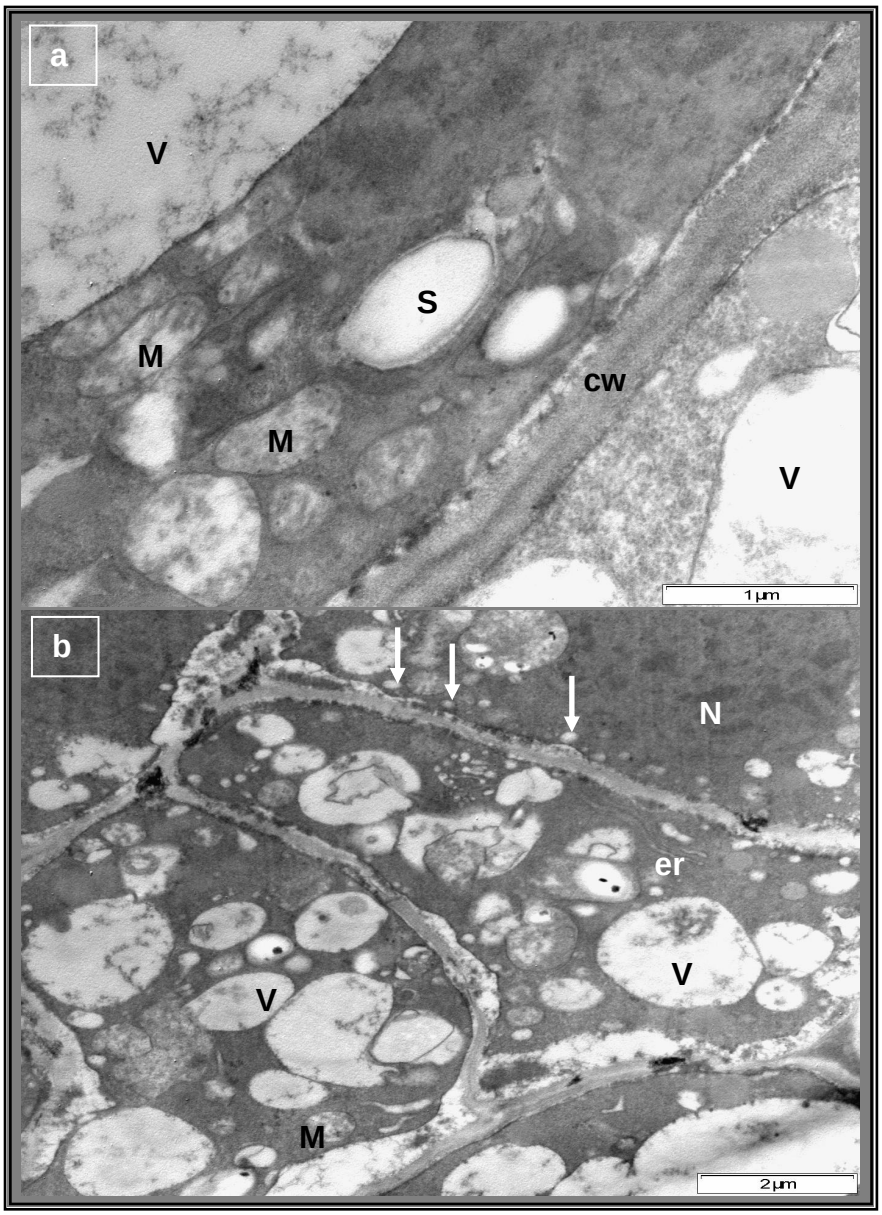

Fig. 3.37a & b: The ultrastructure of shoot meristems excised from soaked (1% AsA+CW solution for 30 min), flash dried (240 min), cooled (in nitrogen slush) and rewarmed zygotic *Q. robur* explants. Numerous mitochondria (M) (Figs 3.37a & b) were noted throughout the cytomatrix. The cytoplasm contained regular shaped, frequently dispersed vacuoles (V) (Fig. 3.37b). The nuclei (N) (Fig. 3.37b) were well developed with patches of heterochromatin evident. Figure 3.37b shows short profiles of endoplasmic reticulum (er) and vesicle activity (indicated by arrows).

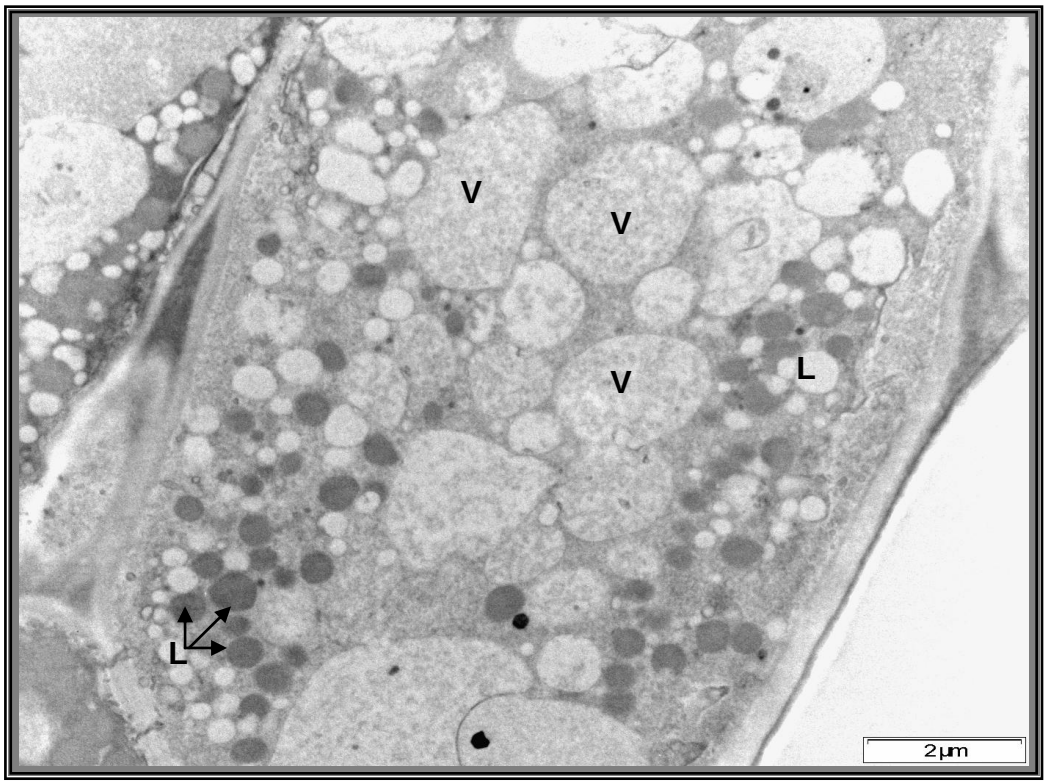

Fig. 3.38: The ultrastructure of shoot meristems excised from soaked (1% AsA+CW solution for 30 min), flash dried (20 min), cooled (in nitrogen slush) and rewarmed zygotic *S. gerrardii* explants. The ultrastructure showed structural organisation with numerous, large vacuoles (V) and compacted, discrete lipid bodies (L).

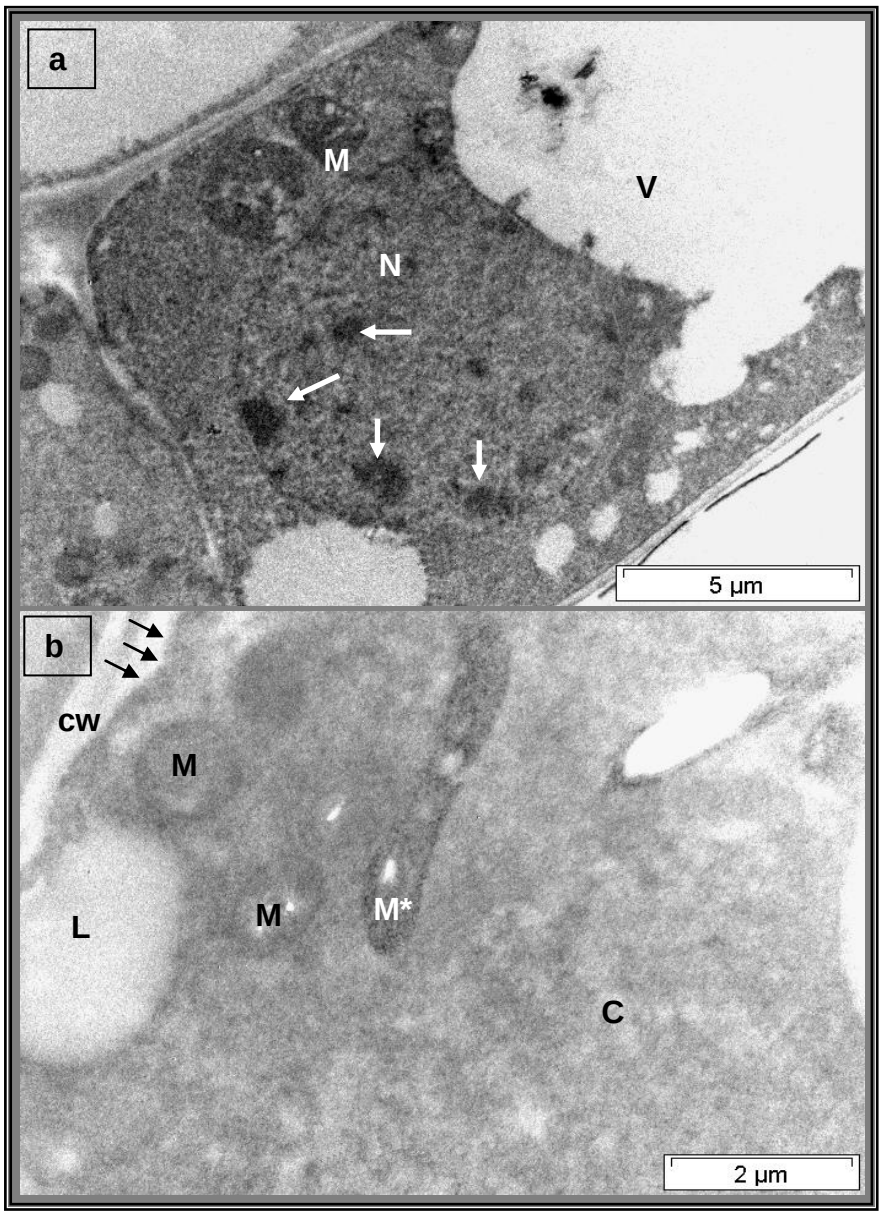

Fig. 3.39a & b: The ultrastructure of shoot meristems excised from soaked (1% AsA+CW solution for 5 min), flash dried (30 min), cooled (in nitrogen slush) and rewarmed zygotic *L. sinensis* explants. Figure 3.39a shows well developed nuclei (N) with prominent patches of heterochromatin (indicated by arrows in Fig. 3.39a). Single, large vacuoles (V) were present in the cytoplasm (Fig. 3.39a). Mitochondria (M) were present throughout the cytoplasm (Figs 3.39a & b), however, some electron translucent mitochondria were noted (M* in Fig. 3.39b). Plasmolysis was apparent (indicated by arrows in Fig. 3.39b).

3.5 Assessment of relationships amongst physiological characteristics, stress biomarkers and explant viability following the various procedural steps involved in cryopreservation

3.5.1 Explant morphological and physical characteristics after excision and soaking and the relationship with post-cryo viability

Table 3.22 below shows post-soaking WC and morphological characteristics of explants of each species and their respective post-cryo viabilities. Explant fresh mass, shedding WC (see Table 3.1. for data on both these parameters) and post-soaking WC (Table 3.22) did not show significant correlations with post-cryo viability. However, explants of *T. dregeana* possessed significantly higher shedding WC (Table 3.1) and post soak WC (Table 3.22) than explants of the remaining species and were the only ones to exhibit no post-cryo viability. Explants of *S. gerrardii* possessed significantly lower fresh mass (Table 3.1) than explants of the remaining species and exhibited the highest post-cryo viability (Table 3.22).

Anatomical differences in explant types (Table 3.1; Fig. 3.1) observed in the connections between meristematic regions and cotyledons, and noted in the cotyledonary/endospermic composition of the explant had an influence on post-cooling viability.

Explants of *T. dregeana* were the only species to show an integrated connection between shoot meristem and cotyledons (Fig. 3.1a). During the final excision of explants of this species, portions of the cotyledons were physically separated from the shoot meristematic region (Fig. 3.1a; F) which may create lesions in the embryo. The shoot meristem in explants of this species was the closest in proximity to a wound site created upon excision, compared with explants of the remaining species. Anatomical positioning of the shoot meristem and cotyledons observed in explants of *T. dregeana* were completely dissimilar from the structure of explants in any of the other species, and as mentioned above, were the only explants to exhibit no post-cryo survival.

Of the remaining species, explants of *L. sinensis* exhibited the next lowest viability, where cryopreserved explants produced 40% roots and callus at shoot apices. Anatomically, these explants were the smallest in length but had a significantly higher fresh mass (Table 3.1) compared with explants of the other species. Root and shoot meristems of the embryo were completely concealed by endospermic tissue (Fig. 3.1c; P). While these structures remained

protected from physical injury during cryopreservation, the thick endosperm which was responsible for the high mass of the explant may have influenced the cooling rate at which cells froze. The anatomy of this explant was completely dissimilar from the explant selected from the other endospermic seed species investigated in this study (*S. gerrardii*), which had the highest post-cryo survival (Table 3.22).

Cryopreserved explants of *Q. robur* exhibited the second highest viability (60% seedling survival) and had exposed root meristems with partially concealed shoot meristems within cotyledons (Fig. 3.1d; F). Explants of this species were the only ones to be excised without using incisions to prepare the final explant (Fig. 3.1d; F). Detaching the loosely connected explant from the seed was minimally injurious, with wound sites being the smallest compared with explants across species. The positioning of the shoot meristem in *Q. robur* explants was in complete contrast to those of the non-endopsermic species in this study (*T. dregeana*). While both were measured to be comparable in length (4-6 mm) and not significantly different in fresh mass, explants of *Q. robur* with partially concealed shoot meristems and minimal excision injury showed high post-cryo viability (60%).

Explants of *S. gerrardii* possessed papery thin cotyledons, which remained loosely attached to conceal the shoot meristem of the embryo (Fig. 3.1b; P), making their anatomy unique across explants of all investigated species. The incision point made during final excision of the explant was halfway down the length of the cotyledons and therefore not in close proximity to either the shoot or root meristems (Fig. 3.1b; F). The anatomy and physiology of explants of this species is in contrast with explants of the other endospermic species in this study (*L. sinensis*). Explants of *S. gerrardii*, exhibiting 70% post-cryo survival, were significantly lighter in fresh mass compared with those of *L. sinensis* which exhibited incomplete post-cryo survival (Table 3.1). The explants of *S. gerrardii* were also completely free of endospermic tissue while those of *L. sinensis* exhibited complete attachment of the endosperm to the embryo.

Endospermic seeded species showed variable post-cryo survival, with *S. gerrardii* exhibiting 70% seedling production and *L. sinensis* exhibiting 40% incomplete survival. Anatomical and physical observations of explants of these species indicate that fresh mass and tissue composition of the explant influence post-cryo survival. Both non-endopsermic seeded

species exhibited differences in survival as well, with *Q. robur* showing 60% seedling production and *T. dregeana* exhibiting no survival at all. Explants of both these species have similar mass and lengths but differ in the concealment of the shoot meristem and the frequency of wound sites on the explant. These physical characteristics influence post-cryo survival.

Table 3.22: Physiological characteristics of soaked explants across species.

Species	Explant WC after soak (g g^{-1})	Explant morphology cotyledon/endosperm	Post-cryo viability (%)
T. dregeana	2.18 ± 0.30^{a}	Root meristem exposed; shoot meristem physically separated from cotyledons	0
L. sinensis	1.36 ± 0.02^{b}	Root and shoot meristem completely concealed within endosperm	40
Q. robur	1.13 ± 0.02^{b}	Root meristem exposed; shoot meristem partially concealed within thick cotyledons	60
S. gerrardii	1.32 ± 0.30^{b}	Root meristem exposed; shoot meristem concealed by remnants of papery thin cotyledons	70

n=20 for post-cryo viability and n=10 for explant WC after soaking. Values labelled with different letters are significantly different across species ($p<0.05$, ANOVA).

3.5.2 Explant drying characteristics and the relationship with post-cryo viability

The following section summarises some of the physical drying characteristics of the zygotic explants investigated here in relation to their post-cryo viabilities.

Explants of the four species differed in terms of how rapidly water was lost during their respective drying periods, which also differed considerably (20-240 min) across species (Table 3.23). Explants of *T. dregeana* had the highest post-soak WC and those of *Q. robur* had the lowest. Water contents achieved after rapid drying were significantly highest in explants of *T. dregeana* and comparable across the remaining species. The significantly higher post-soak and post-drying WC in *T. dregeana* explants corresponded to the lowest (and in this case, zero) post-cryo viability. Despite the differences described above, no significant correlation was found between drying rate of explants and post-cryo viability ($r = 0.400$; $p>0.05$, Spearman correlation), explant target WC and post-cryo viability

(r = -0.200; p>0.05, Spearman correlation) or explant post-soak WC and post-cryo viability (r = -0.800; p>0.05, Spearman correlation). The amount of water lost to reach target WC from explants of each species was also variable, but explants of all species could be dried to WC within the range of 0.35-0.41 g g^{-1}. The amount of water removed from explants to reach target water content was significantly negatively correlated with explant post-cryo viability (r = -0.959; p<0.05, Spearman correlation) when data for all species were pooled for analysis (Fig. 3.40).

Table 3.23: Drying characteristics and post-cryo viability of *T. dregeana*, *Q. robur*, *S. gerrardii* and *L. sinensis* explants.

Species	WC after soak (g g^{-1})	Target WC for cryogen immersion (g g^{-1})	Drying time (min) required to reach target WC	Water removed to reach target WC (g g^{-1})	Water removed to reach target WC (%)	*Drying rate(% H_2O loss min^{-1}) calculated for target WC	**Drying rate (g H_2O g^{-1} DM min^{-1}) calculated for target WC	**Viability post-cryo (%)
T. dregeana	2.18±0.30[a]	0.41±0.01[a]	120	1.76	81	0.67	0.014	0
L. sinensis	1.36±0.02[b]	0.35±0.005[c]	30	1.01	74	2.46	0.033	40
Q. robur	1.13±0.02[c]	0.37±0.01[bc]	240	0.76	67	0.28	0.003	60
S. gerrardii	1.32±0.30[b]	0.38±0.01[b]	20	0.94	71	3.56	0.047	70

n=10 for water content (WC); water loss and drying rates which were calculated for selected drying periods. Post-cryo viability (n=20) is shown for each species. Water content values followed by different letters are significantly different when compared across species (p<0.05, ANOVA).

*Drying rates (% H_2O loss min^{-1}) were calculated for zygotic explants by expressing the decrease in WC (WC after soaking – WC after drying) as a percentage of the WC after soaking over the drying period required to reach the target WC.

**Drying rates (g H_2O g^{-1} DM min^{-1}) were calculated for zygotic explants by expressing water loss (g) over the drying period required to reach the target WC.

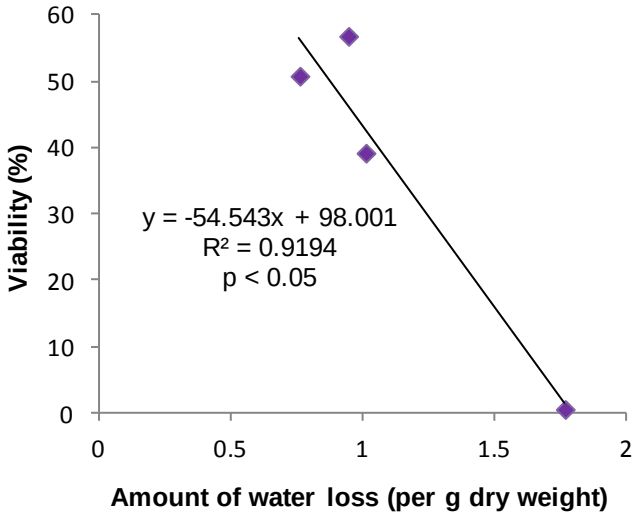

Fig. 3.40: Negative linear correlation between post-cryo viability and water loss in zygotic explants after partial dehydration to target WC for cryopreservation ($r = -0.959$; $p<0.05$, Pearson correlation).

3.5.3 Explant redox metabolism

Results presented thus far have shown that cryogenic procedures differentially influence the selected stress biomarkers and viability across species. The interaction between each biomarker and viability has been described for each procedural step. These results are important in optimising cryopreservation protocols for each of these species and address some the first major aims of this study: to elucidate, compare and contrast some of the stresses associated with the various procedural steps involved in cryopreservation across four recalcitrant-seeded species. The results presented thus far also partly address the second aim which was to investigate approaches towards ameliorating some of these stresses, particularly those associated with uncontrolled ROS production. However, to fully address this second aim it is necessary to draw comparisons across procedural steps, within species and within procedural steps, across species; also, to characterise the relationships, if any, amongst stress biomarkers such as ROS, TAA, respiratory activity and viability (Figs 3.41-3.44). The results presented in this sub-section are the product of these comparative and correlative analyses.

Importantly, these results also feature data on the effect of the different procedural steps on an oxidative stress ratio, viz., TAA:ROS, and viability (Figs 3.45-3.48). This ratio involved expressing the total aqueous antioxidants during each procedural step as a ratio of the

corresponding cumulative ROS level ($^{\cdot}O_2^-$ + H_2O_2). This balance between ROS and antioxidants, or lack thereof, is used as an indicator of oxidative stress.

3.5.3.1. Comparisons of explant O_2^- production across procedural steps of cryopreservation within each species

Figure 3.41 shows $^{\cdot}O_2^-$ production in explants across all species to significantly decrease from levels measured in the control compared with those measured after soaking. Furthermore, across all species, $^{\cdot}O_2^-$ levels following soaking were the lowest across all procedural steps. The highest viabilities across all species were measured after soaking and this declined progressively with each procedural step, except in *L. sinensis* explants which retained 100% viability after soaking+FD (Fig. 3.41b).

When all the steps applied for cryopreservation of explants are considered, $^{\cdot}O_2^-$ production exhibited a significant increase with a concomitant decrease in explant viability with each procedural step in *T. dregeana* (Fig. 3.41a) and *Q. robur* (Fig. 3.41c). In explants of *L. sinensis*, no significant change in $^{\cdot}O_2^-$ production was observed in soaked and soaked+FD explants with 100% viability being maintained after both these steps (Fig. 3.41b). However, $^{\cdot}O_2^-$ production was significantly higher in soaked+FD+C+RW explants compared with soaked+FD explants in this species, with a corresponding decline in viability (60% decline relative to 100%) in cryopreserved explants (Fig. 3.41b). In explants of *S. gerrardii*, $^{\cdot}O_2^-$ production significantly increased in soaked+FD explants compared with levels in soaked explants, with a corresponding decline in viability of 25% (Fig. 3.41d). In soaked+FD+C+RW explants, $^{\cdot}O_2^-$ production did not change significantly from that measured in soaked+FD explants and a 5% decline in viability in cryopreserved explants was noted in this species (Fig. 3.41d).

No significant correlations were measured between $^{\cdot}O_2^-$ production and viability when data for all procedural steps were pooled for analysis for each species (Fig. 3.41). However, a significant correlation was measured between $^{\cdot}O_2^-$ production and viability when data for all procedural steps were pooled for analysis across species (Table 3.24).

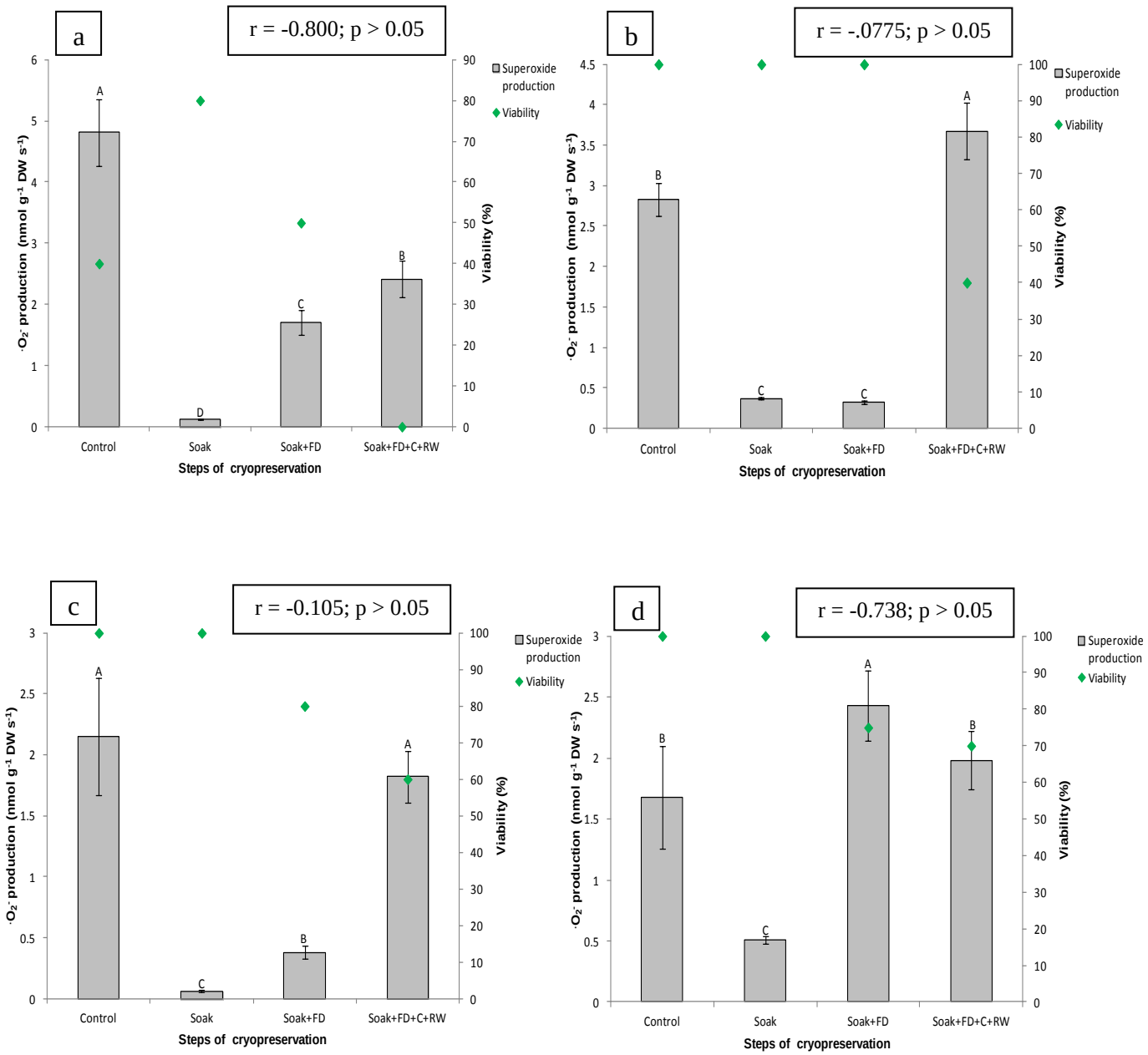

Fig. 3.41: Extracellular $\cdot O_2^-$ production and viability in control and treated zygotic explants of *T. dregeana* (a), *L. sinensis* (b), *Q. robur* (c) and *S. gerrardii* (d). Treatments correspond to the various procedural steps applied during cryopreservation: soaking (soak), a combination of soaking and flash drying (soak+FD), and soaking, flash drying, cooling and rewarming (soak+FD+C+RW). Values represent mean±SD for levels of $\cdot O_2^-$ (n=6). Bars labelled with different letters are significantly different when compared across treatments ($p<0.05$, ANOVA). *r*- and p-values given for each species represent the results of correlation analyses between $\cdot O_2^-$ production and viability (Spearman correlation).Viabilities (diamonds) are also shown for each step (n=20).

3.5.3.2 Comparisons of explant H_2O_2 production across procedural steps of cryopreservation within each species

Figure 3.42 shows the effect of procedural steps of cryopreservation on H_2O_2 production and viability in explants across species. In *T. dregeana* and *Q. robur* explants, H_2O_2 production was observed to significantly decline after procedural steps relative to the control. In *L. sinensis* explants, H_2O_2 production significantly increased after steps applied relative to the control, which was observed to be the trend in *S. gerrardii* explants as well. However, in *S. gerrardii* explants there was no significant change in H_2O_2 production in cryopreserved explants relative to the control. The trend here was observed to be different from that seen in $^{\cdot}O_2^-$ production (Fig. 3.41), where $^{\cdot}O_2^-$ levels decreased after soaking in explants of all species except *S. gerrardii* (Fig. 3.41d).

When all the steps applied for cryopreservation of explants are considered, a significant decline in H_2O_2 production in soaked+FD and soaked+FD+C+RW explants compared with those that were soaked only in *T. dregeana* (Fig. 3.42a), *L. sinensis* (Fig. 3.42b) and *Q. robur* (Fig. 3.42c) was observed. Hydrogen peroxide production did not differ significantly between soaked+FD and soaked+FD+C+RW explants in any of these species. Explants of *S. gerrardii* showed a significant increase in H_2O_2 production in soaked+FD explants compared with soaked explants, however, these levels decreased significantly relative to soaked+FD explants upon cooling and rewarming (Fig. 3.42d). Viabilities consistently declined with progressive steps regardless of increases or decreases in H_2O_2 production.

No significant correlations were measured between H_2O_2 production and viability when data for all procedural steps were pooled for analysis for each species (Fig. 3.42) or across species (Table 3.24).

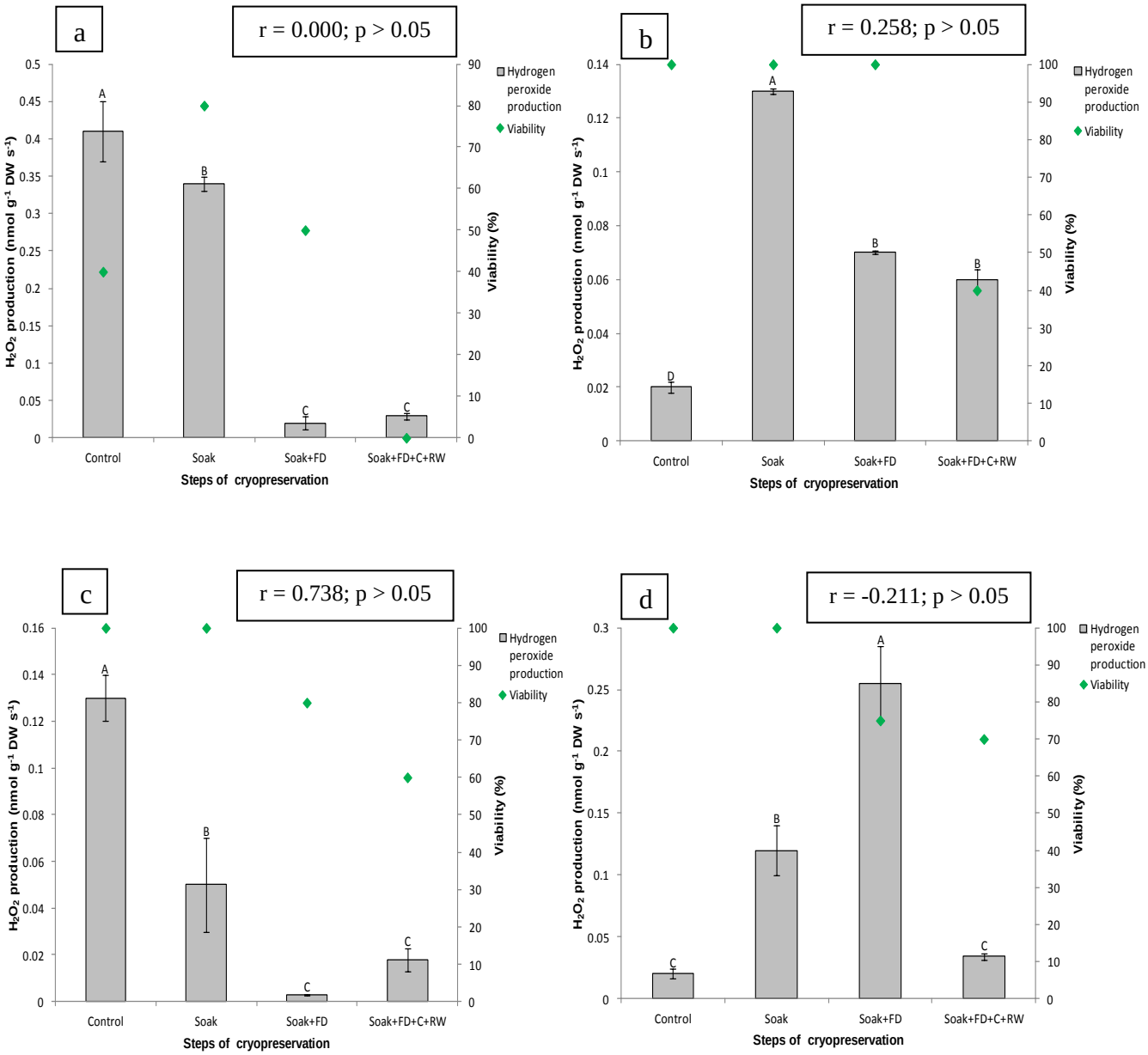

Fig. 3.42: Extracellular H_2O_2 production and viability in control and treated zygotic explants of *T. dregeana* (a), *L. sinensis* (b), *Q. robur* (c) and *S. gerrardii* (d). Treatments correspond to the various procedural steps applied during cryopreservation: soaking (soak), a combination of soaking and flash drying (soak+FD), and soaking, flash drying, cooling and rewarming (soak+FD+C+RW). Values represent mean±SD for levels of H_2O_2 (n=6). Bars labelled with different letters are significantly different when compared across treatments ($p<0.05$, ANOVA). *r*- and p-values given for each species represent the results of correlation analyses between H_2O_2 production and viability (Spearman correlation).Viabilities (diamonds) are also shown for each step (n=20).

3.5.3.3 Comparisons of explant TAA capacity across procedural steps of cryopreservation within each species

Figure 3.43 shows the effect of procedural steps on TAA capacity and viability in explants across species. In explants of *T. dregeana* (Fig. 3.43a) and *Q. robur* (Fig. 3.43c), TAA levels decreased significantly in all steps applied during cryopreservation compared with levels in control explants. Viability also decreased in these explants in all steps subsequent to soaking. In explants of *T. dregeana* (Fig. 3.43a), TAA capacity showed no change during all procedural steps applied. This corresponded to the lowest viability retention in *T. dregeana* explants across species during all procedural steps (Fig. 3.43a).

When comparing TAA capacity in explants of *L. sinensis* (Fig. 3.43b) and *Q. robur* (Fig. 3.43c) during the steps applied for cryopreservation, TAA levels progressively decreased during cryopreservation of *L. sinensis* explants and increased in *Q. robur* explants. During the steps applied during cryopreservation to *Q. robur* explants, viability declined in line with TAA capacity. In explants of *L. sinensis*, viability was maintained at 100% in soaked explants and soaked+FD explants but declined after cooling, which was also the step after which TAA capacity declined significantly. In explants of *Q. robur*, the significant increase in TAA capacity in soaked+FD and soaked+FD+C+RW relative to soaked explants corresponded with high viabilities of 80 and 60%, respectively (Fig. 3.43c). This in contrast to the decline in TAA levels measured in soaked+FD+C+RW explants of *L. sinensis* which was accompanied by 40% root production and callus formation (Fig. 3.43b) after cryopreservation.

Explants of *S. gerrardii* (Fig. 3.43d) showed the significantly highest TAA levels across steps applied for cryopreservation after soak+FD and retained 75% viability. TAA levels in explants of *S. gerrardii* were equally high after cooling as those levels measured after soaking (Fig. 3.43d). In explants of *Q. robur*, TAA levels after cooling significantly increased compared with levels measured after soaking (Fig. 3.43c). Explants of both these species (Fig. 3.43c & d) were the only ones to show seedling production post-cooling (70 and 60% respectively). Cryopreserved explants of *T. dregeana* (Fig. 3.43a) showed TAA levels comparable with those after soaking, as the case in explants of *S. gerrardii* (Fig. 3.43d). However, there was no viability retained in soaked+FD+C+RW explants of *T. dregeana* (Fig. 3.43a) whereas explants of *S. gerrardii* (Fig. 3.43d) after the same treatments showed the

highest viability retention across species. In cryopreserved explants of *L. sinensis* (Fig. 3.43b), which showed only root growth, TAA levels significantly declined compared with levels after soaking and soaking+FD.

No significant correlations were measured between TAA capacity and viability when data for all procedural steps were pooled for analysis for each species (Fig. 3.43). However, a significant correlation was measured between TAA capacity and viability when data for all procedural steps were pooled for analysis across species (Table 3.24).

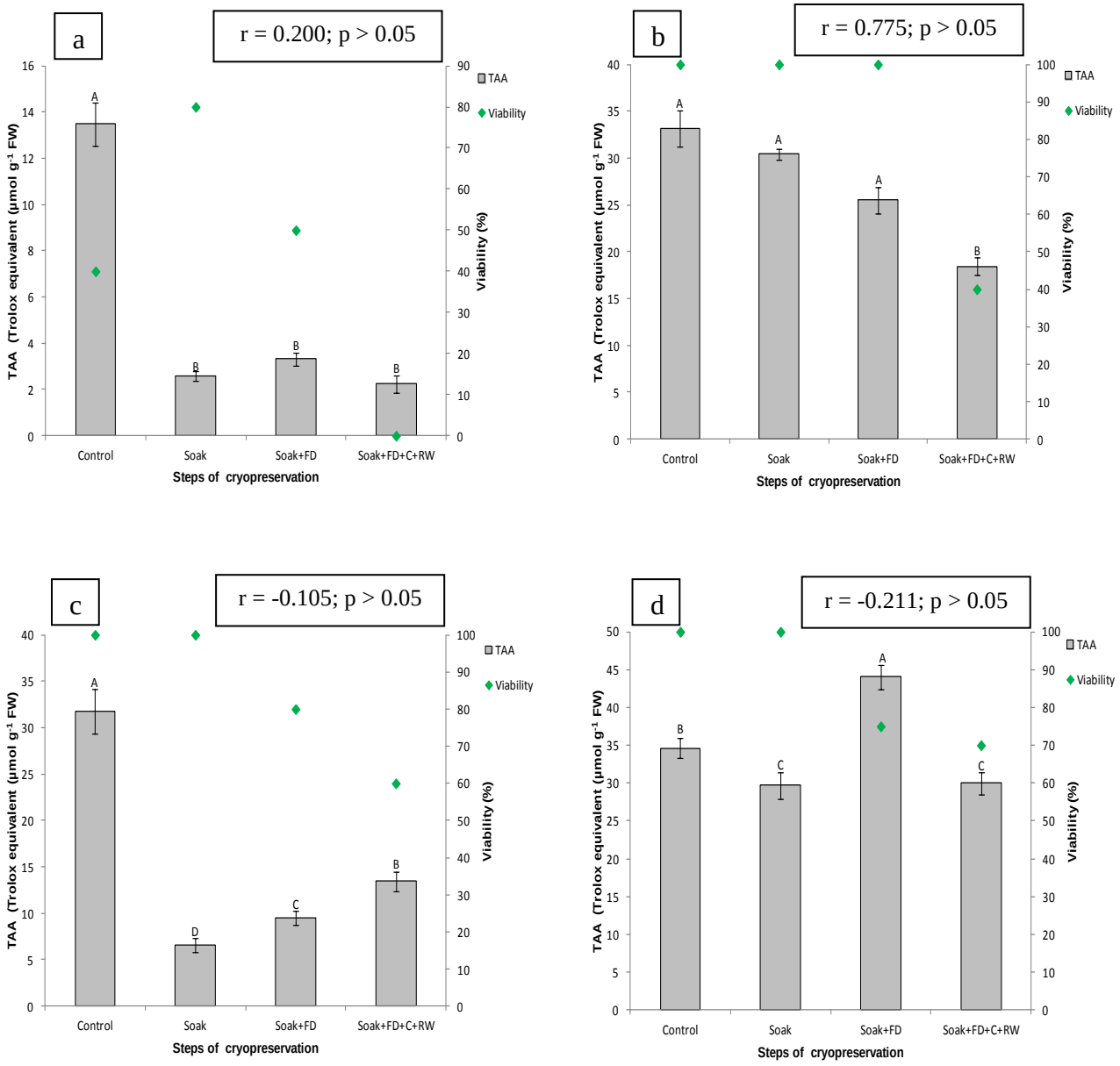

Fig. 3.43: Total aqueous antioxidant capacity and viability in control and treated zygotic explants of *T. dregeana* (a), *L. sinensis* (b), *Q. robur* (c) and *S. gerrardii* (d). Treatments correspond to the various procedural steps applied during cryopreservation: soaking (soak), a combination of soaking and flash drying (soak+FD), and soaking, flash drying, cooling and rewarming (soak+FD+C+RW). Values represent mean±SD for levels of TAA (n=8). Bars labelled with different letters are significantly different when compared across treatments ($p<0.05$, ANOVA). *r*- and p-values given for each species represent the results of correlation analyses between TAA production and viability (Spearman correlation).Viabilities (diamonds) are also shown for each step (n=20).

3.5.3.4 Comparisons of explant respiratory activity across procedural steps of cryopreservation within each species

Figure 3.44 shows the effect of procedural steps on respiratory activity and explant viability across species. When steps within each species were compared, respiratory activity was significantly highest in soaked explants of *T. dregeana* (Fig. 3.44a), control and soaked explants of *L. sinensis* (Fig. 3.44b) and control explants of *Q. robur* (Fig. 3.44c) and *S. gerrardii* (Fig. 3.44d).

When all the steps applied for cryopreservation of explants are considered, *T. dregeana* explants showed a significant decline in respiratory activity along procedural steps and a corresponding decline in viability (Fig. 3.44a). A significant decline in respiratory activity in soaked+FD explants compared with soaked explants and a significant increase thereafter in soaked+FD+C+RW explants compared with soaked+FD explants in *L. sinensis* (Fig. 3.44b) and *S. gerrardii* (Fig. 3.44d) was observed. This was the opposite to the case in *Q. robur* explants which showed a significant increase in respiratory activity in soaked+FD explants relative to soaked explants and a significant decrease in respiratory activity in soaked+FD+C+RW explants relative to soaked+FD explants (Fig. 3.44c).

No significant correlations were measured between respiratory activity and viability when data for all procedural steps were pooled for analysis for each species (Fig. 3.44) or across species (Table 3.24).

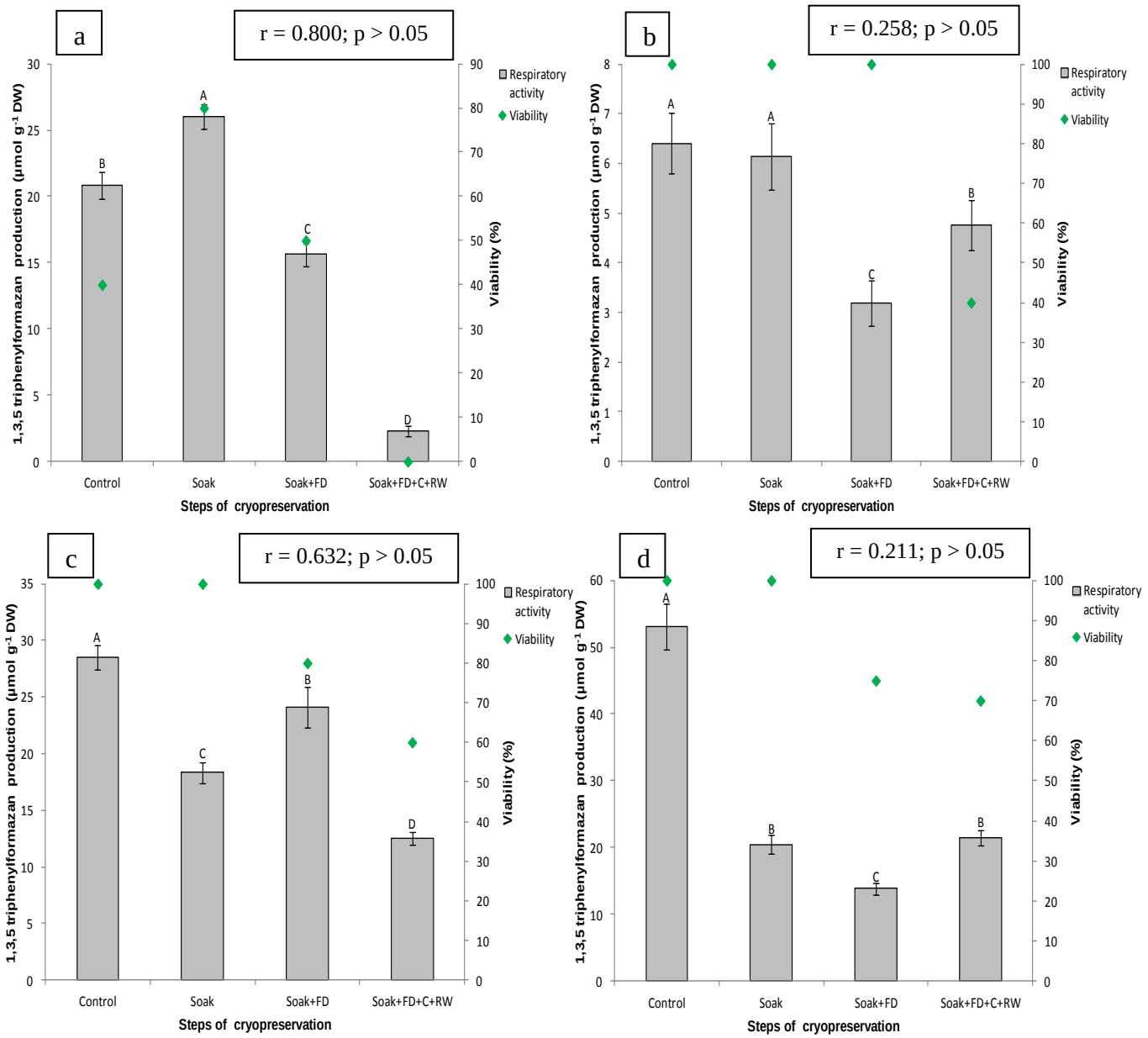

Fig. 3.44: Extracellular respiratory activity and viability in control and treated zygotic explants of *T. dregeana* (a), *L. sinensis* (b), *Q. robur* (c) and *S. gerrardii* (d). Treatments correspond to the various procedural steps applied during cryopreservation: soaking (soak), a combination of soaking and flash drying (soak+FD), and soaking, flash drying, cooling and rewarming (soak+FD+C+RW). Values represent mean±SD for levels of TTZ (n=8). Bars labelled with different letters are significantly different when compared across treatments ($p<0.05$, ANOVA). *r*- and p-values given for each species represent the results of correlation analyses between TTZ production and viability (Spearman correlation).Viabilities (diamonds) are also shown for each step (n=20).

3.5.3.5 Comparison of TAA: ROS in explants across procedural steps of cryopreservation within each species

The following graphs depict the influence of procedural steps of cryopreservation on the TAA: ROS ratio and viability for each species.

In *T. dregeana*, TAA: ROS was highest after soaking (**5.1:1**) and lowest after soaking+FD+C+RW (**0.9:1**). Values for this ratio declined progressively with each step applied during cryopreservation and this decline corresponded with declining germination capacity (Fig. 3.45) of 30% after soak+FD and 80% after soak+FD+C+RW relative to soaked explants. Notably, the ratios calculated for *T. dregeana* explants were much lower, almost by a factor close to ten, when compared with the ratios calculated for the other three species (Figs 3.46-3.48), and possessed relatively lower antioxidant capacities (2-14 µmol g^{-1} FW) than the remaining species (Fig. 3.43).

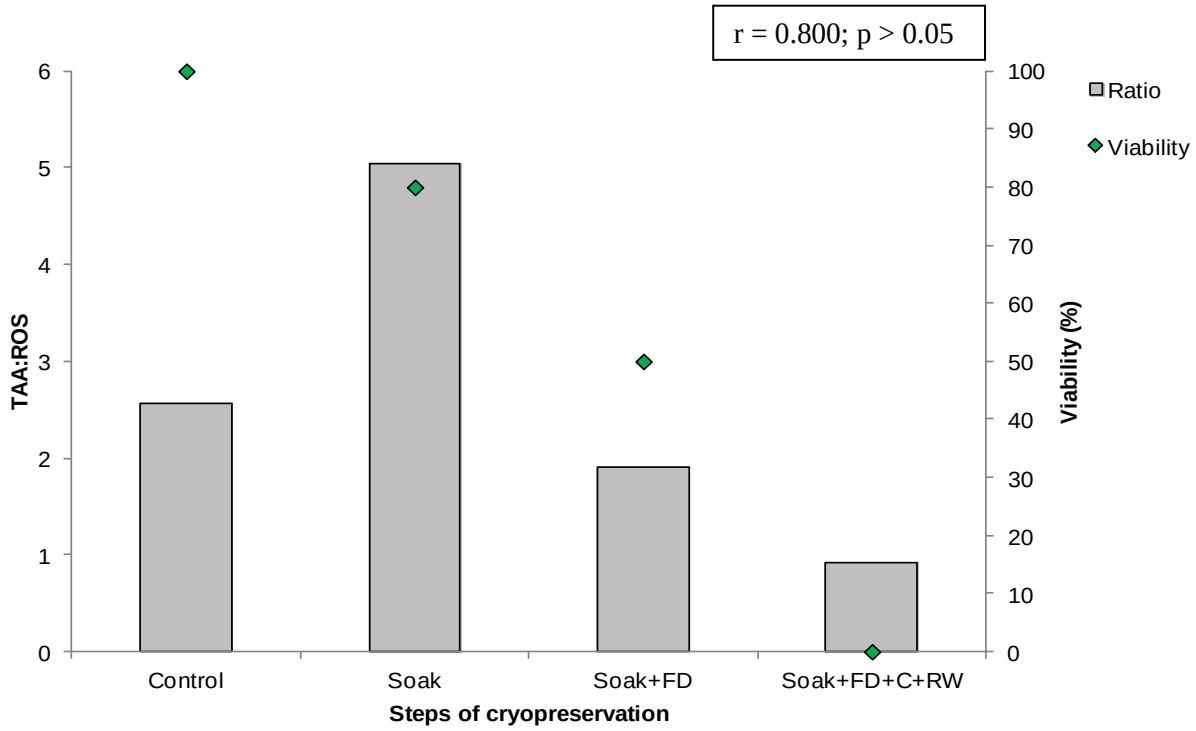

Fig. 3.45: Ratio of TAA to ROS (TAA: ROS) and the corresponding viabilities of *T. dregeana* explants after exposure to soaking (soak), and a combination of soaking and flash drying (soak+FD) and soaking, flash drying, cooling and rewarming (soak+FD+C+RW). *r*- and p-values represent the results of correlation analysis between TAA: ROS production and viability (Spearman correlation).

In *Q. robur*, TAA: ROS was highest after soaking (**59.4: 1**) and lowest after soak+FD+C+RW (**7.3: 1**). As in the case of *T. dregeana* explants (Fig. 3.45), values for this ratio declined progressively with each step applied during cryopreservation and this decline corresponded with declining germination capacity of 20% after soak+FD and 40% after soak+FD+C+RW (Fig. 3.46) relative to soaked explants. However, *Q. robur* explants possessed relative higher antioxidant capacities (9-32 µmol g^{-1} FW) than *T. dregeana* explants (Fig. 3.43).

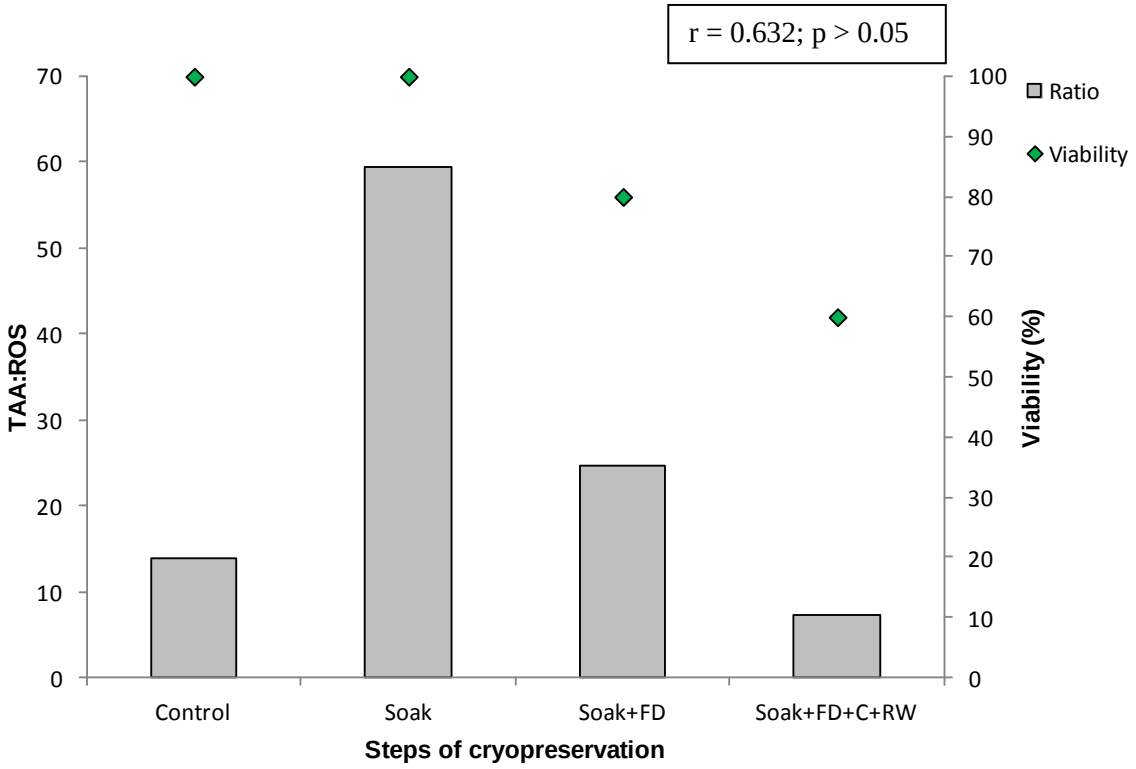

Fig. 3.46: Ratio of TAA to ROS (TAA: ROS) and the corresponding viabilities of *Q. robur* explants after exposure to soaking (soak), and a combination of soaking and flash drying (soak+FD) and soaking, flash drying, cooling and rewarming (soak+FD+C+RW). *r*- and p-values represent the results of correlation analysis between TAA: ROS production and viability (Spearman correlation).

In *S. gerrardii*, TAA: ROS was highest after soaking (46.4:1) and lowest after soak+FD+C+RW (14.8:1). Values for this ratio declined progressively with each step applied during cryopreservation and this decline corresponded with declining germination capacity after soaking+FD (by 20%) and after soaking+FD+C+RW (by 25%) relative to soaked explants (Fig. 3.47). Antioxidant capacities of *S. gerrardii* explants (30-44 µmol g^{-1} FW) were relatively higher than those across all other species (Fig. 3.43).

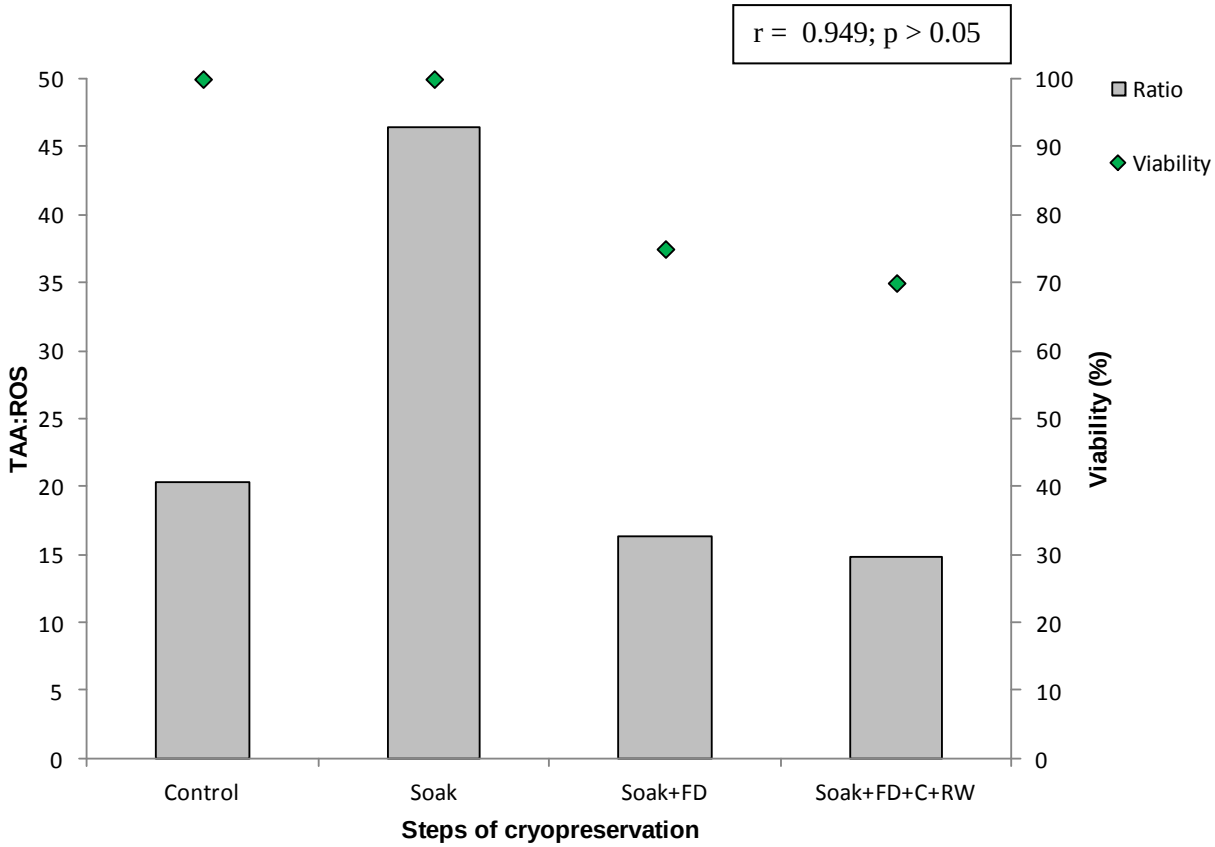

Fig. 3.47: Ratio of TAA to ROS (TAA: ROS) and the corresponding viabilities of *S. gerrardii* explants after exposure to soaking (soak), and a combination of soaking and flash drying (soak+FD) and soaking, flash drying, cooling and rewarming (soak+FD+C+RW). *r*- and p-values represent the results of correlation analysis between TAA: ROS production and viability (Spearman correlation).

In *L. sinensis*, TAA: ROS was highest after both the steps of soaking and soak+FD (**64:1**), and lowest after soak+FD+C+RW (**5:1**). TAA: ROS and viability did not differ significantly between soaked and soaked+FD explants. The declining ratios from soaked+FD explants to soaked+FD+C+RW explants corresponded to a decline from 100% seedling production in dried explants to 40% root production in cryopreserved explants. Antioxidant capacities of *L. sinensis* explants (18-34 µmol g^{-1} FW) were relatively higher than those of both *T. dregeana* and *Q. robur* explants (Fig. 3.43).

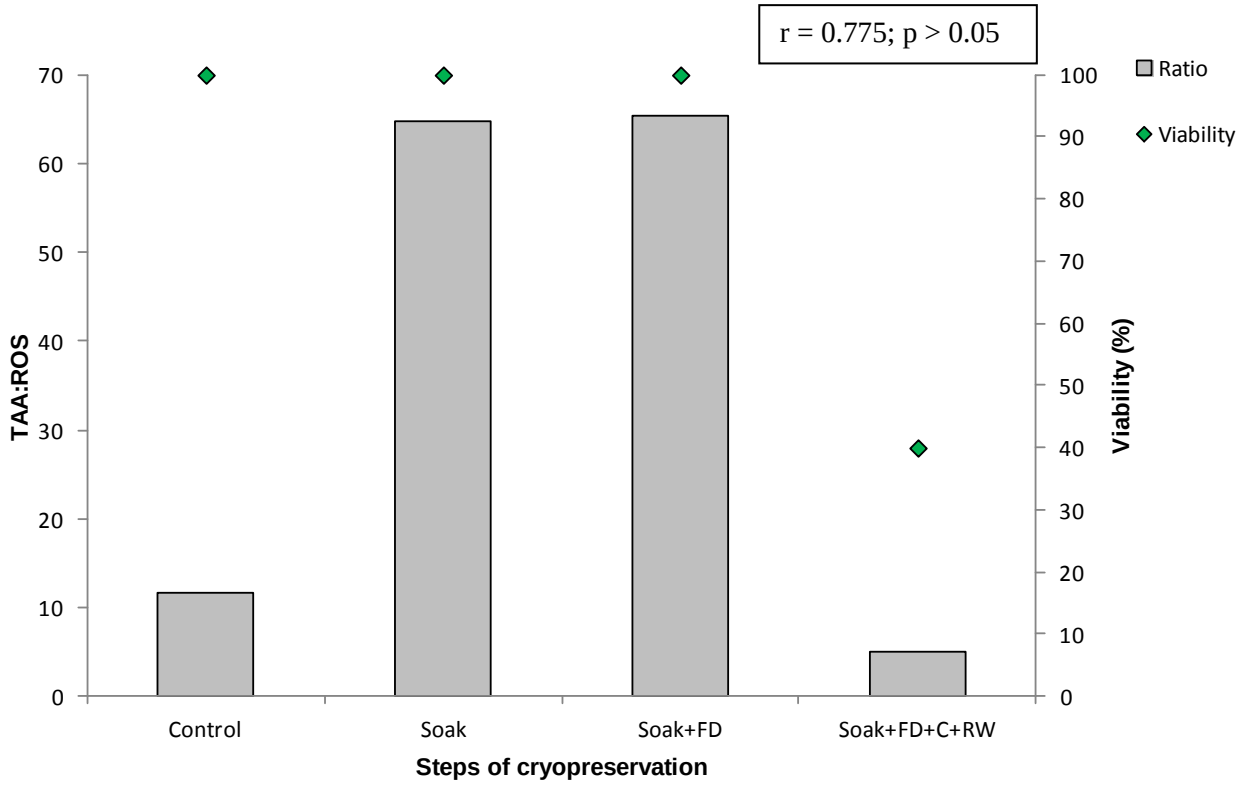

Fig. 3.48: Ratio of TAA to ROS (TAA: ROS) and the corresponding viabilities of *L. sinensis* explants after exposure to soaking (soak), and a combination of soaking and flash drying (soak+FD) and soaking, flash drying, cooling and rewarming (soak+FD+C+RW). *r*- and p-values represent the results of correlation analysis between TAA: ROS production and viability (Spearman correlation).

No significant correlations were measured between TAA: ROS and viability when data for all procedural steps were pooled for analysis for each species (Figs 3.45-3.48). However, a significant correlation was measured between TAA: ROS and viability when data for all procedural steps were pooled for analysis across species (Table 3.24).

Table 3.24: Correlations between cumulative data recorded for biomarkers and viability during all assessed steps of cryopreservation across species (Pearson correlation).

Correlations	Pearsons r	P
H_2O_2 vs viability	-0.083	>0.05
Respiratory activity vs viability	0.325	>0.05
TAA vs viability	0.544	<0.05
$^{\cdot}O_2^-$ vs viability	-0.514	<0.05
TAA: ROS vs viability	0.675	<0.01

CHAPTER 4: Discussion

The germplasm conservation of many horticulturally important and endangered recalcitrant-seeded species is hampered by the challenges associated with the short- to medium-term storage of their seeds and the lack of cryopreservation protocols for the long-term storage of their germplasm (Pammenter & Berjak, 1999; Berjak & Pammenter, 2008; Engelmann, 2011; Berjak & Pammenter, 2013a). Successful cryopreservation incorporates relevant engineering principles with developments in cellular, physiological and developmental biology (Baust *et al*., 2009) which is why studies aiming to establish the causes of explant death must consider each of these aspects. For zygotic explants excised from recalcitrant seeds, cryopreservation offers stable, durable and low cost storage which shields specimens from disease, pathogens and detrimental environmental conditions (Reed *et al*., 2012). However, many cryopreservation studies on recalcitrant-seeded zygotic explants have shown that cryopreservation procedures impose both physical (Chandel *et al*., 1995; Berjak *et al*., 1999b; Goveia *et al*., 2004; Engelmann, 2011; Hajari, 2011; Sershen *et al*., 2016) and oxidative stress (Anchordoguy *et al*., 1987; Varghese & Naithani, 2008; Berjak *et al*., 2011; Naidoo *et al*., 2011; Kaczmarczyk *et al*., 2012; Naidoo *et al*., 2016) to be contributing factors towards cell death. Therefore, the development of strategies to curb stresses associated with cryopreservation in this study considered ameliorating damage during physical manipulations by using the least injurious methods, and regulating ROS generation by the inclusion of antioxidants at all procedural steps. In this study, stress biomarkers were measured after implementation of these ameliorative measures to understand the impact of procedural steps on ROS ($^{\cdot}O_2^-$ and H_2O_2), total aqueous antioxidants (TAA), respiratory activity and cellular ultrastructure and the consequent impact on survival of recalcitrant-seeded zygotic explants during cryopreservation.

Stress responses can be characterised as a "eustress" or "distress" condition, where the former condition induces or enhances protective functions and the latter cannot be resolved by adaptation or coping mechanisms and could lead to cell death (Kranner *et al*., 2010). Both types of responses were observed in this study which aimed to elucidate, compare and contrast some of the stresses associated with cryopreservation of zygotic explants from recalcitrant seeds of *Trichilia dregeana, Quercus robur, Lychee sinensis* and *Strychnos*

gerrardii by assessing the impact of procedural steps on the selection of stress biomarkers stated above. This study identified the stressful procedural steps from previous investigations on cryopreservation of recalcitrant-seeded zygotic explants to be excision, partial dehydration, cryoprotection, cooling, rewarming and decontamination before germination. These steps can impose both physical and oxidative injury to explants (Kioko, 2002; Whitaker *et al*., 2010; Berjak *et al*., 2011; Berjak *et al*., 2014). Therefore, with the aim of ameliorating stresses during all steps of cryopreservation to either improve or retain viability, specific measures to counteract physical damage of explants and oxidative stress were undertaken. Results presented on optimisation of cryopreservation techniques such as explant excision (Fig. 3.1), exogenous application of antioxidants (Figs 3.2 & 3.3; Table 3.4), selection of a suitable drying technique and dehydration period (Figs 3.16-3.23) addressed the specific aim of investigating approaches to ameliorate associated stresses and improve development of certain procedures of cryopreservation protocols. The results of this study will be discussed with a focus on the optimisation of cryopreservation procedural steps and the role of explant anatomy and physiological responses on viability. The discussion follows with the analysis of stress biomarkers in response to these steps and finally, the relationships between explant physical characteristics, stress biomarkers and viability.

The study began by analysing the relationship between explant characteristics such as morphology, tissue architecture, fresh mass and shedding WC with physiological responses such as viability and WC after soaking, cryoprotection and partial dehydration during cryopreservation (Fig. 3.1, Table 3.1; Table 3.9; Figs 3.16-3.23). Results presented in this regard addressed the specific aim of comparing explant anatomy and physiological characteristics across different recalcitrant-seeded species and establishing the influence of these variables on the ease of obtaining explants free of injury, and on physiological responses to cryoprotection and partial dehydration. Such responses that are linked to explant anatomy and physiology are poorly understood (Ballesteros *et al*., 2014) and may have direct implications on survival during cryopreservation.

4.1 Disparities and commonalities in explant morphology, *in vitro* procedures and responses to different antioxidant treatments across species

Pammenter *et al*. (2011) showed that topography and morphology of recalcitrant-seeded zygotic explants influence techniques applied during cryopreservation and the consequent physiological responses by explants to cryogenic manipulations such as excision. It is accepted that the explants excised for cryopreservation should be as small as possible to generate a large surface area to volume ratio which will allow for rapid desiccation and cooling (Berjak *et al*., 1996; Wesley-Smith *et al*., 1999; Berjak & Pammenter, 2001; Wesley-Smith *et al*., 2001b). The smallest explant representing genetic diversity is the zygotic embryo with cotyledons or endosperm removed. In recalcitrant-seeded species where such is possible, explant viability is not reduced after excision, as exemplified by *Cocos nucifera* (Assy-Bah & Engelmann, 1992), *Amaryllis belladonna* (Sershen *et al*., 2012a) and *Haemanthus montanus* (Sershen *et al*., 2012b). In these species, embryos can be easily removed from the surrounding seed tissue without incurring any injury and no viability loss is induced. However, in some recalcitrant species, like *T. dregeana* investigated here, excision related injury inhibits complete seedling production after excision (Goveia, 2007; Naidoo, 2012). In comparison, *S. gerrardii*, *Q. robur* and *L. sinensis* embryos were removed from the seed tissue, without injury to meristematic regions and exhibited complete seedling production after excision. Particularly in the case of *T. dregeana*, obtaining a suitable explant for cryopreservation is challenging and it has been reported that when cotyledons were removed flush with the explant no shoots were produced after excision (Goveia, 2007; Naidoo *et al*., 2011).

The anatomical variability in explants across species is apparent (Fig. 3.1), with differences occurring in explant length, shedding WC and fresh mass (Table 3.1). However, these differences are unlikely to underlie survival directly after excision and have more impact on physiological responses during subsequent procedural steps. Physical injury affected the type of *in vitro* growth after excision in *T. dregeana* explants, with only 40% of the explants producing shoots (Table 3.1). The method of excision of the final explant of this species (Fig. 3.1a; F) using hypodermic needles was implemented as an ameliorative measure to minimise physical injury to shoot meristems. The shoot meristem in this species is not only contiguous with the cotyledons, but embedded within it (Fig. 3.1a) and complete detachment

causes physical wounds to this region (Goveia *et al*., 2004; Naidoo *et al*., 2011). While the excision technique minimised physical injury, the wounded site created by the excision of cotyledons lies within close proximity to the shoot meristem (Ballesteros *et al*., 2014), which is unique to this species anatomy compared with others in this study. The inability for freshly excised explants of *T. dregeana* to exhibit 100% shoot production, even prior to stresses induced by cryopreservation, indicate that it is either physical damage, oxidative damage during wound response (Doke *et al*., 1996; Roach *et al*., 2008) or both, which inhibit shoot development.

Explants of *S. gerrardii* (Fig. 3.1b; F), *L. sinensis* (Fig. 3.1c; F) and *Q. robur* (Fig. 3.1d; F) were excised to the smallest possible size with minimal physical injury to the embryo. In each of these explants, the incision sites (in the case of *Q. robur*, the attachment site), were a distance away from the shoot meristematic region, with both areas being partitioned by endosperm or cotyledon tissue. The seed anatomy of these species influence the structure of the final explant, and allow for the shoot meristem to be kept at a distance from the excision sites and associated ROS bursts, much like the embryo anatomy of recalcitrant *A. belladonna* and *Syzygium cordatum* (Pammenter *et al*., 2011), where complete seedling production occurred after excision despite the incidence of an accompanying ROS burst (Roach *et al*., 2008; Berjak *et al*., 2011). This is a characteristic anatomical difference between explants of these three species and *T. dregeana* explants which is suggested to influence the difference in survival after excision. The inability of explants to produce seedlings due to excision related injury is common in tropical-recalcitrant seeded dicotyledonous species having fleshy cotyledons such as *Ekebergia capensis* (Peràn *et al*., 2006), *Trichilia emetica* and *Protorhus longifolia* (Naidoo, 2012).

A post-excision antioxidant soak was employed in this study to ameliorate excision associated oxidative injury which was observed to facilitate normal seedling production in explants of *T. dregeana* when cotyledons had been completely removed (Naidoo, 2012). Additionally, that study reported the influence of using an antioxidant solution throughout cryopreservation as a means to reduce ROS production in explants of *T. dregeana*. Before considering responses to the optimisation of an antioxidant soak, commonalities and disparities amongst explants during *in vitro* procedures will be discussed.

Decontamination of explants is essential for the removal of external and internal microflora (Mycock & Berjak, 1990) which can proliferate during seed handling prior to asceptic processes. A 0.5% sodium dichloroisocyanurate (NaDCC) applied for various durations (Table 3.2) to eliminate potential contaminants proved to be just as efficacious as, and possibly less toxic than the other chemical agents tested in this study (Table 3.2). This was the first study to test and optimize the use of NaDCC as an explant decontaminant for *S. gerrardii*, *Q. robur* and *L. sinensis*. Berjak *et al*. (2014) reported the efficacy of NaDCC as an effective decontaminant for explants of *T. dregeana*. The published effects of NaDCC as a decontaminating agent with lower phytotoxicity than hypochlorites such as sodium hypochlorite, calcium hypochlorite and mercuric chloride (Berjak *et al*., 2014) supports the suggestion that the use of this decontaminant is a factor that may contribute to survival of explants during steps of cryopreservation in the present study.

Following excision and appropriate decontamination procedures, it was crucial to culture zygotic explants on medium which facilitates full seedling development prior to manipulations for cryopreservation (Dumet *et al*., 1997). Germination of control explants presented in Table 3.1 was obtained on medium including growth regulators/additives (Table 3.3) specific to each species. Explants across all species required additional phytohormones or antioxidant additives to improve shoot development as complete seedling development did not occur on MS medium alone (Table 3.3). While whole plants have sufficient levels of endogenous cytokinins, small organs and tissue isolated in tissue culture require additional sources of cytokinin to sustain growth (Staden *et al*., 2008). This is often the case with dicotyledonous tissue and so it is not uncommon to frequently add cytokinins to basal medium during cryopreservation of recalcitrant-seeded germplasm (Berjak *et al*., 1999b; Kioko, 2002; Normah & Makeen, 2008; Hajari *et al*., 2011; Kistnasamy *et al*., 2011). These studies have shown the benefit of growth hormones or additives in the improvement of survival during cryopreservation of zygotic explants in species such as *E. capensis*, *Landolphia kirkii* and *T. emetica* (Goveia, 2007; Hajari, 2011; Kistnasamy *et al*., 2011). The requirement of different additives to germination medium for explants across species (Table 3.4) in this study highlights the variability in their *in vitro* responses prior to various cryopreservation procedures.

The addition of benzylaminopurine (BAP) initiated 40% shoot development of *T. dregeana* explants (Table 3.1). As no shoot development was observed prior to addition of BAP, this growth regulator was essential to initiate shoot meristem morphogenesis in explants of this species. *Trichilia dregeana* explants were also the only to have possibly incurred physical/oxidative injury to the shoot meristem during excision, which could explain the lack of total shoot development.

Explants of *Q. robur* and *L. sinensis* both required cytokinins, viz., BAP and 2 iP + trans-zeatin respectively, to stimulate differentiation of cells in shoot meristems for complete shoot development. Ascorbic acid was an also an essential addition to the growth medium for explants of *Q. robur* to facilitate full shoot development, and is known to control browning of explants caused by the oxidation of phenols (Chanana & Gill, 2008; Das & Rahman, 2016), the occurrence of which was observed when explants were cultured on MS alone. Ascorbic acid and other additives such as citric acid and charcoal have been added to media or to blotting paper on which embryos were accumulated after the excision step in other species of *Quercus*, such as *Q. rubra* and *Q. gambelli* (Xia *et al*., 2014) to counteract exudations in response to wounding. Explants of *L. sinensis* exhibited higher percentage shoot development on *in vitro* medium containing natural growth hormones 2 iP and trans Zeatin, compared with MS medium containing the chemical analogue BAP (Table 3.3). The effect of cytokinins may vary based on the variety or species from which the explant is derived (Staden *et al*., 2008), therefore the suitability of natural cytokinins for *in vitro* germination of *L. sinensis* explants may be simply a consequence of species specificity.

Explants of *S. gerrardii* did not require the addition of cytokinins but showed improved shoot production on medium containing activated charcoal (AC) regardless of the concentration of MS. However, 100% shoot production was obtained on full strength MS medium supplemented with AC (Table 3.3). One of the characteristic features of *S. gerrardii* explants is the production of phenols upon excision. Activated charcoal is reported to act as adsorption agent to extract phenolic exudations in the medium (Thomas, 2008) and has been widely reported in tissue culture protocol for woody species (Minocha & Jain, 2000; Silva *et al*., 2016).

The ROS burst in response to explant excision has been documented in recalcitrant-seeded species such as *A. belladonna* (Pammenter *et al*., 2011), *T. dregeana* (Whitaker *et al*., 2010) *Castanea sativa* (Roach *et al*., 2010) and *S. gerrardii* (Berjak *et al*., 2011). To investigate the effect of an antioxidant soak, particularly on $\cdot O_2^-$ and H_2O_2 levels associated with excision, cryoprotection, partial dehydration and cooling, trials were conducted on explants of *Q. robur* (Figs 3.2a & b) and *T. dregeana* (Figs 3.3a & b) during selected steps of cryopreservation. These trials compared the effect of the post-excision application of the non-antioxidant CaMg solution (Mycock, 1999) and cathodic water (Berjak *et al*., 2011) on ROS levels in explants of *Q. robur* (Figs 3.2a & b). Thereafter the effect of similar application of cathodic water and a 1% AsA+cathodic water (1% AsA+CW) on ROS levels in explants of *T. dregeana* (Figs 3.3a & b) was compared to establish the most effective antioxidant soak. Based on these trials, 1% AsA+CW was the most effective antioxidant treatment to reduce levels of ROS and was selected to use during cryopreservation. This result was significant in the optimisation of cryopreservation procedures as multiple studies have indicated high ROS levels, specifically after excision and during pre-cooling desiccation, to correspond to a decline in explant viability (Roach *et al*., 2008; Roach *et al*., 2010; Whitaker *et al*., 2010; Berjak *et al*., 2011; Naidoo *et al*., 2011).

Once it was established that an antioxidant soak decreased ROS production after excision and other procedural steps, the physiological response of explants to the soak was assessed. The duration of exposure to 1% AsA+CW had to be optimised for explants across species. Germination and vigour results showed that this treatment applied for 30 min, had a remediating effect on explants of *T. dregeana* which showed an increase in seedling production by 40% (Table 3.4). It was further observed that the application of this antioxidant solution improved vigour, indicated by the lesser time taken for 50% root emergence, in explants of all species. For explants of *S. gerrardii*, *L. sinensis* and *Q. robur*, though soaking was not necessary to improve germination (Table 3.1), it did improve vigour (Table 3.4). Trials on the effect of exogenously applied antioxidants on ROS production during steps of cryopreservation (Figs 3.2-3.3) showed the beneficial effects of this treatment. It has also been documented that a variety of methods for priming of plant tissues before exposure to stresses can possibly abate oxidative stress and act as stress protectants. This has been illustrated by the use of agents such as melatonin on *Zea mays* L. and *Vigna radiata* L. seeds

(Janas & Posmyk, 2013), cryopreserved *Rhodiolo crenulata* callus (Zhao *et al*., 2011), and cryopreserved *Ulnus americana* L. shoot tips (Uchendu *et al*., 2013); ascorbic acid and vitamin E on cryopreserved *Rubus* shoot tips (Uchendu *et al*., 2009), and cathodic water on desiccated and cryopreserved *S. gerrardii* embryos (Berjak *et al*., 2011). Based on these reasons, 1%AsA+CW was applied to explants across species immediately after excision, as the first procedural step of the cryopreservation protocol. Explants of *L. sinensis* required the shortest exposure period of 5 min (Table 3.4) to improve vigour. The smallest size of this explant compared with others (Table 3.1) could have facilitated the action of 1%AsA+CW to occur during a shorter duration.

4.2 Physiological and oxidative responses of explants to procedural steps

Post-excision soaking

Control and soaked explants represent hydrated tissue and analysis and interpretation of levels of stress biomarkers are considered in the context of high WC. Fresh, excised explants (control) exhibited generally high levels of $^{\cdot}O_2^-$ across species, relative to levels measured in recalcitrant embryos isolated without injury such as *Amaryllis belladonna* (below 0.5 nmol g^{-1} DW^{-1}; Pammenter *et al*., 2011). Whilst one cannot significantly compare basal levels of $^{\cdot}O_2^-$ across species, the trend suggests that explants exhibiting more cut surfaces during excision (Fig 3.1) also exhibited higher $^{\cdot}O_2^-$ levels (Fig 3.4). However, treatment of explants with antioxidant soaking showed significantly reduced $^{\cdot}O_2^-$ levels (Fig. 3.4) and TAA levels (Fig. 3.6) relative to the control in explants across all species. Explants of *T. dregeana* and *Q. robur* exhibited the highest reductions in both stress biomarkers compared with *L. sinensis* and *S. gerrardii* explants (Tables 3.5 & 3.7). Reduced TAA levels after an antioxidant soak were in contrast to Berjak *et al*. (2011), where those authors reported enhanced antioxidant capacity in explants of *Boophane disticha* during steps of cryopreservation. However, those authors reported elevated antioxidant levels after treatment with cathodic water alone relative to a CaMg treatment, not with untreated explants, as in this study.

Present results indicate that at the initial step of cryopreservation, the availability of external antioxidants acted to quench the excision induced $^{\cdot}O_2^-$ burst and the transient levels being produced thereafter during the 30 min soak. Responses of cells to oxidative stress may not

always involve increasing levels of endogenous antioxidants (Halliwell, 2006); external provision of protection can act in inhibiting ROS-producing systems and enhancing other mechanisms such as chaperones for transport of antioxidants (Halliwell, 2006). The significant reduction in endogenous antioxidants seen across species after soaking could be a response to prevent overtly high levels of reductants from potentially reacting with the beneficial levels of ROS that are required to modulate programmed cell death, growth, development and stress adaptation which are important biological processes for optimal cellular function (Gadjev *et al*., 2008; Bouayed & Bohn, 2010). That being said, the levels at which $^{\cdot}O_2^-$ and H_2O_2 exist to provide beneficial effects, differ between each ROS and across species (Tables 3.5 & 3.6). While soaking lowered levels of $^{\cdot}O_2^-$ in explants across species, H_2O_2 levels significantly increased in *L. sinensis* (by 550%) and *S. gerrardii* (by 500%) and significantly decreased in *Q. robur* (by 61%) and *T. dregeana* (by 17%) relative to the respective controls (Fig. 3.5; Table 3.6). The antagonistic behaviour in both ROS measured across species to the same stress exhibit inconclusive patterns at this stage.

The reduction of $^{\cdot}O_2^-$ at the onset of cryopreservation is a notable result in terms of assessing contributing factors towards viability. Superoxide is known to inactivate a spectrum of enzymes that are fundamental in energy and amino acid metabolism (Halliwell, 2006), and must be regulated at levels such that inhibition of metabolic pathways crucial to growth and repair is avoided. Since TAA decreased across explants (Fig. 3.7), it is possible that the exogenously applied antioxidant quenched extracellular $^{\cdot}O_2^-$. Reduction of this free radical species upon excision in response to the antioxidant soaking is crucial in reducing oxidative stress at the early stages of explant preparation. In *S. gerrardii* explants it was reported that post-cryo survival did not occur in the absence of an antioxidant soak (Berjak *et al*., 2011), confirming the ameliorative effects of this treatment. Hydrogen peroxide however exhibited an inconsistent pattern after soaking (Fig. 3.5). While reduction of $^{\cdot}O_2^-$ corresponded to high explant viability across species, there was no relationship exhibited between H_2O_2 and viability after soaking. However, despite the disparate behaviour of both ROS, high viability was achieved after this step which indicates that either endogenous or exogenously applied antioxidants acted to modulate ROS levels.

Reactive oxygen species production and the elicited antioxidant responses are metabolic events (Birben *et al*., 2012) and can influence the metabolic state of explants which in this

study, is reflected by respiratory competence. The TTZ test is indicative of viable (respiratory) and non-viable (non-respiratory) tissues (Shrivastava, 2013), but in the present contribution, this biomarker was assessed to establish the effect of procedural steps of cryopreservation on metabolic capacity and the subsequent implications on *in vitro* survival. Relative to the control, *L. sinensis* showed a small decrease in respiratory activity after soaking (by 6%; Table 3.8), while *S. gerrardii* and *Q. robur* were observed to have significantly reduced respiratory activity (by 61% & 35% respectively; Table 3.8). *Trichilia dregeana* explants showed an increase in respiratory activity by 25% (Table 3.8) which corresponded to an increase in *in vitro* viability by 40% compared with the control (Fig. 3.7). The decrease in metabolic activity in explants of *S. gerrardii* and *Q. robur* corresponded with full viability retention in this species. Such inconsistent data presents a difficulty in quantifying a relationship between respiratory activity and viability in soaked explants relative to the control. However, explants of *L. sinensis*, *S. gerrardii* and *Q. robur* had a lower metabolic state relative to the control which might have implications for the subsequent steps. Puntarulo *et al*. (1991) showed a strong dependency of ROS formation during stress on high respiration rates. Therefore, higher metabolic activity and possible dysfunction of antioxidant systems in recalcitrant embryos may pre-dispose explants to more oxidative damage during partial dehydration as suggested by Berjak *et al*. (1989) and Leprince *et al*. (1999), and cooling (Sershen *et al*., 2012a) . Assessment of the stress biomarkers after soaking showed that this treatment significantly decreased $\cdot O_2^-$ and TAA in explants across species, with the percentage decrease in $\cdot O_2^-$ being higher relative to decreases in TAA (Tables 3.5 & 3.7). The minimally injurious manner of explant excision adopted across species is considered to be a factor contributing towards full seedling development in explants of *L. sinensis*, *S. gerrardii* and *Q. robur*. However, there is a possibility of physical and oxidative injury to the shoot meristem in *T. dregeana* explants due to anatomical attributes, and it is suggested that the action of AsA+CW in decreasing $\cdot O_2^-$ levels minimised biochemical stress related injury and improved shoot development in this species.

Assessment of the physiological responses of explants, indicated by viability and ultrastructure, was compared between soaked and control explants. Ultrastructural analysis of control (Figs 3.8a &b; 3.9a & b; 3.10a and 3.11a & b) and soaked explants (Figs 3.8c & d; 3.9c & d; 3.10b & c and 3.11c & d) across species showed a well preserved cellular state in

shoot meristems. This confirmed that soaking as a procedural step maintained a state of cellular integrity comparable to that of control explants and corresponded with full viability retention in the three of the four species. In *T. dregeana* however, only 40% shoot production occurred in control explants, which improved to 80% after soaking (Figs 3.4-3.7). In all explants, cellular characteristics such as matrix dense mitochondria, spherical nuclei with visible patches of heterochromatin, profiles of endoplasmic reticulum and plasma membrane associated vesicles, indicate robust metabolism and high levels of subcellular development (Douce, 1985; Berjak & Pammenter, 2000) that is characteristic of desiccation sensitive embryos, as reported for *L. kirkii* (Berjak *et al*., 1992), *Camellia sinensis* (Berjak *et al*., 1993) and *T. emetica* (Kioko *et al*., 2006).

The presence and development of specific organelles in explants of each species prior to desiccation and cooling provided a comparison for the effect of these subsequent procedural steps on ultrastructure. It further indicates that explants across species are in a state of active metabolism. This presents challenges that are not faced during cryopreservation of orthodox material that undergo a period of metabolic shutdown after development, and are therefore less likely to be predisposed to damage after cooling (Leprince *et al*., 1999). High incidence of mitochondria with defined cristae was visible in control and soaked explants of *T. dregeana* (Fig. 3.8b & d), *Q. robur* (Fig. 3.9a & d) and *L. sinensis* (Fig. 3.11b & d). Profiles of endoplasmic reticulum were evident only in *Q. robur* explants (Fig. 3.9b & d). Both organelles are often associated with high respiratory activity and ongoing membrane synthesis (Berjak & Pammenter, 2000). Shoot meristems across species also featured one or more of the organelles indicative of an inherent accumulation of insoluble reserve material such as starch bodies, lipid bodies and plastoglobuli (Fig. 3.8d; 3.9c, 3.10a & b, 3.11a). Large prominent vacuoles were a common feature across species (Fig. 3.8c & d, 3.9b, 3.10a-c, 3.1b & c) which is typical of desiccation sensitive tissue (Farrant *et al*., 1997). Vacuoles and the presence of reserve material are reported to afford protection against potential mechanical stress during cryopreservation (Farrant *et al*., 1997; Mello *et al*., 2010). This conveys that explants across species do inherently possess ultrastructural characteristics to minimise damage caused during dehydration and possibly cooling. The ultrastructural state across species indicates no occurrence of stress in shoot meristems after excision and soaking and therefore supports that the techniques employed for explant selection to be non-injurious.

Cryoprotection

When developing cryopreservation protocols that incorporate the use of cryoprotectants, it is necessary to assess the effect of cryoprotection on growth and WC of explants. As soaking was considered an essential step, the physiological responses reported in Table 3.9 reflect the state of control explants and those exposed to soaking alone and soaking+CP. Results showed that explant WC increased during exposure to soaking+CP and explant viability decreased relative to untreated and soaked explants (Table 3.9) across species, with the exception of *T. dregeana* explants where viability relative to the control remained unchanged. Cryoprotection negatively impacted viability compared with soaking (Table 3.9). Water content in this study was measured to increase (Table 3.9) relative to the control, which is a contradictory event to the removal of water from cells. The increase in WC could be explained by the influx of DMSO and glycerol into cells. The glycerol molecule contains three carbon atoms that have a hydrophilic, alcoholic hydroxyl group attached to each (Pagliaro & Rossi, 2008). This allows binding between glycerol and other substances that have hydroxyl groups, i.e., water or other glycerol molecules. This property causes the influx of water upon glycerol uptake via aquaglyceroporins (Agre, 2006) during cryoprotection, which may explain the increase in explant WC observed here. Benson (2007) stated that the permeability and toxicity of cryoprotectants are germplasm and species specific, supporting the variability in explant viability and WC across species. Colligative cryoprotectants such as DMSO and glycerol, used in this study, increase the overall osmolality of the cell by replacing water as the cell dehydrates (Benson, 2007), and increases viscosity that may impede the efflux of water upon drying. While this may be beneficial upon cooling to minimise osmotic damage, relatively slower dehydration may exacerbate desiccation-induced damage by increasing the drying period (Pammenter *et al*., 1998; Varghese *et al*., 2011). This was the case presently and will be discussed further, below.

Though the WC of soaked explants also increased relative to control explants (Table 3.9), which would technically increase the dehydration period compared with untreated explants, a soaking treatment induced a beneficial physiological response in terms of vigour and/or viability (Tables 3.4 & 3.9). Explants from a variety of species of *Lychee* have been cryoprotected by other additives with limited success after cooling (Fu *et al*., 1990; Sudarmonowati, 2000). Cryoprotection of explants of *S. gerrardii* were not previously

adopted as part of a cryopreservation protocol (Berjak *et al*., 2011). Explants of *Q. robur* were previously cryoprotected with increasing concentrations of sucrose and glycerol prepared separately, however those studies showed no seedling development after cooling (Chmielarz, 1997; Berjak *et al*., 1999b). A reduction in viability of cryoprotected explants relative to the control, are sometimes related to cryoprotectant toxicity. There are some concerns with the toxicity of DMSO to tissues at high concentrations for prolonged periods (Bajaj *et al*., 1970; Yu & Quinn, 1994; Wolfe & Bryant, 1999). It must be noted however that DMSO used in this study was applied in combination with glycerol. According to Heber *et al*. (1971) the synergistic effect of combined cryoprotectants can cause the concentration of the mildly toxic cryoprotectant in the unfrozen portion of water during freezing to be reduced by the colligative effect of the other cryoprotectant, which in this case was glycerol (Finkle *et al*., 1985). The effect of these cryoprotectants in cells that have not been exposed to cooling, may still work synergistically to reduce the mole fraction of the other such that the respective target sites of each cryoprotectant is not adversely affected. Therefore any reduction in viability seen at this step can possibly be ruled out as being a consequence of DMSO toxicity. Cryoprotection may have influenced stress biomarkers such that levels of ROS and TAA induced oxidative stress, which may have contributed to viability loss during this step and will be discussed further.

Cryoprotectants used in this study were prepared in cathodic water, therefore explants were consistently exposed to an antioxidant during the step of soaking+CP. Naidoo (2012) showed that the preparation of cryoprotectants in an antioxidant solvent promoted seedling development in explants of *T. dregeana* and significantly reduced H_2O_2 production, compared with levels measured in explants exposed to cryoprotectants made up in distilled water, the latter being the conventional method for preparation of DMSO and glycerol as cryoprotectants (Normah & Chin, 1986; Naidoo, 2012). Survival after cryoprotection was suggested to be a direct consequence of the scavenging of H_2O_2 by cathodic water (Naidoo *et al*., 2016); such action of cathodic water was reported by Hanaoka (2001). No studies have investigated the physiological or biochemical responses of recalcitrant embryos exposed to cryoprotectants where additional antioxidants have been incorporated. Assessment of the biochemical biomarkers showed that $^{\cdot}O_2^-$ (Fig. 3.12) and H_2O_2 (Fig. 3.13) production decreased significantly in explants of *L. sinensis*, *Q. robur* and *T. dregeana* after

soaking+CP. In contrast, a significant increase in H_2O_2 production (Fig. 3.13) was noted in explants of *S. gerrardii* with a small increase in $^{\cdot}O_2^-$ production (Fig. 3.12). Antioxidant capacity significantly decreased in explants of *Q. robur* and *T. dregeana* with minimal changes in explants of *L. sinensis* and *S. gerrardii* (Fig. 3.14). Respiratory activity significantly decreased in explants of all species except in those of *L. sinensis* where a significant increase was noted (Fig. 3.15). The combined treatments of soaking+CP show variable effects on these biomarkers, with no consistent increase or decreases in TAA, ROS or respiratory activity being relatable to viability (Tables 3.10-3.13). However, the implication of cryoprotection on biochemical biomarkers with consideration of explant physiology may be discussed.

An important characteristic of these cryoprotectants is the increased membrane permeability to aid in water removal from cells and facilitate protective dehydration during freezing (Fuller, 2004). While the use of cryoprotectants can alter membrane permeability to avoid excessive dehydration (Phelps *et al.*, 1999), the occurrence of increased membrane permeability allows for an influx of ROS, particularly H_2O_2 which is the chemical species that can permeate membranes (Halliwell, 2006). Therefore the regulation of H_2O_2 during cryoprotection is important to minimise damage to sites of oxidation. This mode of action was especially apparent in *T. dregeana* explants reported by Naidoo (2012).

In terms of TAA, this biomarker was significantly reduced in explants of *Q. robur* and *T. dregeana* with negligible changes in explants of *L. sinensis* and *S. gerrardii* (Fig. 3.14). It is possible that prolonged exposure to antioxidants, such as the case with cryoprotection, can alter the activity or levels of endogenous antioxidants, such that an increase in one would result in a compensatory decrease in another; however in this case responses appear to be species-specific. Compensatory changes such as these may not be measurable in overall antioxidant activity (Poljsak, 2011) which may be the case in *L. sinensis* and *S. gerrardii* explants. Changes such as these would not show a measurable difference in overall antioxidant activity, which could be the case in explants of both the latter species. The antioxidant activity of cathodic water and the effects of its uptake still remains uncharacterised, so compensatory changes of endogenous antioxidants within the cell upon exposure is unknown.

Soaking+CP decreased explant viability relative to the control in all species except *T. dregeana* where viability was comparable to the control explants. It is presumed that CP may have resulted in damage including disruption of the cytoskeleton (Kioko, 2002), but events such as these were not investigated in this study.

Cryoprotection can sometimes cause cellular disorganisation which may affect the respiratory activity of cells but not necessarily result in complete cell death (Finkle *et al*., 1985) which may be reflected in reduced viability here. The increase in respiratory activity in explants of *L. sinensis* by 70% relative to the control did correlate with the highest viability retention in explants across species after soaking+CP (Table 3.13).

Partial dehydration

Desiccation is a pre-treatment which is highly beneficial in increasing the survival of plant tissues after cryopreservation (Reed, 1996; Engelmann, 2011; Berjak & Pammenter, 2014). However, explants from recalcitrant-seeded species are invariably desiccation sensitive and partial or complete loss of viability when dried to WC within the range of 0.2-0.6 g g^{-1}, is inevitable (Chaitanya & Naithani, 1994; Vertucci & Farrant, 1995; Pammenter *et al*., 1998; Varghese & Naithani, 2002; Sershen *et al*., 2007; Berjak & Pammenter, 2008; Varghese *et al*., 2011; Sershen *et al*., 2016). Additionally, varying degrees of sensitivity has been reported across recalcitrant-seeded species. For example, embryos from seed species such as *A. belladonna* (Sershen *et al*., 2012a), *H. montanus* (Sershen *et al*., 2012b) and *L. kirkii* (Kistnasamy *et al*., 2011) can maintain high viability at the lower end of the WC range, i.e., between 0.29-0.33 g g^{-1} for amaryllid species, and approximately 0.28 g g^{-1} for *L. kirkii* embryos. At the other end of the continuum, embryos from species such as *T. dregeana* and *T. emetica* lose viability at approximately 0.42 g g^{-1} (Naidoo, 2012) when rapidly dried and around 0.8 g g^{-1} when dried slowly (Varghese *et al*., 2011). Such a wide range of desiccation sensitivity amongst recalcitrant germplasm can be attributed to anatomical, physiological and biochemical differences (Vertucci & Farrant, 1995; Berjak & Pammenter, 1996) across species, the variability in ecological conditions of their origin (temperate or tropical; Tweedle *et al*., 2003) and environmental characteristics of their micohabitats (Joët *et al*., 2016).

A vital part of cryopreservation of recalcitrant-seeded zygotic explants is the optimisation of partial dehydration before exposure to LN (Reed, 1996, Engelmann, 1998; Benson, 2000; Berjak & Pammenter, 2013a). During desiccation, the hydration shells of molecules are gradually lost (França *et al*., 2007) and the ability to withstand further drying without losing viability after this point will determine how desiccation sensitive the zygotic explant is. When optimising the flash drying period for explants of each species, a trade off has to be made between freezing damage upon ice crystallisation at higher WC and desiccation stress due to water reduction at lower WC. Flash drying curves are generally generated as a means to analyse how rapidly water is lost from zygotic explants during desiccation, and the subsequent impact on viability retention (Berjak *et al*., 1990; Pammenter *et al*., 1991; Berjak *et al*., 1999a). The drying period selected for explants of each species was based on the duration necessary to sufficiently reduce WC within a range that is low enough to minimise intracellular ice formation upon cooling (Sershen *et al*., 2012; Wesley-Smith *et al*., 2014) but high enough to avoid desiccation induced damage (Varghese *et al*., 2011). This range is usually between 0.2 – 0.4 g g^{-1} for recalcitrant-seeded zygotic explants (Vertucci *et al*., 1991; Dereuddre *et al*., 1992; Wesley-Smith *et al*., 1992; Sershen *et al*., 2012a; b) and was the selected target WC range for explants across species. It is crucial to reach the target WC in the shortest possible drying period to curb the accumulation of damage that is associated with aqueous- based reactions at intermediate WC (Pammenter *et al*., 1998; Walters *et al*., 2001; Varghese *et al*., 2011). To reach the target WC, flash drying was suitable for explants of *Q. robur*, *T. dregeana* and *S. gerrardii*. This method was applied as an appropriate partial dehydration technique for explants of the latter two species in previous reports (Goveia *et al*., 2004; Berjak *et al*., 2011), and is widely utilised as a partial dehydration method of embryos of other recalcitrant-seeded species, either towards their cryopreservation or investigations on responses to drying (Kioko, 2002; Ajayi *et al*., 2006; Hajari *et al*., 2011; Kistnasamy *et al*., 2011; Varghese *et al*., 2011; Sershen *et al*., 2012a; b; Naidoo *et al*., 2016; Sershen *et al*., 2016).

Quercus robur explants were partially dried using desiccation over silica gel within closed Petri dishes (Chmielarz, 1997) and flash drying using a flash drier (Berjak *et al*., 1999b) in previous reports. Both techniques were employed in this study, but no difference in the drying rate was observed. Based on post-cryo viability being higher using flash drying

(Berjak *et al*. 1999b) than conducting desiccation over silica gel (Chmielarz, 1997), flash drying was selected for partial dehydration of *Q. robur* explants in this study. Explants of *L. sinensis* were also partially dried using two techniques, i.e., flash drying and vacuum flash drying. Vacuum flash drying facilitated significantly faster drying of explants of this species compared with flash drying (Fig. 3.19) but viability did not differ between both techniques. As alluded to above, it is essential to bypass intermediate WC as quickly as possible during partial dehydration and based on the facilitation of a quicker drying period, vacuum flash drying was deemed the preferred method for *L. sinensis* explants. The drying curves constructed for soaked (Figs 3.16-3.19) and soaked+CP (Figs 3.20-3.23) explants across species showed no correlation between viability at the target WC (0.37-.42 g g^{-1}) and drying period to reach target WC. As viability was observed to be lower in soaked+CP+FD explants compared with soaked+FD explants across species (Figs 3.20-3.23), cryoprotection was deemed unsuitable to apply as a procedural step prior to cooling and investigations in this regard were not taken further. The responses of soaked+FD explants will be discussed henceforth.

A great emphasis has been placed on shedding WC, rate of drying and associated viability loss in recalcitrant-seeded material (Pammenter *et al*., 1998; Varghese *et al*., 2011; Ballesteros *et al*., 2014; Sershen *et al*., 2016). Undried explant WC in all four species fell within the range of 1.13 (in *Q. robur*) to 2.1 g g^{-1} (in *T. dregeana)* across species; however, drying periods to reach target WC amenable for cooling ranged from 20 to 240 min. The rapidity at which water is lost during partial dehydration can be in part be related to the interplay between explant physiological and anatomical characteristics such as size, fresh mass and explant tissue composition (Ballesteros *et al*., 2014). *Strychnos gerrardii* explants had the lowest fresh mass across species (Table 3.1) with a portion of papery thin cotyledons attached and reached target WC in the shortest period (20 min; Fig. 3.18). *Lychee sinensis* explants dried in 30 min to reach target WC, despite having the heaviest fresh mass and endosperm tissue surrounding the embryo. However these explants were the smallest in size (Table 3.1), which is a contributing factor towards explant drying rate (Vertucci & Farrant, 1995; Ballesteros *et al*., 2014). Explants of *T. dregeana* required 120 min to reach target WC while those of *Q. robur* required double this period (240 min). Explants of these species were similar in length but larger than explants of *S. gerrardii* and *L. sinensis*. Explant tissue in both

these species consisted of embryos and cotyledon tissue that were thicker than these structures in *S. gerrardii* explants and the embryo and endosperm tissue in *L. sinensis* explants. These factors are suggested to contribute to the longer period of drying taken to reach target WC amenable for cryopreservation in *T. dregeana* and *Q. robur*. With specific regard to *Q. robur* explants, the drying period was substantially longer relative to those applied to explants of the remaining species, with a small loss in viability (20%) relative to the control or soaked explants.

The extent of desiccation stress in recalcitrant-seeded material is characterised by stress intensity (water loss) and stress duration (period of drying) (Pammenter *et al*., 2003), which are important factors in explant physiological responses to drying (Vertucci, 1989; Varghese *et al*., 2011). Physiologically, desiccation damage induced viability loss during partial dehydration is caused by: (i) the lack of insoluble reserve deposition, particularly in vacuoles, that would prevent against tissue collapse upon volume reduction and turgor pressure when water is lost (Iljin, 1957; Pammenter & Berjak, 2013) and (ii) compact arrangement of macromolecules and organelles which ultimately cause the rupture of the plasmalamella allowing entry of extracellular hydrolases (Walters *et al*., 2002a; Farrant *et al*., 2007). Pammenter *et al*. (1998) described a quicker period of drying to preferentially dehydrate the outermost tissue cells causing these cells to contract, while the core cells within the explant remain unaffected. As such, a longer period of drying affects the distribution of remaining water across the cells, where germinative cells from the core have been noted to experience greater dehydration, often resulting in increased desiccation damage to shoot meristems (Wesley-Smith *et al*., 2001a). Membranes are often cited as a major region of structural and chemical induced desiccation injury (Senaratna & McKersie, 1986) which is caused either by the physical loss of water or oxidative stress respectively (Vertucci & Farrant, 1995; Walters *et al*., 2001). At the water content range to which explants were desiccated, both types of damage or a combination of both are possible.

As alluded to above, drying of explants to target WC is a prerequisite but this is not without some or substantial loss in viability. For example, explants of *T. dregeana*, *S. gerrardii* and *Q. robur* showed a decline in viability (30, 25 and 20% respectively) relative to undried explants, while those of *L. sinensis* retained 100% viability even when dried to target WC (Figs 3.16-3.19). There are some factors to consider when interpreting the impact of

desiccation on viability of recalcitrant explants. Sensitivity to desiccation stress can be regarded as a continuum, where factors such as size and topography of the explant, rate of drying (Varghese *et al*., 2011), stage of development and cellular differentiation and metabolic status will influence the response to drying (Vertucci & Farrant, 1995; Ballesteros *et al*., 2014; Sershen *et al*., 2016). The consideration of some of these factors when looking at the response to desiccation in terms of viability showed that each species has a characteristic set of advantages for survival, similar to those that would affect drying rate.

When comparing survival within the target WC (0.37-0.42 g g^{-1}) range, explants of *L. sinensis* exhibited the highest survival after desiccation (Fig. 3.19) being the smallest explants across species, with the added advantage of both shoot and root meristem being enclosed within endospermous tissue (Fig. 3.1c; F) which protected these regions from direct physical injury. The enclosure of the meristems within endosperm may also result in WC of the meristematic regions being higher than that of bulk embryo WC. Ballesteros *et al*. (2014) showed that when *Castanospermum australe, T. dregeana, S. gerrardii* and *A. belladonna* zygotic embryos were flash dried to target WC (0.3-0.4 g g^{-1}), shoot and root meristems were measured to be at a relatively higher WC than bulk embryo WC. However, since meristematic regions are entirely concealed only in *L. sinensis* explants, it is likely that the differentiation in embryo bulk WC and WC of the meristematic regions was much higher in this species relative to others. The implication of this might be that dried *L. sinensis* explants, while exhibiting full viability at such lower WC may not necessarily be more desiccation tolerant than those of the other species. Explants of *Q. robur* and those of *S. gerrardii* had partial and complete concealment of the shoot meristem by cotyledons (Fig. 3.1d; F) which could have protected over-drying of this region during desiccation, much like the situation in *L. sinensis* explants. Another factor to be considered here is the significantly lower shedding WC of *Q. robur* explants compared with those of other species (Table 3.1), which could confer higher desiccation tolerance. The pattern of decreasing shedding WC and increasing desiccation tolerance has been reported for *Q. robur* (Finch-Savage, 1992a) and other recalcitrant seeds of temperate origin such as *Acer pseudoplatanus* (Hong & Ellis, 1990) and *Aesculus hippocastanum* (Farrant *et al*., 1997).

The tolerance of extended periods of drying or lack thereof could also be attributed to the geographical origin and location of the plant species. *Quercus robur* explants are inherently conditioned to withstand desiccation due to its origin being of temperate variety (Xia *et al*., 2014). It is conjectured that *Q. robur* explants could tolerate drying for extended periods because of their temperate origin whereas the remaining species in this study were sub-tropical in origin. Xia *et al*. (2014) reported that other *Quercus* species, which were cold acclimated as a consequence of their natural environment, showed higher desiccation tolerance than those species that inhabited warmer regions, therefore inherent mechanisms acquired as a consequence of ecotype will vary across species. While *Q. robur* explants exhibited behaviour suggesting a higher tolerance to desiccation duration than explants of the other investigated species, target WC was similar to those of *S. gerrardii* and *L. sinensis*. Therefore the tolerance to desiccation intensity is comparable with both these species. The factors mentioned above discuss explant anatomical and physiological factors that could have influenced drying rate and viability across species. Below follows a discussion on the impact of partial dehydration on stress biomarkers.

Oxidative stress induced viability loss is the most deleterious consequence of water depletion in desiccation sensitive tissues (Senaratna & McKersie, 1986; Chaitanya & Naithani, 1994; Vertucci & Farrant, 1995; Varghese & Naithani, 2002; Bailly, 2004; Varghese *et al*., 2011). Biomarkers assessed in soaked+FD explants were viewed within the context of the relatively low target WC (Figs 3.16-3.19). As seen in Figs 3.24 to 3.27 each species exhibited a different interplay between stress biomarkers, which when integrated with the physiology of the explant will determine how much water loss is tolerated before viability is reduced. It is documented that ongoing metabolism in explants from recalcitrant seeds at the target water content achieved during desiccation, leads to impairment of the mitochondria and therefore enhanced leakage of electrons from the mitochondrial electron transport chain (Inzé & Van Montagu, 1995; del Rio *et al*., 2002; Kranner & Birtić, 2005). Even if target WC achieved is low enough to induce a vitrified state of the cytoplasm, these deleterious reactions may still occur, albeit at a slower rate (Sun & Leopold, 1997; Kranner & Birtić, 2005). The mitochondria, which are responsible for the conversion of 1-2% of consumed oxygen to $^{\cdot}O_2^-$ (Boveris & Chance, 1973), are a major source of ROS production (Moller, 2001) and this is enhanced at low water contents (Hendry, 1993; Finch-Savage *et al*., 1994a; Côme &

Corbineau, 1996; Varghese & Naithani, 2002; Pukacka & Ratajczak, 2006; Berjak & Pammenter, 2008). However an increase in both ROS after partial dehydration relative to the control was only exhibited by *S. gerrardii* explants, with dried explants of the remaining species all exhibiting a decrease in $^{\cdot}O_2^-$ production relative to the control (Figs 3.24 & 3.25). Hydrogen peroxide production only decreased in dried explants of *Q. robur* and *T. dregeana*, but significantly increased in dried explants of *L. sinensis* and *S. gerrardii* (Fig. 3.25). Antioxidant capacity across explants exhibited the same trend as $^{\cdot}O_2^-$ production, where dried explants only of *S. gerrardii* exhibited an increase (Fig. 3.26). With the exception of *S. gerrardii* explants, the decrease in $^{\cdot}O_2^-$ levels after drying can be a consequence of the decrease in respiratory activity relative to the control in the remaining species (Fig. 3.27). Dried explants of *S. gerrardii* also exhibited reduced respiratory activity, in fact the highest across species (75%) but exhibited contrasting trends in ROS and TAA production. The decrease in $^{\cdot}O_2^-$ production in explants of species that exhibited a decline in ROS after desiccation to low WC may better be explained by the possible inactivation of enzymes responsible for the production of $^{\cdot}O_2^-$ and a lack of substrates and reductants (Roach *et al*., 2008; Varghese *et al*., 2011). Despite the different responses in oxidative biomarkers, viability of soaked+FD explants decreased relative to the control in *S. gerrardii* and *Q. robur* explants. In *L. sinensis,* explant viability was retained at 100% and in *T. dregeana* explants there was a 10% increase in viability relative to the control. In the case of *T. dregeana*, it is likely that an increase in viability in soaked+FD explants occurred due to exposure to an antioxidant soak, which was not applied to control explants. Partial dehydration did in fact reduce viability when soaked+FD explants (Fig. 3.24) are compared with soaked explants (Fig. 3.4).

The downregulation of metabolism is associated with survival of desiccation in orthodox tissue (Hoekstra *et al*., 2001) and could be a coping mechanism for survival in these recalcitrant explants under stress. In *Q. robur* and *T. dregeana* explants, viability decreased in dried *Q. robur* explants and increased in dried *T. dregeana* explants relative to the control (Figs 3.24-3.27). In both of these species, TAA decreased by 70 and 75 % respectively in relative to the control (Table 3.16). Explants of *L. sinensis* retained full viability after desiccation and rehydration which corresponded to only a 23% reduction in TAA relative to the control (Table 3.16). No other studies to date have reported full viability retention of

explants of *L. sinensis* to the target WC range in this study. Explants of *L. sinensis* showed the lowest respiratory activity relative to the control prior to desiccation treatment (i.e., after soaking; Fig. 3.7) across all species, with *Q. robur* and *S. gerrardii* explants also exhibiting declines in this biomarker prior to drying (Fig. 3.7). This should be considered when assessing explant viability retention after dehydration in these species, as high metabolic activity is often associated with increased desiccation sensitivity (Berjak & Pammenter, 2013a) and lower activity is associated with a greater resilience to water loss (Berjak & Pammenter, 2000). In this regard, soaked *T. dregeana* explants, the only explants to exhibit an increase in respiration relative to the control, also exhibited the lowest viability after partial dehydration.

There is a plethora of literature which has reported on increased ROS production in tissues from recalcitrant seeds during desiccation (Hendry *et al*., 1992; Chaitanya & Naithani, 1994; Varghese & Naithani, 2002; Roach *et al*., 2010; Whitaker *et al*., 2010; Varghese *et al*., 2011). Often, oxidative stress upon drying of recalcitrant tissue is manifested by the ongoing aqueous based metabolism, which causes an imbalance stemming from persistent and enhanced ROS production, and failure of antioxidant systems. This is termed metabolism-linked damage (Walters *et al*., 2001) which accrues with longer drying periods (Varghese *et al*., 2011; Sershen *et al*., 2016). Therefore explants such as *T. dregeana* and *Q. robur* which exhibit behaviour of inherently "holding on" to water and takes the longest drying periods to reach target WC (120 min and 240 min respectively) should theoretically incur the most metabolism-linked damage, while a species such as *S. gerrardii* was expected to better cope with this stress due to a shorter desiccation period (20 min). Flash dried *S. gerrardii* explants showed an increase in TAA relative to the control, which shows that in explants of this species, the antioxidant capacity increased with increased ROS. Varghese *et al*. (2011) suggested that in the case of *T. dregeana*, reduced viability at low WC is potentially a non-oxidative event as the antioxidant status remained stable throughout rapid drying regardless of WC; however a complete failure of antioxidant enzymes and glutathione was reported by those authors when the axes were dried slowly. It is important to note that while explants may exhibit relatively high viability after desiccation, the act of dehydration in desiccation sensitive tissue pre-disposes explants to incur damage at the cooling step (Sershen *et al*., 2012b). The extent of damage upon post-cryo retrieval and re-warming may to a large extent,

apart from other factors, be influenced by the period of drying, which is one part of the parameters that define desiccation stress.

Explants of *L. sinensis* were the only to retain full viability after partial dehydration. These viability results were obtained in conjunction with an oxidative profile that showed a significant decrease of 89% in $\cdot O_2^-$; 250% increase in H_2O_2 production; 23% significant decrease in TAA and 50% significant decrease in respiratory activity (Tables 3.14-3.17). The decrease in TAA was the lowest in explants of *L. sinensis* across species that showed a decline in antioxidant activity after desiccation, but there was a significantly high increase in H_2O_2. Queiroz *et al*. (2015) showed that by-products of *Lychee* i.e. fractions of the seed and peel, had significantly high levels of bioactive compounds such as ascorbic acid, beta-carotene and phenols that promoted high antioxidant activity. That study showed that seed fractions have very high levels of lycopene, a lipid-soluble carotenoid that has potent antioxidant activity (Story *et al*., 2010). This may reflect in antioxidant capacity measured in *L. sinensis* embryos.

Viability data after desiccation of excised explants and whole seeds of *Q. robur* (Finch-Savage, 1992b) and oxidative data after desiccation of excised explants have been documented (Hendry *et al*., 1992; Finch-Savage *et al*., 1994a; Finch-Savage *et al*., 1996; Pukacka *et al*., 2011). However, none of these studies used a flash drying method for rapid desiccation. In those studies, zygotic explants were dried (i) within whole seeds under either ambient or lamina flow air; (ii) individually in a desiccator over silica gel or (iii) individually under special airflow conditions with oxidative measurements taken immediately after drying. These studies show a casual link between the oxidative state and viability during desiccation. The data presented in this study shows that after rapidly desiccating soaked explants to a WC of approximately 0.38 g g^{-1}, ROS, TAA and respiratory activity decreased. Oxidative data corresponded to 80% seedling establishment *in vitro*, which is higher than viability obtained in the most recent study on desiccation in *Q. robur* where explants were desiccated to approximately the same WC (28% WC on a fresh mass basis; Pukacka *et al*., 2011) and showed an increase in $\cdot O_2^-$ and H_2O_2 production in comparison to the control. Total aqueous antioxidants decreased by 70% in comparison to the control, but this was never-the-less a lower reduction than the ROS levels that declined. *Quercus robur* explants show high

phenolic production *in vitro*. Phenols can directly scavenge molecular species of active oxygen (Michalak, 2006). Pukacka *et al*. (2011) showed that guaiacol peroxidase (POX) uses phenols as electron donors to scavenge H_2O_2 in desiccated explants of *Q. robur*. The presence of phenols could also be a contributing factor that enhances antioxidant defences without being reflected as an increase in the actual measurement of antioxidants. This would also apply to any other species that contains phenols, with *L. sinensis* being the other example specific to this study.

Physiological responses to desiccation at the ultrastructural level depict a state corresponding to viable explants across species. *Trichilia dregeana* showed the lowest survival after desiccation across the four species. In the shoot meristematic region of this species (Fig. 3.28a & b), a number of developed organelles consistent with high metabolic activity was observed such as membrane associated vesicles, profiles of endoplasmic reticulum, numerous mitochondria with dense matrices and a nucleus with visible patches of heterochromatin. A high degree of vacuolation was observed in these explants, comparable to the situation in soaked tissue (3.8b & c). A high degree of vacuolation also featured in *S. gerrardii* explants (Fig. 3.30). This generally precedes autophagy and is indicative of a stress response to desiccation as reported for other recalcitrant-seeded embryos (Berjak *et al*., 1989; Farrant *et al*., 1989; Berjak *et al*., 1999b; Wesley-Smith *et al*., 2001b). However, after partial dehydration vacuoles were smaller and less regular and defined in structure in *T. dregeana* explants. The extent of vacuolar differentiation may contribute to the lowest survival of shoot meristems (Berjak *et al*., 1989) after desiccation in *T. dregeana* compared with the other species. However, vacuoles in *S. gerrardii* are similarly differentiated (Fig. 3.30) but success was achieved during cryopreservation of this species so it cannot be vacuole differentiation alone that prevents survival after cryopreservation. Explants of *Q. robur* (Fig. 3.29a & b), *S. gerrardii* (Fig. 3.30) and *L. sinensis* (Fig. 3.21a & b) showed the presence and integrity of all organelles featured in soaked explants. In explants of *Q. robur* long profiles of endoplasmic reticulum indicated the normal resumption of intracellular metabolism after drying (Sershen *et al*., 2012a). In recalcitrant embryos, dissolution of the tonoplast and mixing of vacuolar contents are usually signs of lethality for e.g., in slow dried axes of *Artocarpus heterophyllus* (Wesley-Smith *et al*., 2001a). This was in contrast to vacuoles observed in shoot meristems across species, where these organelles exhibited integrity of the tonoplast. Explants across

these species showed high viability retention after drying and no artefacts of damage were noted in the ultrastructure of the shoot meristems. Notably, explants across these species maintained intact lipid bodies, starch bodies and large vacuoles which could minimise the extent of cellular collapse upon water removal (Berjak & Pammenter, 2000), thereby serving as a contributing factor for survival.

Some conclusions can be drawn upon consideration of all physiological factors that influence the ability to endure desiccation stress in this study. Desiccation stress is a product of desiccation intensity (target WC) and duration (drying period to reach target WC). Assessment of these characteristics across species indicate that the duration or intensity of desiccation alone show no relationship with viability after partial dehydration, but may be influenced by explant characteristics. Explant topography, size and fresh mass, are however recognised to contribute towards survival. The partial or complete concealment of shoot meristems (*L. sinensis*, *Q. robur* & *S. gerrardii*), a small fresh mass (*S. gerrardii*), small dimensions of the explant (*S. gerrardi & L. sinensis*) and minimal incisions during explant excision (*L. sinensis*, *Q. robur* & *S. gerrardii*) contributed to survival during partial dehydration of explants in these species. In *T. dregeana* explants, the partial exposure of shoot meristems upon cotyledon removal, relatively higher fresh mass and length and relatively long drying period of explants of a sub-tropical recalcitrant-seeded variety are identified as contributing factors towards the reduction in viability relative to undried explants. The presence of organelles representing reserve deposition material such as vacuoles, lipids and starch bodies that were observed across species also contributed to survival during desiccation by preventing intracellular collapse upon water removal. The presence of these organelles can afford tolerance to mechanical damage during drying. Compared with explants of *S. gerrardii* and *Q. robur*, where viability loss at target WC was 25 and 20% relative to undried explants, percentage viability loss in *T .dregeana* explants relative to undried explants was comparable (30%). The substantially lower viability in *T. dregeana* explants after drying compared with the dried explants of the remaining species is also a consequence of reduced explant viability in this species prior to dehydration, in contrast to full viability retained by undried explants of the remaining species. This highlights the importance of considering viability of explants in the previously applied step when

assessing the effects of procedural steps during cryopreservation, as this ultimately impacts post-cooling survival.

Cooling and post-cryo survival

Once the zygotic explants are prepared for cryopreservation, exposure to LN, rewarming and subsequent re-establishment becomes critical. Each step of cryopreservation predisposes explants to more damage in the subsequent steps, such that viability retention after each step is reflective of cumulative stress, not merely stress conferred by individual treatments (Berjak *et al.*, 2011). Cooling in itself is substantially injurious to recalcitrant tissue that is sensitive to chilling and freezing (Berjak & Pammenter, 2013b). Such is the extent of injury at this stage that zygotic explants of recalcitrant-seeded species can sometimes maintain significant viability pre-cooling and exhibit total cell death after retrieval from the cryogen for e.g., cryopreserved *T. dregeana* explants which exhibited 50% survival after drying and complete cellular death after cooling. This is most likely a consequence of cumulative physical injury (due to excision, dehydration and/or freezing) and oxidative imbalance. Zygotic explants are more amenable to rapid cooling and re-warming techniques than to slow cooling and re-warming, as rapid application of these steps minimises the time for development of intracellular ice and the coalescing of small ice crystals into larger ones during cooling and rewarming respectively (Sershen *et al*., 2007; Reed, 2008). In this study, rapid cooling was conducted by immersing naked, soaked+FD explants into nitrogen slush followed by rapidly re-warming explants in a solution of 1% AsA+CW. This method served to potentially minimise deleterious ice crystal formation and growth and additionally combat unregulated ROS activity by the presence of antioxidants upon re-warming. There has never been post-cooling seedling survival reported for explants of *T. dregeana*, whilst there have been reports on variable types of growth as survival for *L. sinensis* explants (Fu *et al*., 1993), *Q. robur* explants (Chmielarz, 1997; Berjak *et al*., 1999b) and *S. gerrardii* explants (Berjak *et al*., 2011). Some studies have reported the physiological responses, biochemical responses or both, to various steps of cryopreservation of *T. dregeana* explants (Kioko, 2002; Goveia, 2007; Whitaker *et al*., 2010; Naidoo *et al*., 2011; Varghese *et al*., 2011; Sershen *et al*., 2016), *S. gerrardii* (Goveia, 2007; Berjak *et al*., 2011) and *Q. robur* (Chmielarz, 1997; Berjak *et al*., 1999b). Procedures applied to explants in those studies are similar to the techniques employed in the present one. However, studies on the responses to cryopreservation of *L.*

sinensis zygotic explants were more deviated than methods employed currently (Fu *et al*., 1993; 1994).

Pammenter & Berjak (2014) deemed successful cryopreservation as the retrieval and recovery of explants from a cryogen that subsequently develop and mature into fully functional plants. In light of this characterisation, there are a handful of recalcitrant species that can be considered as successfully cryopreserved where the zygotic explant has been used as the selected explant such as *Hevea brasiliensis* (Normah *et al*., 1986; Normah & Chin, 1995), *Coffea liberica* (Normah & Vengadasalam, 1992), *Artocarpus heterophyllus* (Chandel *et al*., 1995), *Q. robur* (Berjak *et al*., 1999b), *Zizania palustris* (Touchell & Walters, 2000), *Coffea* (Abdelnour-Esquivel & Engelmann, 2002), *Zizania texana* (Walters *et al*., 2002b), various species of Amaryllidaceae (Sershen *et al*., 2007); *E. capensis* (Hajari *et al*., 2011), *L. kirkii* (Kistnasamy *et al*., 2011), *S. gerrardii* (Berjak *et al*., 2011), *Acer saccharinum* (Wesley-Smith *et al*., 2013), and *Acer pseudoplatanus* (Pukacki & Juszczyk, 2015). To summarise the common physiological problems that have been suggested as influencing death or incomplete germination (greening, callus, root elongation, or development of only roots or shoots) during cryopreservation of zygotic explants, studies have identified the following: (i) excision damage to the shoot meristem upon separation of the explant from the cotyledons (Goveia *et al*., 2004; Whitaker *et al*., 2010; Hajari *et al*., 2011 Naidoo *et al*., 2011; Pammenter *et al*., 2011); (ii) osmotic dehydration and damage through cryoprotection and cooling (Finkle *et al*., 1985; Benson, 2008); (iii) turgor loss and destabilisation or loss of macromolecular integrity (Vertucci & Farrant, 1995; Walters *et al*., 2002a); intracellular ice formation upon cooling and re-crystallisation upon warming (Wesley-Smith *et al*., 1995; 2014; 2015), and (iv) membrane related injury through freezing and thawing rates (Ashmore, 1997; Wesley-Smith *et al*., 2004). In the protocol development for explants of the selected recalcitrant-seeded species, optimisation had been made by employing various methods to reduce these common physiology related problems, i.e., explant selection and excision incorporated a portion of cotyledon/endosperm that provided varying levels of protection to the meristematic regions; an optimum desiccation procedure was implemented after testing a variety of drying methods to attain the quickest drying rate (flash drying, air drying over silica gel and vacuum flash drying); and the fastest cooling and rewarming methods were applied to minimise freezing related injury. These efforts which were aimed at ameliorating

the physical and mechanical injury associated with excision, drying and cooling, facilitated high seedling production after excision and partial dehydration which thereafter decreased variably in cryopreserved explants across species.

As the results discussed thus far shows, each species was affected differently by each procedural step. At the final steps of cooling and rewarming, oxidative imbalance may have persistently occurred in some species, and therefore the cumulative damage here is often lethal. The discussion that follows addresses the influence of oxidative state on survival after retrieval from cooling. Cryogenic storage must obviate biochemical, chemical and free radical reactions, however the continued activity of ROS is likely to occur at sub-zero temperatures (Symons, 1982), bearing in mind that the water dynamics at freezing temperature will differ in every species and therefore permit variable levels of activity. In this respect, regardless of the levels of non-freezable water, oxygen is more soluble in water at lower temperatures which contribute to free radical damage during cooling (Benaroudj *et al*., 2001).

After C+RW, $^{\cdot}O_2^-$ and H_2O_2 production exhibited inconsistent patterns across species (Figs 3.32 & 3.33). Antioxidants (Fig. 3.34) and respiratory activity (Fig. 3.35) decreased in all species. There are few studies that showed the oxidative state of recalcitrant zygotic explants after retrieval from cryopreservation (Varghese & Naithani, 2008; Wen *et al*., 2011). Again at this stage, there is no definitive trend to suggest that an increase or decrease in ROS or TAA is directly responsible for survival. Survival of 70% seedling production in explants of *S. gerrardii* was accompanied by an increase in both ROS (Tables 3.18 & 3.19; 18 and 74% for $^{\cdot}O_2^-$ and H_2O_2 respectively) and a decrease (13%) in antioxidants relative to the control (Table 3.20) whereas in explants of *T. dregeana* no survival was accompanied by a 49% and 93% decrease in $^{\cdot}O_2^-$ (Table 3.18) and H_2O_2 (Table 3.19) respectively and an 83% decrease in antioxidants (Table 3.20) in comparison to the control. By comparing the oxidative profiles of surviving cryopreserved explants to those that did not, it was noted that percentage increases or decreases in levels of ROS and TAA after a treatment as compared with the respective controls or with each other, is not indicative of whether tissue will survive a process or not. Similarly, the oxidative profiles of the species that displayed intermediate levels of survival, i.e., *L. sinensis* and *Q. robur*, are not comparable to each other or to either of the other two species. It was however observed that explants of *T. dregeana* which showed

no survival, experienced the highest decrease in antioxidant activity compared with the control across all species after retrieval from cooling; explants of *S. gerrardii* which showed high post-thaw viability experienced the lowest percentage decrease in antioxidant activity across species (Table 3.20). An increase in ROS levels does not necessarily translate into oxidative stress (Kranner *et al*., 2010). It is suggested that ROS produced after all steps of cryopreservation can negatively impact viability, however the extent to which explants recover and cope with ROS production is dependent upon the detection and response to ROS by antioxidants, which results presented here have shown, differed across species. Specifically after cooling and rewarming, stress has accumulated. In explants that do not survive, such as those of *L. sinensis* and *T. dregeana*, ROS production may be so severe that the system cannot recover and has possibly experienced a steady decline in the protective and repair mechanisms, causing irrevocable damage to a number of cells at critical locations which leads to necrosis and death (Kranner *et al*., 2010). To establish if this has occurred in explants of these species, correlative analyses between biomarkers and viability was undertaken and is discussed further below (Section 4.4).

In certain species, explants that are retrieved upon cooling that do exhibit high survival, often show increased antioxidant activity (Lynch *et al*., 2011), whereas other studies show that upon intensive desiccation, the activity of antioxidant enzymes drastically decrease during post freeze-thaw cycles and this correlates with diminished viability (Wen *et al*., 2010). These findings support the correlation between adequate antioxidant systems and post-cooling survival (Table 3.24). Explants of *S. gerrardii* have been successfully cryopreserved previously with inclusion of cathodic water at cryogenic steps (Berjak *et al*., 2011). Despite the precluded survival of cryopreserved *T. dregeana* explants, the optimised use of these antioxidant treatments have promoted viability retention up to the step of desiccation and studies have been conducted to show that without the incorporation of antioxidants, explants do no retain such viability (Naidoo *et al*., 2011; 2016). Antioxidants have not previously been incorporated during the selected steps of cryopreservation used here for *Q. robur* and *L. sinensis* explants. Results from the present contribution supports the suggestions of others that exogenous application of antioxidants promotes viability during procedural steps of cryopreservation and should form part of standard protocol development (Uchendu *et al*., 2010a; 2010b; Reed *et al*., 2012; Reed, 2014) for explants excised from recalcitrant seeds.

At the ultrastructural level, cryopreserved explants of *T. dregeana* exhibited complete ultrastructural degradation (Fig. 3.36a & b) and this was consistent with no *in vitro* survival (Figs 3.32-3.35). Cryopreserved explants of the remaining species featured cellular characteristics in line with post-cryo survival (*Q. robur*, Fig. 3.37a & b), (*S. gerrardii*, Fig. 3.38) and (*L. sinensis*, Fig. 3.39a & b), though there were varying levels of survival across these species (Figs 3.32-3.35). Both desiccation and freezing impacts ultrastructure during cryopreservation and it is important to comparatively assess the cellular state between dried and cryopreserved explants. Often, changes can be induced by drying that are not severe enough to affect viability, but can be accentuated during freezing and induce cell death (Alla-N'Nan *et al*., 2014), for e.g., shoot meristems of soaked+FD *L. sinensis* explants featured ultrastructure indicative of cellular survival after desiccation (Fig. 3.31a & b), but displayed characteristics of damage that could have contributed to cell death during cooling and rewarming (Fig. 3.39a & b). Shoot meristems of cryopreserved *L. sinensis* explants (Fig. 3.39a) featured no changes in the conformation of the nucleus compared with dried explants (Fig. 3.31a), suggesting the maintenance of a high level of nuclear activity during drying and cooling. However, electron translucent mitochondria and plasmolysis of the cell membrane where observed (Fig. 3.39b) which indicates a certain extent of damage. The profound degradation of cellular architecture in shoot meristems of cryopreserved *T. dregeana* explants and features such as the receding of the plasmalamella from the cell wall in *L. sinensis* are indicative of freezing injury (Wesley-Smith *et al*., 1995) to which germplasm from many tropical recalcitrant seeds are susceptible (Kioko *et al*., 2006). The ultrastructure of shoot meristems of cryopreserved *L. sinensis* explants indicate that lack of proper shoot development is caused by damage incurred at a subcellular level. The physiological responses of *L. sinensis* explants, indicated by viability and ultrastructure during drying and cooling, suggest that explants of this species are more cooling than desiccation sensitive.

Trichilia dregeana explants also featured indications of surviving, actively respiring tissue after drying (Fig. 3.28a & b) but displayed complete destruction of cellular architecture indicated by absence of organelles, plasmolysis of the cell membrane, wide periplasmic spaces and the retraction of the cytoplasm into the cell during cooling (Fig. 3.36a & b). All of these features are often irreversible and underlie necrosis and malformation of cells which invariably translate to cell death (Pammenter *et al*., 1998). In this species, viability is reduced

after drying and completely inhibited upon cooling, and ultrastructural assessment after these steps suggest that the cooling step of cryopreservation caused physical damage which was a prominent factor underlying complete cell death in cryopreserved explants. In both species that do not produce plantlets after C+RW (*L. sinensis* & *T. dregeana*), despite ultrastructural integrity after partial dehydration, it is proposed that the stress induced by synergistic interaction of drying and cooling stress are lethal to tissue, as suggested by Berjak *et al*. (1999b) during cryopreservation of *Q. robur* embryos.

Cryopreserved explants of *S. gerrardii* and *Q. robur* exhibited 70 and 60% seedling production, respectively. The decline in viability after C+RW compared with soaked+FD explants of these species was substantially different i.e., by 5% in *S. gerrardii* explants and 20% in *Q. robur* explants. The decline in viability between these steps suggests that explants of *S. gerrardii* were more resilient to freezing stress than those of the temperate *Q. robur*. Ultrastructural images of cryopreserved *Q. robur* explants (Fig. 3.37a & b) featured many well developed mitochondria (Fig. 3.37b), plastids and starch bodies (Fig. 3.37a) which were also present in soaked+FD explants of this species (Fig. 3.29a & b). However, a higher degree of vacuolation with visible inclusions was observed in cryopreserved explants, indicative of a response to stress. This was in contrast to the situation in soaked+FD explants where relatively few vacuoles were dispersed. Cryopreserved explants also featured relatively smaller, stacked endoplasmic reticulum (Fig. 3.37b) compared with the longer profiles observed in soaked+FD explants (Fig. 3.29a & b). Stacking or splitting of the endoplasmic reticulum can sometimes occur following cold stress, as reported in cryopreserved embryos of the recalcitrant-seeded *Cocos nucifera* L. (Alla-N'Nan *et al*., 2014). These are further indications of a greater sensitivity to cooling compared with drying in *Q. robur* explants and also indicate that ultrastructural abnormalities can feature even in explants surviving C+RW (Sershen *et al*., 2012a; b). *Strychnos gerrardii* explants showed a similar frequency and compactness of vacuoles, lipids and starch bodies in soaked+FD (Fig. 3.30) and cryopreserved (Fig. 3.38) explants. No abnormalities or damage was visible in the ultrastructure of explants after either of these steps, consistent with the high viability retention in soaked+FD and cryopreserved explants. The state of cellular ultrastructural after both procedural steps suggests that *S. gerrardii* explants are comparably tolerant to both procedures.

The retention of post-cooling viability by *Q. robur* and *S. gerrardii* explants may be permitted by physiological and anatomical characteristics which are different from those of *T. dregeana* and *L. sinensis*. These factors will be discussed further when assessing relationships between explant characteristics and post-cooling survival.

4.3 Physiological characteristics of explants during cryo-preparative steps and the relationship with post-cryo viability across species

To further understand relationships between physiological responses and post-cooling viability, some factors observed to influence survival after excision and partial dehydration were related to post-cooling survival.

Explant shedding WC, post excision soak WC and fresh mass of zygotic explants (Table 3.1 & 3.22) showed no significant correlations with post-cooling viability across species. However each species exhibited a certain set of characteristics that could be related to the level of survival after cooling. For example, *Trichilia dregeana* explants exhibited the highest shedding and post soak WC across species (Table 3.1 & 3.22). The explant physiology was unique compared with those of other species, as the shoot meristem was exposed during desiccation and cooling. Explants of this species were the only to show no post-cooling viability. Explant viability in *T. dregeana* was the lowest across species throughout various steps of cryopreservation and exhibited a steady decline during all procedural steps far greater than that observed for the other three species in the study. An appraisal of anatomy of recalcitrant-seeded explants that have been successfully cryopreserved such as those investigated in this study (*S. gerrardii* and *Q. robur*) and in others i.e., *C. nucifera* (Assy-Bah & Engelmann, 1992), *L. kirkii* (Kistnasamy *et al*., 2011), *H. montanus* (Sershen *et al*., 2012c), *A. belladonna* (Sershen *et al*., 2012c) and *A. saccharinum* (Wesley-Smith *et al*., 2014) etc. have shown that zygotic embryos exhibiting post-cooling seedling production did not sustain physical injuries to the meristematic region upon excision, unlike in *T. dregeana* explants. Explants of the remaining species all exhibited shoot meristematic regions partially or completely concealed by cotyledons (*S. gerrardii* & *Q. robur*) or endosperm (*L. sinensis*) illustrated in Fig. 3.1 and described in Table 3.22.

There are some other contributing physiological factors towards seedling production in cryopreserved *S. gerrardii* and *Q. robur*. Explants of *S. gerrardii* exhibited the smallest fresh

mass across species (Table 3.1) and comprised of papery thin cotyledons and only a slightly thicker embryo (Fig. 3.1). This combination of anatomical characteristics would facilitate rapid cooling of the explants, as size and mass (fresh and dry) have been reported to affect how rapidly recalcitrant-seeded explants are cooled (Wesley-Smith *et al*., 2013; Xia *et al*., 2014). *Quercus robur* explants, while heavier and larger than *S. gerrardii* explants (Table 3.1) have a greater uniformity in tissue type relative to explants of the remaining species. Cotyledons comprise almost the entire explant with a substantially smaller plumule (shoot meristem) partially concealed within it (Fig. 3.1) Heterogeneity of cellular tissue of the selected explant can affect post-cryo survival (Nadarajan & Pritchard, 2014). While *Q. robur* explants are not as homogenous or undifferentiated as tissues such as shoot meristems or shoot apices, these explants are more homogenous in explant tissue relative to *S. gerrardii*, *T. dregeana* (embryo & portions of cotyledon) or *L. sinensis* (embryo & endosperm tissue) which may limit ice nucleation events during cooling. The tissue composition of the explant and the absence of incisions or cut surfaces during excision are suggested to be contributing factors towards post-cooling seedling production in *Q. robur* explants.

Explants of *L. sinensis* exhibited incomplete growth post-cooling i.e., 40% callus development at the shoot meristems and root production. Physiological and anatomical characteristics suggested to limit survival to this type of growth is the largest fresh mass of these explants (Table 3.1) due to the attachment of endosperm tissue surrounding the embryo and the heterogeneity of the whole explant. Freezing point temperature can vary between mature seed embryo and endosperm tissue (Woltz *et al*., 2005) and if this occurred in *L. sinensis* explants the differential or rather uneven cooling rate may have affected the type of growth.

Drying characteristics during partial dehydration are also suggested to influence post-cryo explant viability. Results on physical characteristics during drying (Table 3.23) showed no significant correlations between explant WC post soak, explant target WC or drying rate with post-cooling viability. It is worth noting that (i) *T. dregeana* explants which exhibited the highest post-soak and target WC, showed no post-cooling growth and (ii) *S. gerrardii* explants exhibiting the quickest drying rate and shortest drying period showed the highest post-cooling survival. These factors, while observed to have an inconsistent influence on post-cooling viability across species, are recognised to play a contributing role in

cryopreserved explant viability of *T. dregeana* and *S. gerrardii*. A significant negative correlation between the amount of water lost during drying to target WC and post-cooling viability was measured (Fig. 3.40). In explants of *T. dregeana*, *S. gerrardii* and *Q. robur*, the increase in the amount of water lost during drying to target WC (Table 3.23) corresponds to decreasing viability in soaked+FD explants. In this respect, the sensitivity of these explants to water loss during dehydration is correspondingly reflected in their sensitivity to cooling. This suggests that post-cooling viability in explants of these species are influenced by the sensitivity to water loss during dehydration. Explants of *L. sinensis* did not show sensitivity to water loss during dehydration to target WC, indicated by full viability retention post-drying. Therefore, the amount of water lost during drying in this species may affect post-cooling viability but did not influence post-drying viability.

4.4 Explant redox metabolism and the relationship with viability during procedural steps of cryopreservation

When considering cryopreservation stresses in terms of redox biology, it is important to ascertain cause and effect. As Benson (2000) stated, all aerobic organisms are dependent upon oxygen, free radical mediated reactions and protective mechanisms in the form of antioxidant defences. However, the endogenous antioxidant capacity of cells is finite, whilst free radicals are unhindered in their ability to perpetually react with and attack cellular macromolecules. Oxidative stress occurs when the shift in balance between antioxidant and oxidants (ROS) moves in favour of the oxidant (Birben *et al*., 2012). To prevent cellular damage, all aerobic organisms should remain in a state of a pro-oxidant/antioxidant balance (Couée *et al*., 2006). Quantifying oxidative stress during cryopreservation is very complicated due to: (i) the numerous cellular reactions ROS takes part in; (ii) complicated spatial distribution of ROS across many organelles in the cell; (iii) the heterogeneity of cells and tissues across explants and species and (iv) the rapidly changing free radical processes according to the physiological state of the tissue (Luschak, 2014). After each procedural step of cryopreservation, no definitive trend in increasing or decreasing antioxidants, ROS or respiratory activity correlated with a consistently corresponding increase or decrease in viability. Each species had a different initial or 'steady state' balance between ROS and antioxidants in control explants. Therefore, absolute levels of ROS, TAA and respiratory activity in this study were compared with their respective controls. Comparisons between

species were only made when these parameters were measured as a percentage increase or decrease of the control. These comparisons within and across species still showed no definite trend in the relationship between the effect of the treatment on viability or the effect of the treatment on ROS, TAA and respiratory activity. It is not uncommon for researchers to have difficulty quantifying oxidative stress in biological systems, as the same stress inducing events can either increase, decrease or have no observable effect on ROS and antioxidants yet a stressed oxidative state is declared (Lushchak, 2014). Furthermore, due to ROS having positive roles in signalling pathways (Bailly, 2004; Mittler, 2016), a measure of ROS levels are not adequate to indicate cellular damage. It is more important to ascertain if the antioxidant system is sufficiently functional to regulate ROS at levels which do not cause damage, and these levels may be specific to explant types and species. Despite high reactivity, the negative physiological impacts of high levels of ROS only occur in the absence of efficient neutralisation systems operating in concert with processes that repair and eliminate ROS-modified molecules (Lushchak, 2014; Das *et al*., 2015). This situation was exemplified by *S. gerrardii* and *L. sinensis* explants after soaking, where excessively high increases in H_2O_2 were measured (Table 3.6) concomitant with small decreases in TAA (Table 3.7) and full viability retention.

In cryopreservation of recalcitrant germplasm it has often been concluded that an increase in ROS is associated with a negative biological response which is almost always characterised as a decrease in viability compared with the control (Roach *et al*., 2008; Varghese & Naithani, 2008; Whitaker *et al*., 2010; Berjak *et al*., 2011; Naidoo *et al*., 2011). In the present study, the effect of cryopreservation procedures on ROS and TAA was assessed after the incorporation of ascorbic acid and cathodic water in the protocol design. Therefore the effect on the oxidative state and viability that is measured here is a reflection of a species' ability to cope with cryogenic stresses with the aid of exogenously applied antioxidants. The viability retention of explants of the investigated species after procedural steps of cryopreservation is higher or equal to those achieved by studies which implemented protocols without the presence of antioxidants. There were variable effects on the oxidative state across species and treatments. Specifically in explants of *S. gerrardii*, an increase in both $^{\cdot}O_2^-$ and H_2O_2 was consistently measured (Table 3.6, 3.10, 3.11, 3.14, 3.15 & 3.18) despite high viability

retention throughout cryopreservation and the highest survival after retrieval from cooling being retained by explants of this species.

In order to ascertain the effect of procedural steps on stress biomarkers and the corresponding influence on viability, comparative and correlative analysis of $^{\cdot}O_2^-$, H_2O_2, TAA, respiratory activity and TAA: ROS within and across species during procedural steps were conducted. The outcomes of these analyses provided a few significant results.

In explants of *T. dregeana*, *L. sinensis* and *Q. robur*, $^{\cdot}O_2^-$ production was the highest after C+RW, the treatment after which viability was the lowest across species (Fig. 3.41a-c). However, in *S. gerrardii* explants this biomarker was the highest after flash drying (Fig. 3.41d), despite the quickest drying period (20 min). Superoxide production showed a significant negative correlation with viability during procedural steps across species (Table 3.28) but not within species (Fig. 3.41), indicating the role of this ROS in viability reduction across investigated species in this study.

There was no significant correlation observed between H_2O_2 production with viability across (Table 3.28) or within species (Fig. 3.42). However, in response to procedural steps, H_2O_2 production was noted to decrease relative to the control in both non-endospermic seed species viz., *T. dregeana* (Fig. 3.42a) and *Q. robur* (Fig. 3.42c) and increase or remain the same in both endospermic species viz., *L. sinensis* (Fig. 3.42b) and *S. gerrardii* (Fig. 3.42d). There are no studies to date to suggest that the different responses of H_2O_2 levels to cryogenic steps between germplasm from endospermic and non-endospermic recalcitrant seeds is a consequence of seed tissue composition. However, this was the response of this biomarker observed in this study.

Total aqueous antioxidant capacity showed a significant positive correlation with explant viability during procedural steps across species (Table 3.28) but not within species (Fig. 3.43). Total aqueous antioxidant capacity across species was observed to decline relative to the control after all procedural steps. This decline was consistently significant in explants of both non-endospermic species (Figs 3.43a & c). In both endospermic species, TAA capacity declined significantly only in cryopreserved explants of *L. sinensis* (Fig. 3.43b) and in both soaked and cryopreserved explants of *S. gerrardii* (Fig. 3.43d). The highest TAA capacity in *S. gerrardii* explants occurred after soaking+FD, which was the same step after which the

highest $^{\cdot}O_2^-$ levels were measured. It was also noted that TAA levels in control explants of *T. dregeana* (Fig. 3.43a) was almost three times lower than TAA levels in control explants of the remaining species. This may implicate inherently lower levels of TAA in *T. dregeana* explants as a factor towards the lowest explant survival across procedural steps across species.

There was no significant correlation observed between respiratory activity with viability across (Table 3.28) or within species (Figs 3.44) during procedural steps. However, the metabolic state of explants across species significantly declined relative to the control in response to partial dehydration and C+RW. Recalcitrant-seeded germplasm is most sensitive to desiccation when metabolic activity is high (Berjak *et al*., 1999a) and a reduction in metabolism specifically during partial dehydration can contribute towards lessening sensitivity to desiccation and facilitating viability across species.

These analyses suggest that increasing levels of $^{\cdot}O_2^-$ production reduce explant viability while elevated levels of TAA promote explant survival during procedural steps of cryopreservation. Hydrogen peroxide production and respiratory activity do not show a significant impact on viability during procedural steps across species but do show certain trends that have not been previously reported.

The measure of TAA: ROS did not significantly correspond to viability within individual species (Figs 3.45-3.48) but did show a significant positive correlation with viability across species when the data was pooled (Table 3.28). However, a decline in viability during cumulative steps of cryopreservation was observed to consistently correspond to a decline in this ratio across species (Figs 3.45-3.48). The exception was soaked+FD *L. sinensis* explants where neither viability nor TAA: ROS ratio declined after soaking (Fig. 3.47). This result further supported the resilience of *L. sinensis* explants towards oxidative and viability changes effected by drying. The highly significant correlation between TAA: ROS and viability during procedural steps (Table 3.28) across species suggest that higher levels of antioxidants, whether induced or constitutive, afford recalcitrant-seeded explants a greater resilience to cryopreservation stresses.

This study has outlined species-specific physiological responses that play a role in maintaining viability during procedural steps of cryopreservation. In terms of the influence of

redox metabolism on viability, $^{\cdot}O_2^-$ production, TAA and the specific balance between TAA: ROS such that adequate antioxidants are present to regulate ROS during cryopreservation are suggested to be contributing factors towards survival during recalcitrant-seeded explant cryopreservation.

CHAPTER 5: Conclusions and recommendations

One of the major products of this study was the development of cryopreservation protocols for zygotic explants of four recalcitrant-seeded species that incorporate steps which ameliorate some of the stresses identified to occur during cryopreservation. The key conclusions of this research are presented below.

The ameliorative measures undertaken to abate physical injury during explant excision and to limit oxidative stress during some of the procedural steps (e.g., excision and partial dehydration) reduced ROS production, and this was accompanied by relatively high post-cryo viability in *S. gerrardii* (70%) and *Q. robur* (60%). However, despite efforts to minimise injury and oxidative stress during cryo-preparative steps, *L. sinensis* and *T. dregeana* explants did not exhibit post-cryo survival (in terms of seedling production).

The procedural steps of excision, partial dehydration and cooling each inflicted stress which was evidenced by variable losses in ultrastructural integrity, antioxidant capacity and uncontrolled ROS production. Though all species survived excision and partial dehydration relatively well (≥50% survival across all species) these steps appear to predispose these explants to even more damage during subsequent steps and eventual post-cryo viability loss. However, the use of an antioxidant soak and techniques employed during excision and partial dehydration prior to rapid cooling and rewarming facilitated post-cryo seedling development of explants from two of the investigated species.

The disparity in extent and type of post-cryo survival achieved across species is largely attributed to differences in physiological and anatomical characteristics such as tissue architecture, incidence of physical injury during excision and loss of water during partial dehydration. Furthermore, explant redox balance was shown to influence explant survival. Total aqueous antioxidant capacity, $^{\cdot}O_2^-$ production and TAA: ROS status showed a strong correlative relationship with explant viability across species during all procedural steps. More specifically, the results suggest that an increase in TAA and TAA: ROS and a decrease in $^{\cdot}O_2^-$ production were positively correlated with explant viability.

Explant shoot meristem ultrastructure did not reveal signs of the stresses induced by explant excision and partial dehydration across species. Explants possessed a high degree of

ultrastructural integrity across species during these cryo-preparative steps. However, as alluded to above, partial dehydration may have predisposed explants to even greater damage during cooling, evidenced by varying levels of derangement in the ultrastructure of cryopreserved explants particularly, those of *L. sinensis* and *T. dregeana*.

The optimised protocols presented for *S. gerrardii* and *Q. robur* in this study include modified explant excision, antioxidant protection and rehydration and rewarming methods. The use of exogenously applied ascorbic acid prepared in cathodic water during procedural steps was particularly important to improve germination in *T. dregeana* explants and improve vigour across species after explant excision. However, these modified methods may have to be further improved to achieve post-cryo survival in *T. dregeana* and *L. sinensis* explants. Cryoprotection was deemed unnecessary and unsuitable for explants across species, owing to the fact that it often increased explant WC prior to dehydration and reduced viability, which would likely predispose explants to further stress during partial dehydration and cooling.

The key contradictions observed in this study were related to the relationship between ROS, antioxidant capacity and viability during procedural steps. Results constantly showed that increases or decreases in these biomarkers did not have a predictable effect on viability across species during procedural steps, i.e., in some cases, high ROS levels were associated with high explant viability whilst in other cases high ROS production was related to low viability. It has often been noted that ROS at low levels function as important signalling molecules and at high levels cause programmed cell death (PCD) (Bailly, 2004). Antioxidants modulate this dual function by either eliminating toxic ROS when levels become highly elevated under a stress conditions or tightly regulate ROS under normal conditions, which theoretically results in a cellular homeostatic state (Bailly, 2004). While this reasoning is widely accepted and supported by physiological, metabolic, proteomic and genomic studies, the absolute levels at which ROS are deemed to be ‘high’ and ‘low’ in seed tissues remains unknown. In the present study, while elevated ROS levels were noted to correspond to high viability in some cases, antioxidant levels increased in parallel. Therefore, the statement that all ROS are harmful to organisms at high concentrations can only hold true if high concentrations overwhelm antioxidant capacity. The TAA: ROS ratio data presented in Chapter 3 lend support to this suggestion in the context of the plant tissues and stresses investigated here. Various aspects of this research were challenged by the fact that working with recalcitrant-

seeded species only remain viable for a short period of time and their availability is seldom consistent across seasons. This limited the levels of replication that could be achieved for a number of the assays which resulted in very few datum points being used in some of the correlations analyses. This reduced the power of many of these statistical analyses and may explain why a number of the correlations were not significant even when trends were evident. This is evidenced by the fact that when data for biomarkers and viability were pooled across species, many of the correlations were significant.

This study proposes recommendations for future research with a focus on a few key aspects. For both *L. sinensis* and *T. dregeana* the lack of survival suggests that the potential of using alternate explants such as shoot meristems from developed seedlings may have to be explored. As tissue architecture and composition of the zygotic explant in both these species were highlighted as contributing factors towards post-cryo death, shoot meristems may be more amenable to cooling due to the homogeneity of cells and substantially reduced size of the explant relative to zygotic embryos. The use of alternate explants, produced using a variety of *in vitro* techniques, for cryopreservation has been successfully applied to recalcitrant-seed germplasm of a few species (*Trichilia emetica*, [Varghese *et al*.,2009]; *Quercus robur* [Chmielarz *et al*., 2011] and *Ekebergia capensis* [Hajari *et al*. 2011]).

The most useful way to relate oxidative metabolism to viability during the various procedural steps in this study was via the used of the TAA: ROS ratio. Therefore it is recommended that future investigations on recalcitrant-seeded species use this ratio to optimise cryopreservation protocols.

In the present study, respiratory activity measured via the TTZ test, assessed the reaction between dehydrogenases and substrates that release hydrogen ions which are subsequently released into the soluble TTZ salt solution. These ions enzymatically reduce the TTZ to 1,3,5 triphenyl formazan (TPF) which accumulates in respiring tissue. The biochemical measurements of these events were used to establish the metabolic activity of explants during procedural steps. The state of metabolic activity is influenced by ROS and antioxidant activity and in this study it was observed during some steps that a reduction in the oxidative biomarkers corresponded to a reduction in metabolic activity and high explant viability. To further establish the relationship between ROS and antioxidants with respiration in explants,

a more current technique, i.e., the polarographic method could be implemented for future studies (Derevyanchuk *et al*., 2016). This may offer more meaningful and conclusive results on the effect of oxidative biomarkers on alternate and main respiration pathways during different procedural steps and further elucidate the mechanisms of plant respiration pathways in regulating ROS and antioxidants.

The TAA assay employed in this study has a short coming of not discriminating between enzymatic and non-enzymatic antioxidants, therefore one cannot identify failures or dysfunction of specific antioxidants within the antioxidant system. Assays that measure the activity of specific enzymatic (catalase, superoxide dismutase and ascorbate peroxidase) and non-enzymatic compounds (ascorbate and reduced glutathione) would provide a more in-depth assessment of failures which can then be used as a basis for more specific ameliorative measures. Furthermore, the influence of the relationship between TAA: ROS and viability could be assessed in conjunction with the influence of procedural steps on the half-cell reduction potential of glutathione [E (GSSG: 2GSH)] in explants, which has been documented as a useful plant stress biomarker (Foyer & Noctor, 1998; Kranner *et al*., 2006; Varghese *et al*., 2011; Moothoo-Padayachie *et al*., 2016).

In keeping with the current use of molecular techniques to elucidate the basis for stress coping mechanisms plants, the next step towards improving recovery of explants after cooling would be to use studies based on desiccation tolerant systems to identify the genes responsible for coping with oxidative stress and to investigate how these may be enhanced in sensitive material. For example, non-phosphorylating alternative respiratory pathways mediated by NAD(P)H dehydrogenase and alternative oxidase have been linked to minimising the formation of destructive ROS during stress in tobacco plants and *Arabidopsis thaliana* (Smith *et al*., 2009; Panda *et al*., 2013). Alternatively, and possibly more feasible, would be the identification of genes that are upregulated during stress in recalcitrant species that show good survival after cryogenic procedures, such as *S. gerrardii* (Berjak *et al*., 2011) and various species of amaryllids (Sershen *et al*., 2007) and to investigate whether these can be over-expressed in less cryo-tolerant species.

Ultrastructural investigations from this study showed that cooling imposed a stresses that was overcome in *S. gerrardii* and *Q. robur* explants but not in *L. sinensis* and *T. dregeana*

explants, indicated by the lack of explant survival. The ability to cope with cooling stress is related to explant anatomical and tissue characteristics, where certain characteristics afford even and rapid cooling which ultimately minimise intracellular ice formation. Wesley-Smith *et al*. (2015) reported that the frequency, location and size of ice crystals formed during freezing influence cell survival and these variables may be related to PCD responses. Further ultrastructural studies should investigate ice formation characteristics during cooling across recalcitrant species that show variability in post-cryo explant viability, to elucidate the possible influence of these events on survival. Thereafter, partial dehydration and cooling methods may be adjusted to manipulate such characteristics in a way that promotes survival.

The outcomes of this study have made significant advances towards the characterising and alleviating some of the physiological and biochemical stresses associated with the cryopreservation of recalcitrant zygotic embryos. The key findings have provided a basis for further avenues of research that, if pursued, may improve our ability to successfully cryopreserve the seed-derived explants of recalcitrant-seeded species.

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