An estimation of oxidative metabolism in relation to desiccation tolerance, chilling sensitivity and hydrated storage lifespan of recalcitrant seeds from tropical and temperate provenances

by

CHANDIKA RAMLALL

208505701

Submitted in fulfilment of the academic requirements of

Master of Science

of Biological Sciences

School of Life Sciences

College of Agriculture, Engineering and Science

University of KwaZulu-Natal

Durban

South Africa

March 2017

PREFACE

The research contained in this dissertation was completed by the candidate while based in the School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, South Africa. The research was financially supported by National Research Fund.

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.

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Signed: Prof. N. W. Pammenter

Date: 31 March 2017

_____

Signed: Dr B. Varghese

Date: 31 March 2017

Deceased_____

Signed: Prof. P. Berjak

Date: 31 March 2017

DECLARATION 1: PLAGIARISM

I, Chandika Ramlall, 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;

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(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;

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Date: 31 March 2017

DECLARATION 2: PUBLICATIONS AND CONFERENCE PROCEEDINGS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, in press and published and give details of the contributions of each author to the experimental work and writing of each publication).

Publication 1: (In preparation) Ramlall, C., Berjak, P., Pammenter, N.W., Varghese, B., 2017. An estimation of oxidative metabolism during hydrated storage of recalcitrant-seeded species at 3 and 16°C.

Publication 2: (In preparation; short communication) Ramlall, C., Berjak, P., Pammenter, N.W., Varghese, B., 2017. Desiccation sensitivity, drying rates and associated oxidative metabolism of tropical and temperate recalcitrant-seeded species.

I carried out all the experimental work and wrote these papers and my supervisors provided input on draft copies.

Conference poster presentation: Ramlall, C. 2016. An estimation of oxidative metabolism during hydrated storage of recalcitrant-seeded species from temperate and tropical provenances at 3 and 16°C. *7th International Workshop on Desiccation Sensitivity and Tolerance Across Life Forms*, Cape Town, South Africa.

Signed: ....

Chandika Ramlall

31 March 2017

ABSTRACT

Recalcitrant seeds are shed metabolically active, are desiccation sensitive and susceptible to chilling and cryo-injury. There are varying degrees of desiccation tolerance, chilling sensitivity and storage lifespan between seeds of tropical and temperate provenance. Since recalcitrant seeds cannot be stored using conventional methods, the only short- to medium-term storage approach, referred to as "hydrated storage", is possible if the water content is maintained as close to that at shedding, and at ambient or slightly reduced temperatures. Due to their ongoing metabolic activity, seeds undergo germinative metabolism in storage, which often results in a mild to increasingly severe water stress from the required additional water not being supplied. The deleterious events associated with this stress result in unbalanced metabolism and generation of reactive oxygen species (ROS), which may reduce storage lifespan and contribute to seed death. Oxidative stress is associated with a loss of water; initial dehydration can lead to an increased mobilisation of antioxidant systems which enables efficient ROS sequestration; however, further dehydration results in un-coordinated metabolism and subsequent loss of viability. This study examined the possible differences in viability retention and the oxidative metabolism of embryonic axes of seeds from tropical and temperate provenances when rapidly dried to various water contents and when stored hydrated at 3 and 16°C for up to 18 months. The results suggest that seeds of tropical origin, *Trichilia dregeana* and *Artocarpus heterophyllus* are more desiccation and chilling sensitive than seeds of temperate origin, *Camellia sinensis* and *Quercus robur*. This is indicated by a substantial loss in viability when rapidly dried to lower water contents (<0.4 g g^{-1} DW) and reduced storage duration (4-6 months) at 3°C, respectively. Drying rates seemed species-specific rather than provenance related, possibly due to the morphology of the axis. *A. heterophyllus* displayed the fastest drying rate of 0.1419 g H$_2$O lost per min, as well as shortest drying time-course of 90 min, whilst *Q. robur* exhibited the slowest drying rate of 0.0147 g H$_2$O lost per min, and required 9 hours of drying to reach a water content of 0.1 g g^{-1} DW. Estimations of oxidative metabolism at shedding, and at selected water contents of 1.0, 0.3 and 0.1 g g^{-1}DW, showed a variable response to desiccation between provenances, however, a reduction in water content resulted in an average increase in ROS production; extracellular superoxide: *C. sinensis*: 5.3 $\pm$ 1.5 nmol g^{-1} DW s^{-1}, *Q. robur*: 3.6 $\pm$ 0.9 nmol g^{-1} DW s^{-1}, *T. dregeana* 11.3 $\pm$ 4.9 nmol g^{-1} DW s^{-1} and *A. heterophyllus* 2.5 $\pm$ 0.8 nmol g^{-1} DW s^{-1}; extracellular hydrogen peroxide: *C. sinensis*: 0.058 $\pm$ 0.041 µmol g^{-1} DW s^{-1}, *Q. robur*: 0.009 $\pm$ 0.008 µmol g^{-1} DW s^{-1},

T. dregeana 0.044 ± 0.020 µmol g^{-1} DW s^{-1} and *A. heterophyllus* 0.003 ± 0.002 µmol g^{-1} DW s^{-1} and total aqueous antioxidant activity *C. sinensis*: 23.3 ± 7.3 µmol g^{-1} FW s^{-1}, *Q. robur*: 24.8 ± 6.6 µmol g^{-1} FW s^{-1}, *T. dregeana* 14.0 ± 3.4 µmol g^{-1} FW s^{-1} and *A. heterophyllus* 19.0 ± 3.8 µmol g^{-1} FW s^{-1}), when axes from freshly harvested seeds were compared with axes dried to 0.1 g g^{-1} DW. Extended periods of hydrated storage, >10 months at 3°C (for *Q. robur*) and 16°C (for *C. sinensis, T. dregeana* and *A. heterophyllus*), resulted in heightened extracellular superoxide production in axes of *Q. robur* seeds stored for 12 months at 3°C: 1.2 ± 0.7 nmol g^{-1} DW s^{-1}, *C. sinensis* seeds stored for 18 months at 16°C: 3.6 ± 0.9 nmol g^{-1} DW s^{-1}, *T. dregeana* seeds stored for 18 months at 16°C: 0.4 ± 0.1 nmol g^{-1} DW s^{-1} and *A. heterophyllus* seeds stored for 15 months at 16°C: 2.5 ± 1.2 nmol g^{-1} DW s^{-1}, when axes from freshly harvested seeds were compared with axes from seeds stored for various time intervals stated above. Estimations of extracellular hydrogen peroxide and total aqueous antioxidant activity showed variable responses throughout storage to both temperature and longevity irrespective of provenance. However, at 3°C, extracellular superoxide and hydrogen peroxide levels in tropical recalcitrant-seeded species displayed a characteristic bell-shaped curve during storage as viability declined to 0%. Fungal proliferation and germination in storage were contributors to reduced storage period for seeds of *Q. robur* and *T. dregeana*, particularly at 16°C, however, *Q. robur* seeds were most affected by fungal contamination at both storage temperatures. The optimum conditions for storage of *Q. robur* seeds are at 3°C, non-hydrated, since hydrated storage resulted in germination at both temperatures. For *C. sinensis* seeds, hydrated storage at 3°C resulted in the highest viability retention after 8 months (>80%). Tropical recalcitrant-seeded species stored hydrated at 16°C for 6 months showed a minimal reduction in viability (>80%), whilst at 3°C there was a complete loss of viability, when compared for the same period. Viability gradually decreased with an increase in storage time when seeds were stored at 16°C, irrespective of provenance. Although the effects of germination-induced free-radical production cannot be ignored, further investigations are required to elucidate the potential effects of oxidative stress while desiccation sensitive seeds are stored hydrated.

ACKNOWLEDGMENTS

"Just because you have taken longer than others,

does not mean you have failed"

Author unknown

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CHAPTER 1: INTRODUCTION

1.1   Biodiversity conservation

South Africa is mega-diverse due to the variety in plant and animal endemism, contributing to 6% of the worlds' plant biodiversity (Berjak *et al.*, 2011a; Raimondo, 2015; Thuiller *et al.*, 2006). Of South Africa's nine terrestrial ecosystem types, the South African National Biodiversity Institute (SANBI) has estimated that 40% are threatened, with 9% Critically Endangered, 11% Endangered and 19% Vulnerable. Although 25% of identified bioregions are well protected, 35% have no protection (Driver *et al.*, 2012; Raimondo, 2015). There are currently 2576 plant species that are threatened with extinction (Raimondo, 2015). There are numerous factors threatening biodiversity loss, such as human-induced factors, increased population growth and urbanisation along with the associated anthropogenic activities to maintain communities, habitat loss and land transformation for agriculture and commercial forestry, over-exploitation of natural resources, particularly by indigenous and local communities for use in traditional medicine, extensive spread of alien invasive species and unpredictable effects of climate change (Berjak *et al.*, 2011a; King *et al.*, 2007; von Ahlefeld and Gordon-Gray, 2003).

In 2010, South Africa endorsed an updated version of the Global Strategy for Plant Conservation and the adoption of Decision X/17, and since has developed 16 outcome-oriented targets in order to improve plant conservation for the 2011-2020 period (Raimondo, 2015). This report details the work necessary, from a taxonomic level, to conservation strategies and finally a socio-economic aspect, in order to conserve plants both *in situ* and *ex situ* (Raimondo, 2015).

Whilst *in situ* and *ex situ* conservation strategies each have their merits, there are fundamental differences between the two. *In situ* conservation involves maintaining populations of plant species under natural conditions, thus enabling evolutionary events to occur, but is more difficult to achieve due to the maintenance cost of national parks/reserves and protected areas in wildlife sanctuaries and is both land and labour-intensive (Engelmann, 2012; Engelmann and Engels, 2002; Maxted and Ambrose, 2001). *Ex situ* conservation strategies involve the preservation and maintenance of regenerative plant propagules harvested from their natural habitat and stored under artificial conditions. These collections are easier to maintain and manipulate, and are more cost-effective, but do require skilled personnel (Paunescu, 2009). Storage of pollen and tissue or cell cultures are often achieved

after collection using *in vitro* manipulations implemented via slow-growth techniques or cryopreservation (Engelmann, 2012; Wang *et al.*, 1993). Conservation of whole plants, seeds and vegetative propagules are maintained and regenerated in botanical gardens, nurseries, and seed banks (Engelmann, 2012; Maunder *et al.*, 2004; Paunescu, 2009; Wang *et al.*, 1993). *In situ* and *ex situ* methods are not exclusive, but should be used in conjunction to facilitate improved conservation strategies of plant biodiversity (Engelmann, 2012; Engelmann and Engels, 2002). Proper selection and adoption of appropriate strategies should consider the biological nature of the species and the feasibility of chosen methods (Engelmann, 2012; Engelmann and Engels, 2002; Li and Pritchard, 2009; Wang *et al.*, 1993).

1.2   Seed banking

Seed banking is one of the cheapest forms of conserving plant germplasm (Engelmann *et al.*, 2003). This type of storage method is determined by, but not limited to, a combination of plant ecology, associations between plant, fruit and seed characteristics, seed size, shedding water content, desiccation and chilling sensitivity and seed classification (Hong and Ellis, 1996). The aim of seed banking is to store a variety of seed material under suitable conditions that will maintain vigour and viability for predictable time periods, and when removed from storage, produce normal seedlings under field conditions (Hong and Ellis, 1996; Sacandé *et al.*, 2004).

1.3   Seed storage behaviour

Seeds are classified into three categories based on their desiccation tolerance and storage behaviour (see section 1.5), namely recalcitrant, intermediate and orthodox (Ellis *et al.*, 1990; Roberts, 1973). During late seed development, most seeds undergo a phenomenon called maturation drying whereby most of the tissue water is lost and seeds equilibrate with the relative humidity of the surrounding atmosphere (Bewley and Black, 1994). Seeds that undergo this process and exhibit desiccation tolerance are referred to as orthodox seeds (Roberts, 1973). It is well established that orthodox seeds do not attain maximum potential longevity until just after seed-filling phase terminates (Hong and Ellis, 1996). There are many mechanisms (protection, detoxification, repair) associated with the acquisition of desiccation tolerance in orthodox seeds that collectively, act to bring about survival in the dehydrated state, and upon rehydration enable maximum germination (see section 1.4) (reviewed by Berjak (2006b); Berjak and Pammenter (2008); Pammenter and Berjak (1999)). Orthodox seeds are shed at low water contents (<0.15 g g^{-1} dry weight [DW] basis), can be further

dehydrated without damage, and can be stored for predictable periods under controlled conditions (Ellis *et al.*, 1990, 1991). Conventional seed storage at low temperature (usually between +5 and -20°C) and low water contents (<0.07 g g^{-1} DW) allow for these seeds to survive for years and even decades (Roberts, 1973; Roos, 1989). This category includes many staple food and horticultural crops such as *Zea mays*, *Triticum aestivum* and *Vigna radiata* etc. (SID, KEW Gardens).

Intermediate seeds are shed at higher water contents (>0.8 g g^{-1} DW) and possess some mechanisms which allow tolerance when dried to a relative humidity of 30-40%, but cannot tolerate extreme desiccation lower than 0.2 g g^{-1} DW and storage below 10°C (Ellis *et al.*, 1990, 1991). The introduction of this category was necessary to incorporate species that appear to tolerate desiccation, but not to the same degree as orthodox seeds, and may be stored hermetically, but are chilling sensitive (Hong and Ellis, 1992b; Hong and Ellis, 1996). There may also be provenance-related differences within this category, *Coffea arabica* is native to dry and cool regions of Ethiopia and displays intermediate seed storage behaviour, while *Coffea liberica* is native to the hotter and more humid regions of Liberia and displays recalcitrant seed storage behaviour (Hong and Ellis, 1995). This category includes many *Citrus* spp., some C*offea* species, *Azadirachta indica, Carica papaya, Elaeis guineensis Jacq.* and possibly *Camellia sinensis* (SID, KEW Gardens).

Recalcitrant seeds are shed at very high water contents (>0.8-4.0 g g^{-1} DW) and lack (or do not express) many or all mechanisms associated with desiccation tolerance (Pammenter and Berjak, 2000a). These seeds do not undergo maturation drying and are metabolically active throughout development and after shedding, and will subsequently germinate shortly after shedding. They are desiccation sensitive and many are susceptible to chilling injury (Berjak *et al.*, 1989; Berjak *et al.*, 2004a; Pammenter and Berjak, 1999). Since recalcitrant seeds cannot tolerate the removal of bound water and cold temperatures without a loss in viability, conventional methods of storage are only possible for a few days (Chaitanya and Naithani, 1994, 1998) weeks (Varghese *et al.*, 2011; Varghese and Naithani, 2002) to months (Pammenter and Berjak, 1999). Recalcitrant seeds represent a category that contrasts orthodoxy, and includes many economically important tropical and forest species such as *Persea* spp., *Mangifera* spp., *Litchi* spp., *Artocarpus heterophyllus*, *Trichilia dregeana*, *Castanea sativa* and *Quercus robur* resulting in varying degrees of desiccation tolerance within the seed recalcitrance spectrum.

There are varying levels of desiccation tolerance expressed by both intermediate and recalcitrant seed categories which result in a further division based on provenance (see

Figure 1.1) (Hong and Ellis, 1996). Natural adaptations to cold environments may confer certain advantages, although this is often species-specific (Berjak *et al.*, 2011a; Pammenter and Berjak, 2000b). Temperate recalcitrant-seeded species are reported to be more cryo- and desiccation-tolerant than tropical recalcitrant-seeded species (Berjak *et al.*, 2011a). A possible explanation is seasonality of fruiting- some tropical recalcitrant-seeded species produce seeds in winter, which subsequently germinate over spring during warmer humid conditions (Berjak *et al.*, 2011a; Pammenter and Berjak, 2000a). Conversely, temperate species shed seeds in summer which germinate over winter; this may explain why seeds are more cold-tolerant (Benson, 2008a; Pammenter and Berjak, 2000a). Additionally, differences between recalcitrant-seeded species of tropical and temperate origin may largely depend on the types and proportions of sugars and the composition of both membrane lipids and lipid bodies (Pammenter and Berjak, 1999); which may vary through seed development (Berjak *et al.*, 1989; Berjak and Pammenter, 2008; Farrant *et al.*, 1993; Nkang *et al.*, 2003).
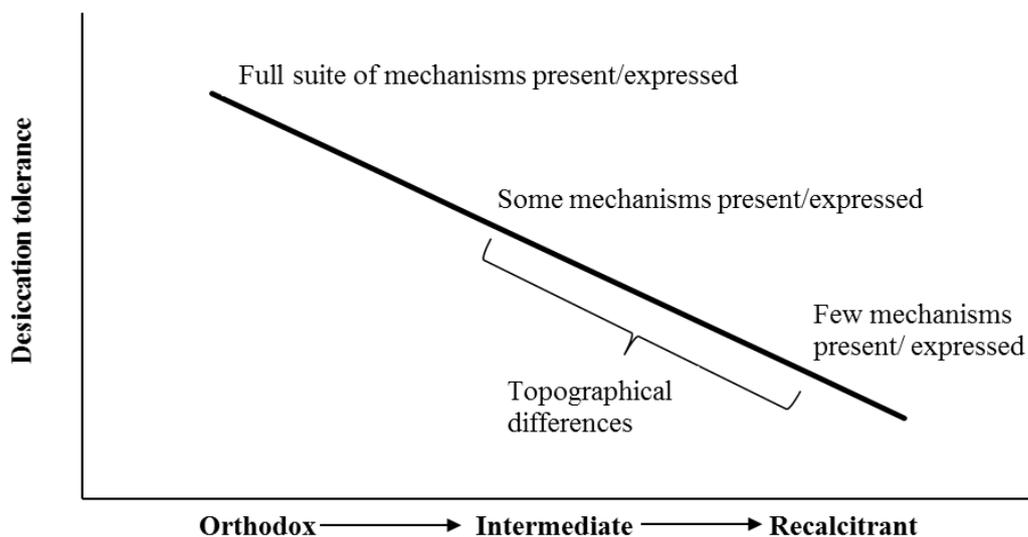
Figure 1.1: Seed storage continuum proposed by (Berjak and Pammenter, 1994; Berjak and Pammenter, 2008; Pritchard, 2004).

Although Pammenter and Berjak (2014) summarised that variability between recalcitrant species is provenance related, variability within a species may be attributed to differences between fruiting seasons, harvest time during fruiting, and even between parent plants. Marcos-Filho (2014) further describes recalcitrant seeds as (a) less recalcitrant -seeds can tolerate a large proportion of water loss before decreases in viability are observed, (b) moderately recalcitrant- seeds which do not tolerate as much water loss and germinate slightly

faster than species in the previous category, and (c) highly recalcitrant- seeds that can withstand little water loss and germinate immediately, even in the absence of additional water (Marcos-Filho, 2014). Recently, Moothoo-Padayachie *et al.* (2016) showed that the degree of recalcitrance may be related to the rate at which water is lost from the seeds and the speed at which it germinates after shedding.

1.4 Desiccation tolerance

The evolution of desiccation tolerance is an advanced characteristic that allowed plants and their propagules to inhabit and disperse into unoccupied niches on land (Pammenter and Berjak, 2000b). In seeds, desiccation tolerance is defined as the ability to withstand low intracellular water concentrations (≤ 0.1 g per gram dry mass) when dried to equilibrium with < 50% relative humidity and subsequent survival at those water contents (Alpert, 2005). There are many protective processes and mechanisms contributing to desiccation tolerance, which develop before or concomitant with maturation drying (Pammenter and Berjak (1999). These include: (1) the maintenance of intracellular physical characteristics upon water removal; (2) intracellular dedifferentiation in order to minimise surface area of membranes and possibly the cytoskeleton; (3) reduced or complete shutdown of metabolic activity, associated with decreased respiratory rates; (4) the presence, and efficient operation, of antioxidant networks to control the reactive oxygen species (ROS) generated during dehydration/respiratory activity; (5) the presence and activities of putatively protective molecules, such as sucrose, certain oligosaccharides and late embryogenic abundant proteins; (6) distribution of certain amphipathic molecules; (7) the presence of a peripheral oleosin layer around lipid bodies; and (8) the presence and operation of repair mechanisms upon rehydration.

1.4.1 Hydration levels

Vertucci (1990) proposed five hydration levels in seed tissues, each associated with a specific range of water content and water potential after sequential removal of water (summarised below). At Hydration level V, there is an abundance of water present that permits normal metabolic reactions to occur as well as germinative events. As tissue is dehydrated (Hydration level IV onwards), the loss of water and subsequent concentration of solutes changes the properties and functioning of many systems within the cell. Upon further dehydration (Hydration level III onwards), vitrification of intracellular contents occur and water behaves as a viscous or glassy solution. Further dehydration (Hydration level II), the

limited amount of water is involved in many deleterious events, and at Hydration level I water is associated strictly with macromolecules, referred to as structure-associated water, with highly limited activities within a cell. A summary of events/properties of water content and ψ at each hydration level is given below (Benson, 2008b; Berjak, 2006a; Vertucci and Farrant, 1995; Walters *et al.*, 2005):

Hydration level V (WC: >0.70 g. g^{-1}; $\psi \geq$-1 MPa):
Free water, cells are fully hydrated, water concentration at its highest, unconstrained metabolic reactions proceed; cell division; germination.

Hydration level IV (WC: 0.45-0.70 g. g^{-1}; $\psi \leq$ -1 MPa):
Free water, slightly lower water concentration; as a result, the cytoplasm begins to concentrate, protein and nucleic acid synthesis unrestricted.

Hydration level III (WC: 0.25-0.45 g. g^{-1}; $\psi \leq c$. -10 MPa):
Freezable water, low water concentration, potential for glass formation, respiration may be hindered; free radical production via unregulated metabolism; metabolism-induced desiccation damage; membrane appression; membrane demixing and bilayer transitions.

Hydration level II (WC: 0.08-0.25 g. g^{-1}; $\psi \leq c$. -100 MPa):
Low freezable water, catabolic activity via enzymes; anaerobic respiration; alcohols emitted; Maillard reactions; water-soluble enzyme degradation; membrane demixing and bilayer transitions.

Hydration level I (WC: <0.08 g. g^{-1}; ψ down to -1000 MPa):
Non-freezable water (structure-associated water), declining glass integrity; carbonyls emitted; free radical production via auto-oxidation; protein structures denatured; membrane structure de-stabilised; photoreceptors functional.

As mentioned earlier, orthodox seeds possess many protective and repair mechanisms which allow cells to migrate through each hydration level just prior to (during maturation drying) and after shedding from the parent plant, and again upon rehydration (Pammenter and Berjak, 1999). In order for excised embryonic axes from recalcitrant seeds to survive desiccation, which is a necessary step prior to cryogen exposure, dehydration rates should allow for cells to pass through various hydration levels rapidly, form and maintain a glassy

state in order to curtail metabolism-linked damage (Pammenter *et al.*, 1998; Pammenter *et al.*, 2002).

1.4.2 Dehydration

The water activity is defined by different states during dehydration: a freezable liquid (Hydration level V), an intermediate glassy state (Hydration level III), and structure-associated water (Hydration level I) (Benson, 2008b). The survival of dry orthodox seeds and partially dehydrated embryonic axes is due to the formation of the glassy state as a result of low water contents and reduced storage temperatures (Walters *et al.*, 2005). Glasses are non-crystalline solids that have physical properties of a solid but lack the organization of its crystal structure (Benson, 2008b). Glasses slow the intracellular molecular mobility, limiting biochemical reactions, and the increased viscosity decreases the chance of water molecules grouping to form ice when exposed to liquid nitrogen (Benson, 2008b; Franks *et al.*, 1990; Karlsson and Toner, 1996). Glass formation has been reported to be essential in achieving success during cryopreservation (Benson, 2008b) of fern spores (Ballesteros and Walters, 2007), seeds (Kim *et al.*, 2002), and shoot tips (Volk and Walters, 2006). Thus, dehydration of embryonic axes from recalcitrant seeds without significant accumulation of stress and loss of viability is of paramount importance. The water content of embryonic axes can be reduced by physical dehydration either by slow dehydration in a laminar airflow and over silica gel (Engelmann and Takagi, 2000), or via rapid drying using fast moving air flow circulating in a flash dryer (Berjak *et al.*, 1990; Pammenter *et al.*, 2002; Wesley-Smith *et al.*, 2001a). Rapid air drying is one of the most effective methods of removing most free water in the cell, in order to form glasses (Ballesteros *et al.*, 2014; Berjak *et al.*, 1993; Pammenter *et al.*, 1998; Varghese *et al.*, 2011; Wesley-Smith *et al.*, 2001a). The glassy state forms when the cell solutes concentrate into a viscous amorphous glass (at ≤ 0.3 g g^{-1} DW); this is called vitrification (Benson, 2008a; Karlsson and Toner, 1996; Mazur, 1984; Wolfe and Bryant, 2001).

Given the abundance of water present in axes of recalcitrant seeds at shedding, it is essential to reduce the amount of water quickly to prevent unregulated metabolism at intermediate water contents (Berjak *et al.*, 2011b; Pammenter and Berjak, 1999; Sershen *et al.*, 2012a, b; Varghese *et al.*, 2011). The rate of dehydration influences the ability of seeds/embryonic axes to survive especially at lower water contents- it is generally accepted that the faster tissues can be dehydrated, the lower the water content that can be achieved before viability is lost (Berjak *et al.*, 1990; Berjak and Pammenter, 2008; Pammenter and

Berjak, 2014; Pammenter *et al.*, 1991; Wesley-Smith *et al.*, 2001b). The principle behind this is that, when rapidly dried, the application of dehydration occurs faster than the dehydration-induced damage can accrue (Walters *et al.*, 2001; Walters *et al.*, 2008). Seeds/embryonic axes that are dehydrated slowly spend more time at water content ranges (Hydration level IV and III) that facilitate unbalanced metabolism which favours the accumulation of the products of harmful oxidative processes, eventually resulting in lethal damage (Berjak *et al.*, 2011a; Pammenter and Berjak, 1999; Varghese *et al.*, 2011). This type of damage is referred to as metabolism-induced or metabolism-linked damage (Berjak *et al.*, 1990; Berjak and Pammenter, 2001; Walters *et al.*, 2001), as opposed to structural damage caused by dehydration, when only structure-associated water remains (Pammenter *et al.*, 1998; Walters *et al.*, 2001). Cherussery *et al.* (2015) showed that *T. cacao* seeds displayed enhanced leakage of electrolytes from the tissues desiccated slowly at room temperature, as well as a significant increase in sucrose content and a decrease in raffinose content. Antioxidant systems of *T. dregeana* were shown to have failed when the embryonic axes were dehydrated slowly (Varghese *et al.*, 2011). However, Magistrali *et al.* (2015) found that application of slow drying outperformed the rapid drying of intermediate *Genipa americana* seeds, possibly due to sufficient time for the induction and operation of protective mechanisms involved in desiccation tolerance being activated under the slow drying conditions.

Irrespective of the drying rates, there appears to be a lower limit to which seeds or embryonic axes can tolerate dehydration without total loss of viability, this may coincide with the level at which the remaining intracellular water is non-freezable (Hydration level I) and damage may be structure associated (Pammenter *et al.*, 1993).

1.5   Seed storage

Knowledge of seed desiccation sensitivity and post-harvest behaviour is a prerequisite for optimum seed storage conditions, and is therefore imperative in seed conservation (Engelmann and Engels, 2002; FAO, 2013; Hong and Ellis, 1996; Joshi *et al.*, 2015). Figure 1.2 describes the protocols and procedures necessary to determine seed storage behaviour (discussed in section 1.2) as well as handling methods prior to investigations. Proper harvest procedure can extend storage lifespan, irrespective of storage category, however, there are many biotic and abiotic factors that need to be considered as well as actual seed compostion (Hong and Ellis, 1996).
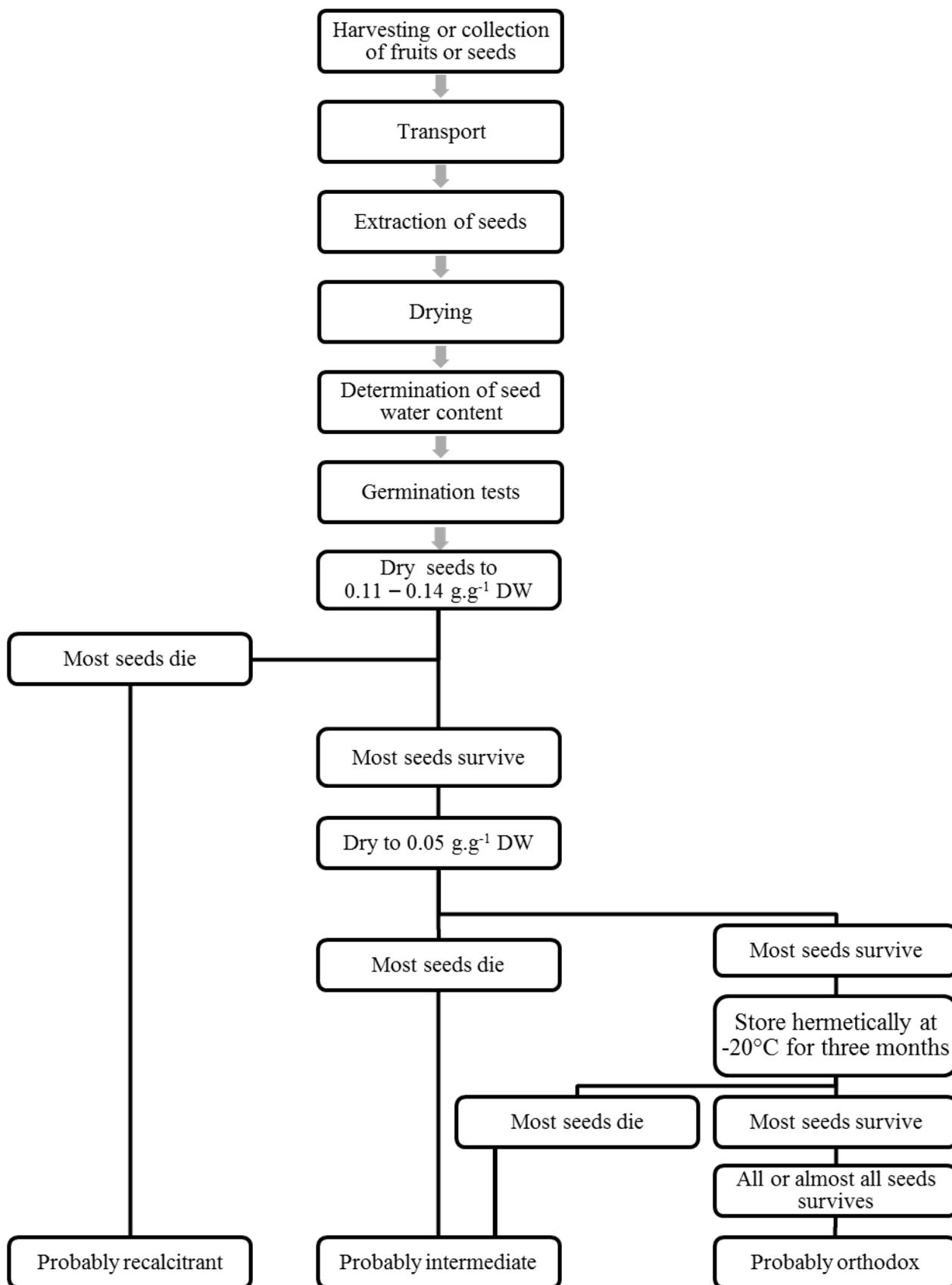
Figure 1.2: Procedures to be adopted prior to seed storage and protocols to determine seed storage behaviour. Adapted from Hong and Ellis (1996).

1.5.1 Hydrated storage

Conventional seed storage affords an efficient *ex situ* conservation method in seed banks, however, recalcitrant seeds cannot tolerate desiccation and storage at reduced temperatures without a loss in viability (Berjak and Pammenter, 1994; Farrant *et al.*, 1986; Pammenter *et al.*, 1994; Roberts, 1973). Furthermore, recalcitrant seeds must be stored at shedding water content, as even mild dehydration has been shown to initiate germinative events which adversely affect viability in storage (Eggers *et al.*, 2007). Hence a method of storage referred to as 'hydrated storage' was developed (Berjak *et al.*, 2004b; Pammenter *et al.*, 1994). This involves storing seed material that has been sterilised on a mesh suspended 100 mm above moistened paper towel inside plastic buckets. The plastic container is then closed to ensure seeds are maintained under saturated relative humidity conditions to prevent water loss. The seeds are stored at ambient (e.g. *Avicennia marina* (Calistru, 2004)) or slightly reduced temperatures of 16°C (*T. dregeana* (Goveia, 2007)) or 6°C (*Amaryllis belladonna, Haemanthus montanus* etc.) depending on their sensitivity to low temperatures.

There are other factors that may reduce storage lifespan (discussed below), and as a result hydrated storage of recalcitrant seeds is strictly a short- to medium-term strategy, possibly for a few days to weeks for seeds of many tropical species, and up to a year or a little longer for seeds of chilling-tolerant temperate species (Pammenter and Berjak, 2014). For some species, hydrated storage may be a suitable strategy for maintaining active collections (collections for short periods), but alternative means such as cryopreservation, need to be developed for long-term storage of a base collection (Pammenter and Berjak, 2014).

1.5.2 Factors affecting hydrated storage

1.5.2.1 Seed collection, transport and processing

Harvesting methods can be either directly from the plant (fruit or seeds are cut with secateurs/clippers or hand-harvested from shorter plants) or freshly shed seeds are collected from the ground. Ideally, fruits or seeds should be harvested at maturity, i.e. when seeds are naturally shed from the parent plant. Assessing the water content during development may be useful in determining harvest timing and maturity of orthodox seeds, however, recalcitrant seeds are shed at high water contents and are harvested just prior to, or just after natural dispersal (Hong and Ellis, 1996; Pammenter and Berjak, 1999). Since recalcitrant seeds are harvested at high water contents, it is safer to transport these seeds within fruits rather than first extracting the seeds. Although water from the fruit tissue may accelerate germinative

processes during transport, extracted seeds may lose water more rapidly in transit (Hong and Ellis, 1996). Recalcitrant seeds should be placed in strong perforated polyethylene bags to allow for aeration, or similar non-airtight containers and transported soon after harvesting preferably at ambient temperature or at 15-20°C (Hong and Ellis, 1996). If temporary storage cannot be avoided after harvesting and seed storage behaviour is unknown, fruits should be stored at 15-20°C in perforated polyethylene bags for as short a period as possible (Hong and Ellis, 1996). Upon arrival in the laboratory, seeds are often extracted by hand and cleaned using a variety of methods such as washing seeds in running tap water under pressure after physical removal of fleshy fruit tissue or soaking for short periods of time to remove gelatinous coverings from seed (Hong and Ellis, 1996). It is important to note that some structures may physically inhibit germination (e.g. the pericarp of *A. marina* seeds (Calistru, 2004), or harbour fungal inoculum (e.g. the aril of *T. dregeana* seeds (Berjak *et al.*, 2004a) which affects storage longevity.

1.5.2.2 Seed maturity and morphology

Seeds should be collected at the same stage of maturity, soon after abscission from plants of a similar age and from a similar vicinity. Seeds of different harvest location and maturity should be stored as different accessions (Hong and Ellis, 1996). Seeds of *Ekebergia capensis* have been shown to exhibit considerable differences in chilling sensitivity when harvested from different provenances (Bharuth *et al.*, 2007). Those authors reported that seeds collected from northern Africa were highly chilling sensitive compared with those collected from South Africa. Research conducted on immature seeds (early harvest) may result in erroneous classification of seed storage behaviour, whilst delayed harvest beyond natural shedding may result in increased desiccation sensitivity due to water loss (Hong and Ellis, 1996). Some recalcitrant seeds are shed before complete maturity is reached, resulting in lag between shedding and germination (e.g. *T. dregeana* and *Strychnos gerrardii*). These seeds may be stored hydrated to allow for development to continue (Berjak *et al.*, 2011b; Goveia *et al.*, 2004), whilst others are shed fully developed (e.g. *A. marina*), and there is a shorter lag between shedding and germination, thus reducing hydrated storage lifespan (Berjak *et al.*, 1989; Berjak and Pammenter, 2008; Farrant *et al.*, 1986).

Most recalcitrant seeds are produced in a humid forest environment, in which evolutionary processes may have selected for the lack of desiccation tolerance due to the availability of water and warm temperatures, thus promoting rapid germination (Pammenter and Berjak, 2000a). Seeds shed from dicotyledonous trees are large, and the cotyledons

comprise about 98-99% of the seed structure, thus the embryonic axis is very small in comparison (Daws *et al.*, 2005; Pritchard *et al.*, 2004). Whilst seed size is not the only factor in determining seed storage behaviour, it is generally accepted that recalcitrant seeds tend to be larger than intermediate seeds, which in turn tend to be larger than orthodox seeds (Hong and Ellis, 1996). Having a large seed size allows for slower water loss when compared with orthodox seeds, and many recalcitrant seeds are shed within a fleshy fruit layer which may confer additional protection from water loss (Marcos-Filho, 2014). Recalcitrant seeds are also shed with thin coverings and a high seed coat ratio. Daws *et al.* (2006a) found that probability of desiccation tolerance decreases as seed mass increases while it increases with a decrease in the seed coat ratio. The shape, size, testa and fruit from recalcitrant species differ greatly. Seeds shed as a multiple fruit, such as *A. heterophyllus* show considerable variation in seed size, shape and maturity despite being within the same fruit (Chaudhury and Malik, 2004). Hard seed coats physically reduce water loss as seen in *C. sinensis* (Berjak *et al.*, 1993), softer seed coats, as in *A. marina*, may harbour fungal spores which proliferate in storage and curtail hydrated storage lifespan (Calistru *et al.*, 2000).

1.5.2.3 Germination in storage

Hydrated storage lifespan is reduced by the microbial (particularly fungal) contamination on or in the seeds (Berjak, 1996; Berjak *et al.*, 2014). At harvest, there is a spectrum of fungal species as well as bacteria present even when freshly mature seeds are hand-harvested (Berjak *et al.*, 2014; Calistru *et al.*, 2000; Mycock and Berjak, 1990). Seeds shed on the ground may harbour soil-borne fungi that may further curtail seed storage lifespan (Murray, 1974). To reduce fungal/bacterial load, seeds are surface decontaminated and seed/fruit coverings are removed prior to storage (Calistru *et al.*, 2000). Seeds are often dusted with an antifungal powder to prevent further fungal proliferation in storage or coated in alginate containing Nipastat™ to reduce the incidence of fungal contamination (Motete *et al.*, 1997). Aside from losing large numbers of seeds due to contamination in storage, fungal proliferation often generates metabolic water, which condenses and aids in increasing germination rates in storage (Calistru *et al.*, 2000; Mycock and Berjak, 1990).

1.5.2.4 Storage temperature

Because of their ongoing metabolic activity, the rate of metabolism and germination can be controlled by storage temperature. However, since many recalcitrant seeds are produced by various tropical/subtropical plant species, chilling sensitivity becomes a major limiting factor of storage lifespan (Pammenter and Berjak, 2014). Therefore, hydrated storage

must be carried out at the lowest temperature (greater than 0°C) at which vigour and viability are not severely compromised. Most recalcitrant seeds are stored hydrated at 16°C at the Plant Germplasm Conservation Research group at UKZN, storage above this temperature results in germination in a short period, e.g. *T. emetica* seeds germinated within 35-40 days at 25°C, whilst storage at 6°C resulted in complete loss of viability within 20 days (Kioko *et al.*, 2006). Most research on chilling sensitivity has been conducted on vegetative tissues or embryonic axes after excision, relatively little data exist for recalcitrant seeds in hydrated storage at low temperatures, for e.g. *E. capensis* (Bharuth *et al.*, 2007), *T. emetica* (Kioko *et al.*, 2006), *Symphonia globulifera* (Corbineau and Côme, 1986) and *Aesculus hippocastanum* (not hydrated storage) (Tompsett and Pritchard, 1998).

1.6   Chilling sensitivity

Reducing storage temperature increases the storage lifespan of recalcitrant seeds due to reduced metabolism and helps prevent water loss as well as curtailing fungal metabolism (Berjak *et al.*, 1989; Calistru *et al.*, 2000). Chilling temperatures range between 0-15°C but is species-specific. Chilling sensitivity may be induced by low temperatures which prevents normal plant growth and causes injury, but not cool enough for ice crystal formation to occur (Levitt, 1980; Raison and Orr, 1990). The nature and severity of chilling injury is a combination of provenance, seed maturity and metabolic state, as well as the duration and severity of the chilling stress (Hong and Ellis, 1996; Raison and Orr, 1990; Saltveit and Morris, 1990). The initial degenerative events associated with chilling stress lead to an alteration of metabolic processes which lead to a series of secondary physiological and physical events that culminate in seed death (Raison and Orr, 1990; Saltveit and Morris, 1990). Lyons (1973) states that the typical chilling stress responses are: membrane phase changes from a flexible liquid-crystalline to a more solid gel structure, resulting in increased permeability and solute leakage; alteration of metabolic processes and respiration activity resulting in oxidative stress (which result in changes in antioxidant activity, protein synthesis and enzyme activity); accumulation of sugars to stabilize membranes, maintain structural conformation of enzymes and assist in metabolic processes; reduction in protoplasmic streaming as a direct consequence of decreased respiratory activity and; rapid disorganisation of cell ultrastructure.

Due to the plethora of degradative processes resulting from cold storage temperature, long-term maintenance of tropical recalcitrant seeds at cool temperatures is not a viable method. Cold moist short-term storage methods have been used for stratification of dormant

recalcitrant seeds of trees such as *Acer platanoides* and *Acer pseudoplatanus* which have been shown to improve germination by 10-30% (Pukacki and Juszczyk, 2015), whilst some *Fagus* spp. require 4 to 20 weeks of moist chilling to break dormancy (Gosling, 1991). Also, some temperate recalcitrant species will tolerate cold storage, e.g. *Q. robur* survived for three years at -3°C (Suszka, 1976), *Castanea sativa* seeds stored for 10 months at 5°C maintained 90% viability and tropical recalcitrant *Hevea brasiliensis* seeds were best stored at 7-10°C (Beng, 1976). Short-term cold storage may be useful for research purposes, however, focus has shifted to cryobiology for long-term storage of recalcitrant-seeded species, irrespective of provenance (Berjak, 2006a; Pammenter and Berjak, 2014).

1.7 Cryopreservation

The only way to conserve germplasm of recalcitrant-seeded species in the long term is by cryopreservation. Cryopreservation is the storage of biological material at ultra-low temperatures, usually in liquid nitrogen (LN) (-196°C) or in the vapour phase of LN (-140 to -160°C) (Benson, 2008a, b; Berjak *et al.*, 2011a; Day *et al.*, 2008; Mazur, 1984). Cryopreserved tissue is said to be in a state of "suspended animation", where the biological clock has been stopped, and theoretically, the tissue should remain in the original preserved state eternally (Benson, 2008b; Day *et al.*, 2008; Mazur, 1984). Metabolic arrest at cryogenic temperatures leads to substantial changes in the physical and chemical integrity of the intra- and intercellular properties of tissues (Benson, 2008b; Mazur, 1984; Mazur *et al.*, 2008). The behaviour of water during cryopreservation is highly influenced by the rapid temperature changes that govern the transition between liquid, glassy and solid states (Benson, 2008a; Karlsson and Toner, 1996). Given the abundance of water present in recalcitrant biological systems, and its unique physico-chemical properties that confer survival, manipulation of the different states of water is essential to ensure successful cryopreservation and storage stability (Benson, 2008a; Day *et al.*, 2008; Karlsson and Toner, 1996).

Recalcitrant seeds are generally large and cannot be rapidly dehydrated to low water contents required to avoid lethal freezing damage without loss of viability (Berjak and Pammenter, 2008). Explants such as embryonic axes are the primary choice, alternatively, shoot apices or meristems derived from *in vitro*-grown seedlings can be used when embryonic axes do not survive cryostorage or are too large to facilitate drying and cooling (Berjak and Pammenter, 2008; Engelmann, 2012). Excised embryonic axes are genetically identical to the seed, can be manipulated *in vitro* to germinate into an independent plant, and, in most species, is small enough to facilitate rapid dehydration (without significant viability loss) in an

airstream (known as flash/rapid drying) to prevent ice crystal damage that may occur during cryopreservation (Pammenter and Berjak, 2014). Ice crystals affect the structural, osmotic and colligative integrity of cells causing physical ruptures and mechanical injury (Benson, 2008b). Wesley-Smith *et al.* (2014) found that intracellular ice formation was not necessarily lethal in embryonic axes of *Acer saccharinum*, but rather if ice crystals are small and localized in the cytoplasm, *in vitro* survival is retained. He further stated that interactions among cooling rate, ice structure, cell structure and water content should be investigated further.

In addition, the combination of excision, dehydration, rapid cooling and subsequent rewarming upon retrieval of the axis imposes many physical, biochemical and intracellular changes, that collectively result in the success or failure of cryopreservation (Berjak *et al.*, 2011b; Pammenter and Berjak, 2014; Sershen *et al.*, 2012a; Wesley-Smith *et al.*, 2014). Procedural steps involved in cryopreservation often elicit a stress response that is evident only after rehydration, when metabolism can potentially be reinitiated (Sershen *et al.*, 2012a). Axes of *T. dregeana* are difficult to successfully cryopreserve (Ballesteros *et al.*, 2014; Naidoo *et al.*, 2011); shoot production occurs only if small pieces of cotyledonary material are left attached to the embryonic axis, since the shoot meristem which is in close proximity to the cotyledonary insertion site, may be injured during excision (Goveia *et al.*, 2004; Whitaker *et al.*, 2010). Similarly, axes of *A. heterophyllus* do not survive cryogen exposure, possibly due to variable maturity levels (Chandel *et al.*, 1995). On the other hand, embryonic axes (Berjak *et al.*, 1999), plumules and embryogenic calli (Chmielarz *et al.*, 2011) of *Q. robur* survive cryopreservation. Embryonic axes of *C. sinensis* appear to be desiccation sensitive which reduces viability after cryogen exposure (Berjak *et al.*, 1993; Wesley-Smith *et al.*, 1992), however, successful cryopreservation of seeds and embryonic axes has been achieved (Kim *et al.*, 2002).

1.8 Oxidative metabolism

1.8.1 ROS and free radical biochemistry

Oxygen plays a pivotal role in aerobic life and the production of ROS has probably been intricately connected since the first appearance of oxygen molecules 2.4-3.8 billion years ago (Benson and Bremner, 2004; Mittler, 2016). ROS are by-products of mitochondrial respiratory metabolism that form from the incomplete reduction of oxygen resulting in the production of superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$) and nonradical molecules such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Bailly *et al.*, 2008; El-Maarouf-

Bouteau and Bailly, 2008; Mittler, 2016; Mittler *et al.*, 2011; Sharma *et al.*, 2012). ROS is a phrase used to describe several reactive molecules and free radicals and for the purposes of this study, the term ROS will be used to describe both radical and non-radical species. The sequential univalent reduction of ground state triplet oxygen results in the formation of intermediate free radical species and their toxic by-products in order to safely reduce oxygen to water. These intermediaries are: superoxide radical (addition of one electron to $^3O_2$) and hydrogen peroxide (addition of two electrons and two protons), hydrogen peroxide may be further broken down via the Fenton reaction to produce hydroxyl radicals, which is possibly one of the most volatile and reactive oxidising agents (Apel and Hirt, 2004; Benson and Bremner, 2004; Kremer, 1999). Whilst a gain of electrons may be responsible for the primary formation of ROS molecules, it is also possible for radicals to react with non-radicals to autopropagate a wave of secondary radical production (Fridovich, 1998). This rapid, although transient, increase in ROS concentration has been described as an 'oxidative burst' and has recently been shown to travel long distance mediated by respiratory burst oxidase homologues (RBOHs), which ensure local and systemic integration of a variety of signal transduction pathways in plants (Apel and Hirt, 2004; Mittler *et al.*, 2011; Suzuki *et al.*, 2011). Failure to control/regulate oxidative bursts and subsequent high levels of ROS results in deleterious events associated with 'oxidative stress' (Berjak *et al.*, 2011b; Foyer and Noctor, 2005a). Primary oxidative stress is a result of initial reactions that generate free radicals which result in perturbed electron-rich domains, whilst secondary oxidative stress occurs as a result of the toxic by-products of those initial reactions (Benson, 1990).

1.8.2 Functions of ROS

Over the years, the role of ROS in plant biology has been studied and reviewed extensively, see reviews by Foyer and Noctor (2005a); Mittler *et al.* (2011); Noctor and Foyer (2016); Shaban *et al.* (2013); Suzuki *et al.* (2011); Wrzaczek *et al.* (2013) both in terms of oxidative stress and damaging biomolecules, and signalling and stress perception. Superoxide is short-lived, has a half-life of 1-4 μs but can travel up to 30nm, and thus may cause damage to a wider range of organelles whilst hydrogen peroxide is somewhat more stable, has a half-life >1ms, can travel longer distances >1μm and consequently pass through cell membranes and disperse into cellular regions further away from the production site (Mittler, 2016). ROS are produced in redox-active compartments in which aerobic metabolic processes occur. These highly active sites include: mitochondria (cellular respiration), chloroplasts (photosynthesis) and peroxisomes as well as plasma membranes and various metabolic

pathways localised in other cellular compartments (Benson and Bremner, 2004; Benson, 1990; Halliwell and Gutteridge, 2015; Noctor and Foyer, 2016) (Figure 1.3). There are a variety of different methods used to assess ROS production which include colorimetric, fluorescent staining, electron spin resonance and high performance liquid chromatography (HPLC) (Moothoo-Padayachie *et al.*, 2016; Noctor *et al.*, 2016; Roach *et al.*, 2008; Varghese *et al.*, 2011).

The main functions of ROS are to control the cell redox status, protect against pathogens and respond to wounding, regulate cellular growth and programmed cell death (Apel and Hirt, 2004; Bailly *et al.*, 2008; El-Maarouf-Bouteau and Bailly, 2008). Previously, it was thought that ROS were damaging to the cells due to their high reactivity within cellular components, induced by a range of stressors in recalcitrant germplasm, but there has been an accumulation of evidence which have shown ROS to be not only beneficial, but also necessary for germination to commence, leading to elongation of the embryonic axis in both orthodox (Müller *et al.*, 2009; Su *et al.*, 2016) and recalcitrant germplasm (Hendry *et al.*, 1992; Moothoo-Padayachie *et al.*, 2016) (Table 1.1).

1.8.3 Antioxidant systems

The movement of intracellular and extracellular ROS in cell signalling is strictly controlled by ROS-scavenging networks that tightly regulate their location and concentration (El-Maarouf-Bouteau and Bailly, 2008; Gechev *et al.*, 2006; Halliwell, 2006). Indeed, it is the uncontrollable generation of ROS during seed storage, dehydration and rehydration that poses the greatest threat to cellular components by bringing about severe oxidative damage to the DNA, proteins and lipids, which can lead to abnormal cell growth and death of tissues (Bailly *et al.*, 2008; Berjak *et al.*, 2011b; Foyer and Noctor, 2005b; Gechev *et al.*, 2006; Pammenter and Berjak, 1999). To counteract oxidative stress, a network of water- and lipid-soluble antioxidants, both enzymatic and non-enzymatic, has evolved timeously to quench ROS (Foyer and Noctor, 2005b; Mittler, 2016; Shao *et al.*, 2008) (Figure 1.3). These antioxidants may be classified into three categories, namely lipid-soluble, water-soluble and enzymatic scavengers (Gill and Tuteja, 2010; Halliwell, 2006; Walker and Mckersie, 1993).

Lipid-soluble membrane associated antioxidants such as α-tocopherol quenches singlet oxygen and sequesters free radicals involved in lipid peroxidation chain reactions, and carotenoids quench singlet oxygen in thylakoid membranes and quench excess chlorophyll excitation energy (Halliwell, 2006; Mittler, 2016). Water-soluble reductants such as glutathione react directly with free radicals or with dehydroascorbate (DHA) to regenerate

ascorbate (ASA). Ascorbate reduces superoxide, hydrogen peroxide and hydroxyl radicals, or quenches singlet oxygen directly (Noctor and Foyer, 1998). Oxidised glutathione (GSSG) is in turn reduced by nicotinamide adenine dinucleotide phosphate (NADPH) from photosystem I (Bailey-Serres and Mittler, 2006; Dietz *et al.*, 2016; Mittler, 2016). Enzymatic antioxidants such as superoxide dismutase (SOD) dismutates superoxide radicals into H_2O_2 and oxygen (Bowler *et al.*, 1992) and catalase (CAT) detoxifies hydrogen peroxide to water and oxygen CAT) (Scandalios *et al.*, 1997). The ascorbate-glutathione cycle, also called the Halliwell-Asada cycle uses ascorbate peroxidase (APX) as the primary enzyme involved in the ascorbate-dependent hydrogen peroxide scavenging system, but also includes monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Noctor and Foyer, 1998). These enzymes are present in chloroplasts, the cytoplasm, mitochondria, peroxisomes and/or the apoplast and are responsible for the regeneration of the powerful antioxidants ASA, GSH and α-tocopherol (Figure 1.3) (Mittler, 2016).

The role of the ascorbic system in seeds has been reviewed by De Tullio and Arrigoni (2003). Glutathione peroxidases (GPX) and other peroxidases (POX) may also catalyse the reduction of hydrogen peroxide and/or hydroperoxides (Eshdat *et al.*, 1997). Various compounds such as polyphenols, flavonoids and/or peroxiredoxins (Aalen, 1999) also have a strong antioxidant function. The use of trolox equivalent antioxidant capacity (TEAC) assay, which is based on the decolourisation of 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid diammonium salt (ABTS), is reactive towards most antioxidants and can be used to determine activities for both hydrophilic and lipophilic antioxidants such as ascorbic acid, α-tocopherol, gluthatione, flavonoids, phenolics and carotenoids (Pinchuk *et al.*, 2012; Re *et al.*, 1999; Walker and Everette, 2009). Functional co-operation among these enzyme-scavenging networks are essential for effective redox homeostasis (Table 1.1). Endogenous antioxidants may also be enhanced by the addition of exogenous antioxidants such as desferrioxamine, dimethyl sulfoxide (DMSO) and cathodic water, which has been previously shown to alleviate ROS-induced damage as well as stimulate the endogenous antioxidants in embryonic axes from recalcitrant seeds (Berjak *et al.*, 2011b; Naidoo *et al.*, 2011; Sershen *et al.*, 2012c).

Table 1.1 summarises experiments conducted over the past 10 years on ROS and antioxidant production in various studies of orthodox, intermediate and recalcitrant seeds. Whilst there exist many other studies, these are pertinent to show not only oxidative status, but the interpretation of these results. Initially, heightened ROS production was associated with oxidative stress, more so when antioxidant capacity was diminished (Table 1.1).

However, more evidence is accumulating in support of the positive role that ROS plays in germination, dormancy alleviation and stress perception (Table 1.1). Most studies showed that ROS levels were heightened in response to chilling, desiccation and storage. Antioxidant response appears variable, based on the type of stress applied and species post-harvest behaviour. Irrespective of the treatment, an increase in ROS production in orthodox seeds appeared to have a beneficial effect by facilitating germinative processes, whilst in recalcitrant seeds there was a loss of viability (Table 1.1).
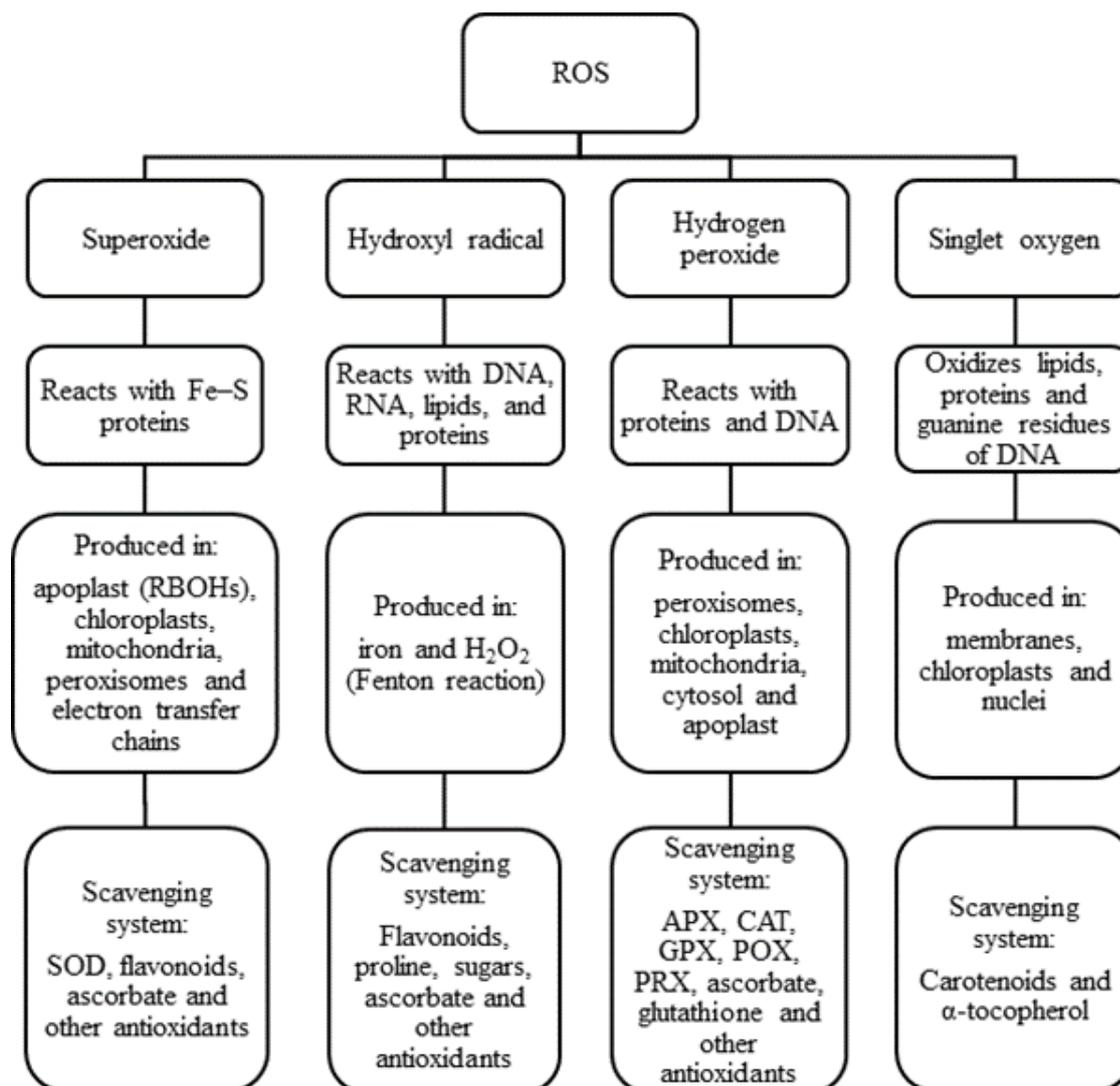
Figure 1.31.8**: Summary of the reactivity, production and scavenging systems of ROS in plant cells. Adapted from Mittler (2016). Abbreviations: APX, ascorbate peroxidase; CAT, catalase; GPX, glutathione peroxidase; POX, peroxidase; PRX, peroxiredoxin; RBOHs, respiratory burst oxidase homologs; SOD, superoxide dismutase.**

Table 1.1: Effect of various treatments/stresses imposed on species belonging to different storage categories and the resulting oxidative status. Directional arrows indicate an increase or decrease in respective values or viability, whilst values in parentheses indicate the % change.

Reference	Species	Seed category	Treatment/Stress	ROS (% ↑/↓)	Antioxidant enzyme (% ↑/↓)	Viability
Pukacka and Ratajczak (2006)	*Acer saccharinum* L.	Recalcitrant	Desiccation: whole seeds, 14 d of drying vs freshly shed	↑ $O_2^{\bullet-}$ (44%) ↑ H_2O_2 (60%)	↑ DHA (44%), GSSG (56%) ↓ ASA (32%), GSH (75%), ASA: DHA (45%), GSH: GSSG (87%)	↓
Pukacka and Ratajczak (2007a)	*Fagus sylvatica* L.	Intermediate	Storage: embryonic axes, 10 years aged vs 2 years aged	↑ $O_2^{\bullet-}$ (27%) ↑ H_2O_2 (20%)	↓ α -Tocopherol (50%), ASA (64%), DHA (43%), GSH (75%) ↓ GSSG (45%)	↓
Cheng and Song (2008)	*Antiaris toxicaria* (Pers.)	Recalcitrant	Desiccation: embryonic axes, 144 hr vs freshly shed	↑ $O_2^{\bullet-}$ (27%) ↑ H_2O_2 (20%)	↑ SOD (21%), CAT (50%) ↓ APX (76%), GR (36%), DHAR (35%)	↓

Abbreviations: $O_2^{\bullet-}$, superoxide; H_2O_2, hydrogen peroxide; DHA, dehydroascorbate; GSSG, oxidised glutathione; ASA, ascorbic acid; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase; DHAR, dehydroascorbate reductase.

Table 1.1: continued ….

Reference	Species	Seed category	Treatment/Stress	ROS (% ↑/↓)	Antioxidant enzyme (% ↑/↓)	Viability
Zheng *et al.* (2009)	*Triticum aestivum* L., cv. Huaimai 17	Orthodox	Germination: whole seeds, saline conditions after 5 d vs ungerminated seeds	↑ $O_2^{\bullet-}$ (49%) ↑ H_2O_2 (56%)	↑ SOD (23%), CAT (31%), POX (23%)	↑
Chen *et al.* (2011)	*Camellia sinensis* (L.) Kuntze	Intermediate	Desiccation: whole seeds 96 hr of drying	↑ H_2O_2 (75%)	↑ SOD (31%), POX (35%), APX (69%), CAT (33%), DHAR (62%), GR (36%)	↓
Pukacka *et al.* (2011)	*Quercus robur* L.	Recalcitrant	Desiccation: embryonic axes, 75 hrs dehydration vs freshly shed	↑ $O_2^{\bullet-}$ (30%) ↑ H_2O_2 (29%)	↑ SOD (10%), POX (5%) ↓ ASA (32%), DHA (75%), APX (33%), GR (28%), α-Tocopherol (48%)	↓

Abbreviations: $O_2^{\bullet-}$, superoxide; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; POX, peroxidase; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; ASA, ascorbic acid, DHA, dehydroascorbate.

Table 1.1: continued….

Reference	Species	Seed category	Treatment/Stress	ROS (% ↑/↓)	Antioxidant enzyme (% ↑/↓)	Viability
Varghese *et al.* (2011)	*Trichilia dregeana* Sond.	Recalcitrant	Desiccation: embryonic axes, 5 hrs dehydration vs freshly shed	↓ $O_2^{\bullet-}$ (48%) ↓ OH^- (71%)	↑ CAT (19%) ↓ SOD (50%), GR (55%), APX (65%), GPX (22%)	↓
Bai *et al.* (2011)	*Baccaurea ramiflora*	Recalcitrant	Chilling: embryonic axes, 2d at 2°C vs control (at 22°C)	↑ H_2O_2 (77%)	↑ APX (14%), MDHAR (25%), DHAR (23%), GR (20%), GSH (23%) ↓ GSH: GSSG (67%)	↓
Sershen *et al.* (2012c)	*Amaryllis belladonna*	Recalcitrant	Cryopreservation: embryonic axes, dehydrated, + glycerol, cryopreserved vs freshly shed	↑ $O_2^{\bullet-}$ (89%)	↑APX (68%) ↓ SOD (37%), CAT (32%), GR (60%)	↓

Abbreviations: $O_2^{\bullet-}$, superoxide; OH^-, hydroxyl radical; H_2O_2, hydrogen peroxide; CAT, catalase; SOD, superoxide dismutase; GR, glutathione reductase; APX, ascorbate peroxidase; GPX, guaiacol peroxidase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GSH, reduced glutathione; GSSG, oxidised glutathione.

Table 1.1: continued….

Reference	Species	Seed category	Treatment/Stress	ROS (% ↑/↓)	Antioxidant enzyme (% ↑/↓)	Viability
Xin *et al.* (2014)	*Glycine max* L. cv. Zhongdou No. 27	Orthodox	Ageing: seeds (mitochondria), 41 d after ageing vs unaged seed	↑ $O_2^{\bullet-}$ (1%) ↓ H_2O_2 (24%)	↑ DHA (50%) ↓ SOD (80%), GR (67%), APX (81%), DHAR (10), ASA (21%), ASA: DHA (71%), GSH (99%), GSSG (71%), GSH: GSSG (68%)	↓
Chandra *et al.* (2015)	*Cicer arietinum* L.	Orthodox	Dehydration: radicals, 24 hrs dehydration vs fully hydrated	↑ $O_2^{\bullet-}$ (56%)	↑ SOD (88%), APX (44%) ↓ POX (46%)	↓
Shalimu *et al.* (2016)	*Punica granatum*	Orthodox	Dormancy alleviation: whole seeds, combination warm + cold after 112 d vs 1 d	↑ H_2O_2 (46%)	↑ SOD (18%), POX (22%) ↓ CAT (14%)	↑

Abbreviations: $O_2^{\bullet-}$, superoxide; H_2O_2, hydrogen peroxide; DHA, dehydroascorbate; SOD, superoxide dismutase; GR, glutathione reductase; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; ASA, ascorbic acid; GSH, reduced glutathione; GSSG, oxidised glutathione; POX, peroxidase CAT, catalase.

Table 1.1: continued….

Reference	Species	Seed category	Treatment/Stress	ROS (% ↑/↓)	Antioxidant enzyme (% ↑/↓)	Viability
Moothoo-Padayachie *et al.* (2016)	*Avicennia marina* (Forssk.) Vierh.	Recalcitrant	Germination: embryonic axes, 3 d after sowing vs freshly shed	↑ $O_2^{\bullet-}$ (23%) ↑ H_2O_2 (51%)	↑ GSSG (19%) ↓ GSH (52%), GSH: GSSG (61%)	↑

Abbreviations: $O_2^{\bullet-}$, superoxide; H_2O_2, hydrogen peroxide; GSSG, oxidised glutathione; GSH, reduced glutathione.

1.9 Aims and objectives

The aim of this study was to determine the role of provenance in the response of recalcitrant seeds and embryonic axes to: (1) dehydration and (2) hydrated storage. In order to assess the importance of provenance, recalcitrant material from tropical and temperate regions were subjected to a range of experimental studies to measure various metabolic (oxidative status) and physiological (dehydration, storage) responses. The objectives of the dehydration study were to establish: (1a) drying time-courses for each species (using rapid drying methods), (1b) assess drying rates and the oxidative status of embryonic axes that have been partially dehydrated to differing hydration levels and (1c) relate these findings to viability retention. The objectives of the storage experiment were to: (2a) assess seed storage longevity, (2b) assess chilling sensitivity when stored hydrated at 3 and 16°C, (2c) determine the oxidative status during storage and (2d) relate the production of ROS and antioxidants to viability retention.

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental design

This study involved two sets of experiments to assess desiccation tolerance and storage longevity at two storage temperatures. The first set involved rapidly dehydrating embryonic axes of *Camellia sinensis* (L.) Kuntze, *Quercus robur* L. (temperate provenance), *Trichilia dregeana* Sond. and *Artocarpus heterophyllus* Lam. (tropical provenance) seeds for various time intervals. Thereafter, at selected water contents of 1.0, 0.3-0.4 and 0.1 g g^{-1} dry weight (DW), the oxidative status of axes was assessed. The second set was designed to investigate the role of oxidative status during hydrated storage and its relationship with chilling sensitivity of each species. Seeds were stored at 3 and 16°C in hydrated storage (see section 2.4.1) and sampled every 28 d for the first eight months, thereafter every 56-84 d until complete loss in viability or 18 months (unless stated otherwise). For both parts of the study, embryonic axes excised from freshly harvested seeds served as a control.

2.2 Species description

Seeds of each species were collected in South Africa, and later, Poland (*Q. robur*), in regions which were similar to their origin. Table 2.1 outlines some of the characteristics of each species as well as the seed descriptions and harvesting locations. Seeds were harvested at approximately the same time each year, and transported to the laboratory in the shortest time possible. Figures 2.2.1-2.2.4 show seeds/fruit of each species and the size of the embryonic axis relative to the cotyledons.

Table 2.1: Seed characteristics and species descriptions of *C. sinensis, Q. robur***,** *T. dregeana* **and** *A. heterophyllus* **based on physical observations and literature.**

Species	*C. sinensis*	*Q. robur*	*T. dregeana*	*A. heterophyllus*
Family	Theaceae[1]	Fagaceae[2]	Meliaceae[3]	Moraceae[4]
Common name	Tea	English oak	Natal Mahogany	Jackfruit
Place of origin	Asia: Myanmar, Irrawaddy River[1]	Caucasus and Europe[2]	Africa: Southern tropics[3]	Asia: India, Western Ghats[4]
Tree life form	Evergreen perennial tree[5]	Deciduous tree[2]	Evergreen tree[6]	Evergreen tree[4]
Conservation status (IUCN Red List)	Not assessed[7]	Least concern[7]	Not assessed[7]	Not assessed[7]
Economical value	Leaves[5]	Wood[2]	Wood and bark[6]	Wood, fruit and seeds[4]
Industry	Beverage[5]	Timber[2]	Timber, cultural[6]	Timber, food[4]
Method of propagation	Grafting/seeds[5]	Seeds[2]	Seeds[6]	Seeds[4]

[1] http://data.kew.org/sid/SidServlet?ID=4331&Num=izH&_ga=1.140210099.1445309976.1470598122; [2] http://www.kew.org/science-conservation/plants-fungi/quercus-robur-englishoak; [3] http://data.kew.org/sid/SidServlet?ID=23333&Num=a4R; [4] https://hort.purdue.edu/newcrop/morton/jackfruit_ars.html; [5] Mondal *et al.* (2004); [6] http://www.plantzafrica.com/planttuv/trichildreg.htm; [7] http://www.iucnredlist.org/search

Table 2.1: continued….

Species	*C. sinensis*	*Q. robur*	*T. dregeana*	*A. heterophyllus*
Seed shedding structures	Enclosed in a fruit, two-four seeds per capsule[5]	Single seed not enclosed by fruit/capsule[2]	Enclosed in a velvety fruit, six seeds per capsule[6]	Enclosed in an aggregate fruit, 100-450 seeds[4]
Seed description	Tough brown testa, light yellow cotyledon (Figure 2.2.1)	Tough pericarp, beige cotyledon (Figure 2.2.2)	Thin black testa, green cotyledon (Figure 2.2.3)	Soft white testa, beige cotyledon (Figure 2.2.4)
Dispersal aid	Green fruit tissue: not edible[5]	None, acorn itself of nutritional value[2]	Red aril: not edible[6]	Fleshy fruit tissue: edible[4]
Seed storage behaviour	Possibly intermediate[1]	Recalcitrant[2]	Recalcitrant[3]	Recalcitrant[4]
Average seed weight (g)	1.5-2.0	3.0-5.0	1.0-3.0	2.0-5.0
Seasonality of shedding	January- April	March-April/ Poland: September-October	March-July	All year; collection: June-September
Location harvested	Nelspruit	Himeville/Poland	Durban	Durban
Transit period	3-5 days	Himeville: 2 days, Poland: 3 weeks	30 min-3 hours	12-16 hours

[1] http://data.kew.org/sid/SidServlet?ID=4331&Num=izH&_ga=1.140210099.1445309976.1470598122; [2] http://www.kew.org/science-conservation/plants-fungi/quercus-robur-englishoak; [3] http://data.kew.org/sid/SidServlet?ID=23333&Num=a4R; [4] https://hort.purdue.edu/newcrop/morton/jackfruit_ars.html; [5] Mondal *et al.* (2004); [6] http://www.plantzafrica.com/planttuv/trichildreg.htm.
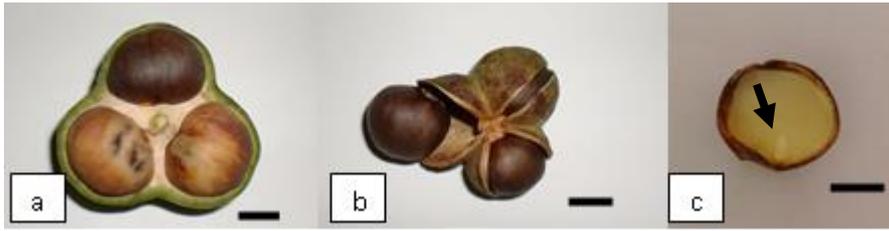
Figure 2.2.1: *C. sinensis* fruit showing (a) immature seeds, (b) mature seeds from dry fruit tissue and (c) longitudinal section showing the position of the axis within the seed. Scale bar = 10 mm.
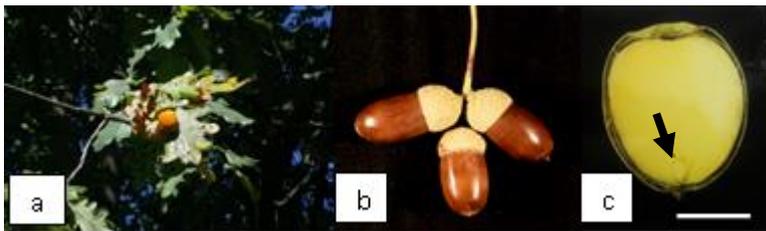
Figure 2.2.2: *Q. robur* acorn (a) whilst attached to the parent plant, (b) when harvested with the cupules attached (photo credit: P. Berjak and N.W. Pammenter) and (c) longitudinal section showing the position of the axis within the seed (Photo credit: P. Berjak and N.W. Pammenter). Scale bar = 10 mm.

Figure 2.2.3: *T. dregeana* seeds (a) whilst attached to the parent plant, (b) when harvested within fruit capsules (Photo credit: P. Berjak and N.W. Pammenter), (c) whole seed and (d) longitudinal section showing the position of the axis within the seed. Scale bar = 10 mm.
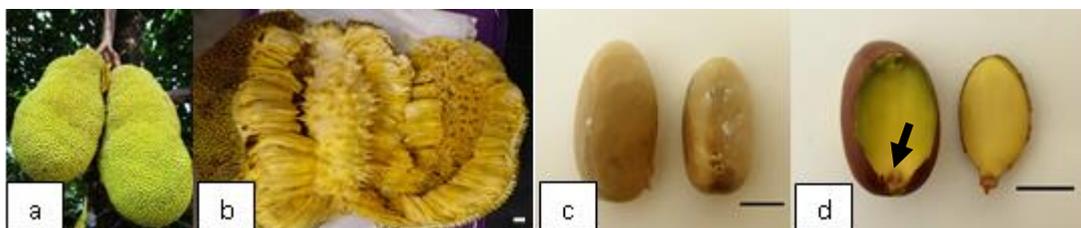
Figure 2.2.4: *A. heterophyllus* (a) whilst attached to the parent plant, (b) longitudinal section showing the fruit enclosing the seeds, (c) whole seeds and (d) longitudinal section showing the position of the axis within the seed. Scale bar = 10 mm.

2.3   Seed procurement and sterilisation

All seeds were collected by hand and transported to the laboratory. Seeds were cleaned within a day of receipt/collection (unless stated otherwise), using an 11 pt. sterile blade, sterilised and rinsed three times with sterile distilled water. Thereafter, the seeds were dried to original batch weight between two layers of paper towel on a laboratory bench at ambient temperature ($\pm$ 22°C) prior to hydrated storage. The details of seed collection and processing for individual species are given below.

2.3.1 *Camellia sinensis*

Mature fruits were collected directly from trees of *C. sinensis* from the Agricultural Research Council in Nelspruit, South Africa (25° 06' 50.62" S; 31° 05' 07.61" E) between February-May (2013-2015). A batch of seeds were collected in December 2013 which yielded mainly immature seeds and was not used for experimentation. The fruits were road freighted at ambient temperature in perforated polyethylene bags (approximately four days in transit). Upon arrival, the fruit tissue was removed, seeds were stored overnight in buckets with a saturated towel covering the seeds and were sterilised the next day. Seeds were selected based on size (> 1cm diameter), colour (dark brown: indicative of maturity, see Figure 2.2.1 for differences in seed colour) and buoyancy by placing seeds in a large container filled with water, seeds that floated were removed and discarded.

The seeds were surface-sterilised by soaking in 1% (m/v) 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 80-100 rpm. The seeds were rinsed once with sterile distilled water and further decontaminated for 30 min in a 0.05% (w/v) sodium dichloroisocyanurate (NaDCC) solution which has been shown to be less phytotoxic (Barnicoat *et al.*, 2011). The seeds were rinsed three times with sterile distilled water, blotted dry with paper towel, dried overnight and dusted with Benomyl 500 WP (active ingredient, benzimidizole; Villa Protection, South Africa).

2.3.2 *Quercus robur*

Acorns of were collected from the ground after natural shedding from mature trees along roadsides in Underberg, South Africa (29° 41' 51.482" S; 29° 31' 33.709" E to 29° 41' 30.797" S; 29° 31' 11.51" E) and Himeville, South Africa (29° 44' 44.58" S; 29° 30' 48.479" E) between April-May (2014-2015). Seeds that were freshly shed were road freighted at ambient temperature in hessian bags (approximately one-two days in storage prior to transit).

Upon arrival, the seeds were stored at 3°C in a temperature controlled room. Seeds were selected based on size (>2.0 cm length), appeared shiny with no visible breaks in the pericarp (Figure 2.2.2), absence of air pockets and buoyancy. The cupules and fruit stalks were removed, seeds were placed in a large container filled with water; seeds that floated were removed and discarded.

Seeds collected in 2014 were surface-sterilised by soaking in 1% (m/v) NaOCl solution containing a few drops of the wetting agent, Tween 20/80® for 30 min on an orbital shaker at 80-100 rpm, rinsed three times with sterile distilled water, dried overnight and dusted with Benomyl 500 WP.

Seeds placed in hydrated storage were periodically sprayed with 10% ethanol weekly, however, this induced fungal proliferation regardless of temperature, and after 6 weeks the seeds were sterilised with a single rinse of sterile distilled water between decontamination, in 0.05% (w/v) NaDCC for 60 min on an orbital shaker at 80-100 rpm, an anti-fungal mixture containing 0.5 ml l^{-1} Early Impact (active ingredients, triazole and benzimidizole; Zeneca Argrochemicals, South Africa) and 2.5 ml l^{-1} Previcur N (active ingredient, propamocarb-HC; AgrEvo, South Africa) for 120 min on an orbital shaker at 80-100 rpm. The active ingredients contained in this mixture have been shown to effectively reduce fungal proliferation during hydrated storage of recalcitrant seeds (Calistru *et al.*, 2000). The seeds were rinsed three times with sterile distilled water, blotted dry with paper towel, dried overnight and dusted with Benomyl 500 WP.

Additionally, seeds were also air freighted in perforated polyethylene bags from Druzyna, Poland (coordinates not provided, near Poznan) due to poor seed quality and fungal proliferation in the batch of seeds collected in 2015 (see results: Fig. 3.2.1 and 3.2.2). The reduced number of seeds resulted in fewer sampling points in storage. Seeds from Poland were sterilised using the same methodology as the batch collected in 2014, were stored non-hydrated at 3°C and used for further analyses.

2.3.3 *Trichilia dregeana*

Opened fruits and seeds were harvested directly from *T. dregeana* trees from the University of KwaZulu-Natal (Westville Campus) (29° 49' 3.188" S; 30° 56' 31.024" E), along municipal roads in Reservoir Hills (29° 48' 16.463" S; 30° 56' 42.871" E to 29° 48' 5.269" S; 30° 57' 31.228" E), and Glenwood (29° 51' 51.288" S; 30° 58' 36.223" E) in Durban, South Africa between April-June (2013-2015). Seeds were selected based on appearance, having a waxy and shiny orange-red aril and no breaks in the seed coat, no visible

damage caused by insects/insect larvae (Figure 2.2.3), no fungal contamination and buoyancy. The seed coat and aril were removed and placed in a large container filled with water, seeds that floated were removed and discarded.

The seeds were surface-sterilised by soaking in 1% (m/v) NaOCl solution containing a few drops of the wetting agent, Tween 20/80® for 30 min on an orbital shaker at 80-100 rpm, rinsed three times with sterile distilled water, thereafter immersed in an anti-fungal mixture containing 0.5 ml l^{-1} Early Impact and 2.5 ml l^{-1} Previcur N for 240 min on an orbital shaker at 80-100 rpm (Calistru *et al.*, 2000). The seeds were rinsed three times with sterile distilled water, dried overnight to original batch weight on a laboratory bench (± 22°C) between two layers of paper towel and dusted with Benomyl 500 WP.

2.3.4 *Artocarpus heterophyllus*

Mature fruits were harvested directly from a single *A. heterophyllus* tree in Clare Estate (29° 48' 47.689" S; 30° 57' 33.692" E) in Durban, South Africa between June-August 2014. The fruit was yellow in colour and had a distinctive aroma. Seeds were removed from the fleshy arils and cleaned of any residual pulp. Thereafter, seeds were rinsed thoroughly in water and left to dry for three hours between four layers of newspaper. Seeds were selected based on size (>2.0 cm length), with no visible breaks in the exocarp (Figure 2.2.4), no visible damage caused by insects/insect larvae and buoyancy by placing seeds in a large container filled with water, seeds that floated were removed and discarded. For studies evaluating desiccation tolerance, seeds were removed from the arils at least 30 min prior to use. These seeds were cleaned of any residual pulp using paper towel and were not rinsed.

The seeds were surface-sterilised by soaking in 0.05% (w/v) NaDCC solution for 30 min on an orbital shaker at 80-100 rpm, then rinsed three times with sterile distilled water. Thereafter, the seeds were immersed in an anti-fungal mixture containing 0.5 ml l^{-1} Early Impact and 2.5 ml l^{-1} Previcur N for 60 min on an orbital shaker at 80-100 rpm. The seeds were rinsed three times with sterile distilled water, blotted dry with paper towel and dried between two layers of paper towel for three hours until the exocarp turned white and dry in appearance. The seeds were then dusted with Benomyl 500 WP.

2.4   Seed storage

2.4.1 Hydrated storage

Decontaminated seeds of all species were placed in two-three layers on a plastic mesh suspended 200 mm above paper towel saturated with sterile distilled water and a few drops of commercial domestic bleach, in white 5 l plastic buckets. Bucket lids were lined with paper towel (to prevent condensate dripping back onto the seeds) before the buckets were closed and stored in 3 and 16°C constant temperature rooms. Both plastic mesh and buckets had been previously disinfected by soaking them in a 1% NaOCl solution for 1 h, subsequently sprayed with 70% ethanol and left to air dry in the laboratory prior to use.

Once in storage, seeds were checked periodically for fungal contamination. Buckets were opened regularly and the seeds were rearranged by gently shaking the plastic mesh. Moistened paper towel was replaced when necessary as well as the paper towel lining around the lid. Seeds that germinated in storage were removed from the buckets and discarded.

2.5   Axis excision

Embryonic axes were excised with an 11 pt. sterile blade or a blunt scalpel, and collected in a relative humidity chamber consisting of a 65 mm Petri dish lid placed on two layers of moistened Whatman® Filter paper (diameter: 90 mm) enclosed in a 90 mm Petri dish. All subsequent axis excisions throughout the experiment were accumulated in the same manner. This was performed to prevent water loss from embryonic axes during ongoing axis excisions.

Axes of *C. sinensis* and *A. heterophyllus* were excised without any cotyledonary segments attached, whilst axes of *Q. robur* and *T. dregeana* were excised with a small cotyledonary fragment covering the shoot apex. Axes which were subject to rapid desiccation were dried with the cotyledonary pieces attached. These cotyledonary pieces were removed prior to water content determination and 5-10 min prior to biochemical analyses, but remained attached for *in vitro* culturing.

2.6   Dehydration

2.6.1 Rapid dehydration

Embryonic axes were rapidly dehydrated in a flash dryer (Berjak *et al.*, 1990; Wesley-Smith *et al.*, 2001b) for various time intervals in order to obtain the selected water contents

(Table 2.2). Flash drying curves were generated in duplicate over two different years of collection (data shown for one harvest). Forty axes were placed in the flash dryer, of which 20 axes were used for viability assessment, 10 axes were used to determine water content and the remaining 10 axes were used to estimate respiratory activity (section 2.11.2).

Table 2.2: Time intervals used to rapidly desiccate embryonic axes in a flash dryer from shedding water content to 0.1 g g⁻¹ DW for each species.

Species	Drying intervals (min)
C. sinensis	15, 30, 45, 60, 90, 120, 150, 180
Q. robur	1, 5, 30, 60, 90, 120, 180, 240, 300, 360, 450, 540
T. dregeana	30, 45, 60, 90, 180, 270, 360, 450
A. heterophyllus	1, 2, 5, 10, 15, 30, 45, 60, 75, 90

2.6.2 Assessment of drying rate

Drying rates were assessed as the rate constant (*b*) of the modified inverse function relating relative water content to time:

$$RWC = b / (b + t)$$

where RWC is the water content relative to the initial water content, and t is drying time *b* being calculated as the slope of a plot of 1/RWC against time (Pammenter *et al.*, 2003).

2.7   Rehydration

After dehydration, axes of *C. sinensis*, *Q. robur*, *T. dregeana* and *A. heterophyllus* were rehydrated (section 2.11) within closed Petri dishes in 25 ml of cathodic water for 30 min (Berjak *et al.*, 2011b) in the dark prior to sterilisation. For *T. dregeana* and *Q. robur*, axes were flash dried with cotyledonary pieces attached, rehydrated in 25 ml of cathodic water for 15 min in the dark and then transferred to a laminar air-flow cabinet. The cotyledonary pieces of *T. dregeana* were removed with a hypodermic needle and a dissecting needle whilst immersed in cathodic water under green light (required 15 min). These axes did not produce shoots (data not shown); hence a second drying curve was performed without

removing the cotyledonary pieces. Similarly, cotyledonary pieces attached to *Q. robur* axes were not removed.

2.8   Preparation of cathodic water

Cathodic water, which is a strong reductant (Hanaoka, 2001), was prepared by the electrolysis of an autoclaved solution of 1 mol L^{-1} $CaCl_2$ and 1 mmol L^{-1} $MgCl_2$ (CaMg solution; (Berjak *et al.*, 1999). The apparatus consisted of two glass beakers, each with 200 ml of CaMg solution. The platinum anode and cathode were immersed in the CaMg solution in two different beakers and the circuit was completed using an agar-based salt bridge (containing saturated potassium chloride in 3% agar). Electrolysis at 60 V for 1 h at room temperature yielded the cathodic (reducing) water at pH c. 11.2, which was always used on the day of preparation or stored at 4°C for not more than a day before being used.

2.9   ROS estimation

2.9.1 Extracellular superoxide assay

Extracellular superoxide production was measured by the oxidation of epinephrine (Sigma, St. Louis, MO) to adrenochrome (Misra and Fridovich, 1972). For each species, four replicates, each comprising of five excised embryonic axes, from fresh (control) and various desiccation and storage treatments, were gently agitated in 2.0 ml Eppendorf tubes on an orbital shaker at 70 rpm in 2.0 ml of 1 mM epinephrine (12.8 mg epinephrine dissolved in 200 µl of 1 M HCl and 9.8 ml of distilled water; pH adjusted to 7.0 using 5 M, 1 M and 0.1 M NaOH), in the dark for 15 min. Thereafter 2 ml of the solution was measured for absorbance at 490 nm spectrophotometrically (ultraviolet-visible spectrophotometer [Shimadzu, UV-2600, Japan]) The embryonic axes were then dried for 72 h in an oven (Gallenkam Incubator 1H150, England) at 80°C. The amount of superoxide produced was calculated using the extinction coefficient, 4.47 $mM^{-1}cm^{-1}$, and expressed as µmol min^{-1} g^{-1} DW.

Confirmatory assay: In order validate the use of this assay for the detection of extracellular superoxide production, 250 U ml^{-1} SOD extracted from solid bovine liver was dispensed into Eppendorf tubes containing the incubation medium described above and axes of each species. The addition of SOD inhibited the oxidation of epinephrine by more than 50% in embryonic axes from seeds stored hydrated and partially dehydrated (Appendix A).

2.9.2 Extracellular hydrogen peroxide assay

Extracellular hydrogen peroxide production was based on the oxidation of Fe^{2+} to Fe^{3+} in the presence of xylenol orange, measured in terms of the Fe-XO complex (Gay and Gebicki, 2000). The working solution comprised of 0.3 ml of reagent A (containing: 25 mM $FeSO_4$, 25 mM $(NH_4)_2SO_4$ and 2.5 M H_2SO_4) and 30 ml of reagent B (containing: 125 mM xylenol orange and 100 mM sorbitol), combined 20 min before use. Four replicates, each comprising of five excised embryonic axes, from each species, at both temperatures during storage/partially dehydrated samples, were gently agitated on an orbital shaker at 70 rpm in 700 µl (*C. sinensis*) or 1.0 ml (*Q. robur, T. dregeana* and *A. heterophyllus*) of Millipore filter sterilised distilled water for 30 min in the dark. Thereafter, 300 µl of the leachate was added to 1.5 ml of the working solution for 30 min in the dark. Thereafter, the solution was measured for absorbance at 560 nm spectrophotometrically as described above. The embryonic axes were then dried for 72 h in foil bags in an oven at 80°C.

A standard curve using 0.05-2.0 µM H_2O_2 (Sigma-Aldrich, Steinheim, Germany) incubated in the working solution was constructed. The absorbance of samples was expressed as H_2O_2 equivalents on a dry weight basis using the standard curve.

Confirmatory assay: In order to validate the use of this assay for the detection of extracellular hydrogen peroxide production, 500 U ml^{-1} CAT extracted from solid bovine liver was dispensed into Eppendorf tubes containing the incubation medium and leachate as described above for each species. The addition of catalase inhibited hydrogen peroxide production by more than 50% in embryonic axes from seeds stored hydrated and partially dehydrated (Appendix A).

2.10   Total aqueous antioxidant activity (TAA)

Total antioxidant activity was determined by measuring the depletion of the 2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical in the presence of an antioxidant extract (Re *et al.*, 1999). For samples taken from hydrated storage, 5 replicates of each species, composed of 7-10 embryonic axes, at each temperature were weighed and snap frozen in liquid nitrogen (LN) and stored at -80°C for later use. Additionally, axes from seeds which germinated in storage in sufficient numbers were also excised and stored in a similar manner as described above. For partially dehydrated samples, five replicates of each species, repeated twice were prepared and stored in a similar manner as described above.

The embryonic axes were transferred to a pre-chilled pestle and mortar and ground finely after the addition of 50 mg of insoluble PVP (Sigma, Germany). The total antioxidants

were extracted in 1 ml of chilled extraction buffer (containing: 1 mM $CaCl_2$, 1 mM KCl, and 1 mM EDTA, pH 7.0). The homogenate was transferred into a pre-chilled Eppendorf tube and centrifuged at 11000 g at 4°C for 20 min. The resulting supernatant was extracted and centrifuged for a further 10 min, transferred into a second Eppendorf tube if necessary, snap-frozen and stored at -20°C for up three days (Johnston et al., 2006). The ABTS solution (containing 7 mM ABTS and 2.45 mM $K_2S_2O_8$ in 1 ml of distilled water) was prepared 12-16 h prior to centrifuging and kept in the dark. The ABTS solution was diluted with phosphate buffer saline (PBS) (containing: 5 mM $Na_2H_2PO_4$, 37.5 mM NaCl, pH 7.4) until an initial absorbance of 0.72 at 734 nm was achieved. One ml of PBS was used as a blank. The decolourization of the working solution was measured before addition of 5, 10, 20, 30, 40, 60 and 80 µl (standardised for each species sampled during hydrated storage/partially dehydrated) of the antioxidant extract 120 s after addition.

A standard curve using 0.05-3.0 µM or 0.05-1.0 mM Trolox™ (Sigma-Aldrich, Steinheim, Germany), dissolved in PBS was constructed. The change in absorbance of samples was expressed as Trolox equivalents on a fresh weight basis using the standard curve.

2.11 Viability assessment

Whole seed germination was performed on 15 freshly harvested seeds, repeated twice per collection. The seed coats of *C. sinensis* and *Q. robur* seeds were scarified and placed in trays filled with vermiculite. The seeds were watered every 3-5 days with 50 ml of distilled water. Seeds of *T. dregeana* (without the aril) and *A. heterophyllus* were germinated between two layers of moistened filter paper in 90 mm Petri dishes. In each case, seeds were germinated in a dark cupboard, at ambient laboratory temperature until radicle protrusion > 1cm.

Twenty excised embryonic axes, from each species, stored at either temperature, or partially dehydrated samples were immersed directly (Perán *et al.*, 2004) in 25 ml of cathodic water (Berjak *et al.*, 2011b) within closed 65 mm Petri dishes for 30 min in the dark. Thereafter, the axes were sterilised and germinated *in vitro* in 65 mm Petri dishes (five axes x three replicates). These cultures were initially placed in the dark at 22°C for up to three weeks or until radical extension growth was observed and moved to a growth room with cool fluorescent lights (50 µmol m^{-2}s^1) and a 16-h photoperiod, at 25 ± 2 °C. Viability was assessed after a further 60 d *in vitro* growth as root and shoot production.

2.11.1 Decontamination and *in vitro* germination

Each species was decontaminated and germinated *in vitro* according to studies published in literature or modified based on techniques developed in our laboratory (cited in Table 2.3). Embryonic axes (from fresh and storage treatments) were excised and placed in a hydrated chamber; once a sufficient number was accumulated, axes were immersed in 25 ml of cathodic water for 30 min prior to being transferred to a laminar air-flow cabinet. Flash-dried axes were directly immersed into cathodic water immediately after removal from the flash drier. Axes were surface-sterilised by serial immersion in 50 ml (25 ml x 2) of various decontaminants (Table 2.3), with a single rinse (1 min) of sterile CaMg solution between each, and finally three rinses with sterile CaMg solution (5 min in total), and blotted dry on Whatman filter paper prior to culturing.

The basic *in vitro* culture media comprised of MS (Murashige and Skoog, 1962) basal medium supplemented with growth hormones (initially dissolved in ethanol or 1 N NaOH and made up in Millipore filter-sterilised water). These growth regulators were stored at 4°C for less than three months and media less than a month. The pH of the media was adjusted to 5.6-5.8 using 1 M NaOH and HCl (Table 2.3).

2.11.2 Triphenyl tetrazolium chloride (TTZ) assay

Embryonic axis viability was measured based on the reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) (Sigma, Germany) to insoluble triphenyl formazan, which stains actively respiring tissues red (Moore, 1962). The assay was based on modifications by Harding and Benson (1995) and Verleysen *et al.* (2004). For each species, partially dehydrated/storage samples, ten excised embryonic axes were incubated in a 2% solution of 0.1 g of TTC dissolved in 5 ml Tris-HCl buffer (pH 7.6; 0.05 M). To each Eppendorf tube, a single embryonic axis was incubated with 300 µl of the 2% solution and 1200 µl of Tris-HCl. After incubating for 12 h at ± 25°C, the embryonic axes were bisected and the formazan extracted in 1 ml absolute ethanol. All steps were carried out in darkness. The ethanol extract was then measured for absorbance at 500 nm spectrophotometrically as described above, at a constant temperature of 25°C. The embryonic axes were then dried for 72 h in foil bags in an oven at 80°C to determine the dry weight. The respiratory activity was calculated using the extinction coefficient, 12.2 $mM^{-1}cm^{-1}$, and expressed as µM Formazan g^{-1} DW.

Due to numerous inconsistencies with this assay in 2013, possibly arising from fungal proliferation, the extracted formazan was not read spectrophotometrically for partially dehydrated/storage samples in 2014-2015. The respiratory activity was assessed based on the

colour of the axes and solution as a quicker method to determine viability retention, since germination *in vitro* required an extended period. Therefore, these results are not reported.

Table 2.3: Media preparation and *in vitro* decontamination of embryonic axes of *C. sinensis, Q. robur*, *T. dregeana* and *A. heterophyllus*.

		Media preparation		
Species	*C. sinensis*	*Q. robur*	*T. dregeana*	*A. heterophyllus*
Reference	Pritchard and Prendergast (1986)	Walker (2000)	Varghese *et al.* (2011)	Govender (2009)
Plant growth medium (MS)	Full strength, 4.4 g l^{-1}	Half strength, 2.2 g l^{-1}	Full strength, 4.4 g l^{-1}	Quarter strength, 1.1 g l^{-1}
Sucrose	30 g l^{-1}	30 g l^{-1}	30 g l^{-1}	30 g l^{-1}
Bacteriological agar	8 g l^{-1}	8 g l^{-1}	8 g l^{-1}	8 g l^{-1}
Auxin	IAA, 0.2 mg l^{-1}	NAA, 0.1 mg l^{-1}	None	NAA, 0.5 mg l^{-1}
Cytokinin	Kinetin, 2 mg l^{-1}	BAP, 2 mg l^{-1}	None	None
Amino acid	None	None	None	Arginine, 0.1 mg l^{-1}
		Serial decontamination		
Species	*C. sinensis*	*Q. robur*	*T. dregeana*	*A. heterophyllus*
Reference	Wesley-Smith *et al.* (1992)	Walker (2000)	Goveia (2007)	Fu *et al.* (1993)
Decontaminant 1	0.05% (w/v) NaDCC, 10 min	0.05% (w/v) NaDCC, 20 min	2% (v/v) Hibitane®, 2 min	0.05% (w/v) NaDCC, 10 min
Decontaminant 2	0.2% (v/v) Hibitane® (Zeneca, South Africa), 10 min	None	70% (v/v) ethanol, 2 min	1% (w/v) Amoxycillin (Adco-amoxycillin, Adcock Ingram Ltd., South Africa), 5 min
Decontaminant 3	None	None	0.05% (w/v) NaDCC, 5 min	None

2.12 Gravimetric determination of water content

Water content was determined gravimetrically for 10 embryonic axes excised from seeds sampled from hydrated storage/immediately after rapid dehydration. Axes were weighed on a 6-place balance (Mettler, MT5, Germany) before and after drying over silica gel in an oven at 80°C for 48 h. Water content was thereafter expressed on a dry weight basis (g H_2O per g DW; g g^{-1}).

2.13 Statistical analyses

The data were analysed using IBM SPSS Statistics 23 (SPSS Inc., Chicago, IL). Preliminary studies were evaluated using One-Way ANOVA or Independent-samples t-test. Germination percentiles were tested using a Pearson Chi-squared analysis. Responses to desiccation were tested on the ranks of the data for water content, root and shoot production via One-Way ANOVA. Drying rates were not analysed due to insufficient replication for reliable statistics Oxidative response (extracellular superoxide, extracellular hydrogen peroxide, and TAA) to desiccation was tested using One-Way ANOVA. Mean separation for all analyses was performed using a Tukey post hoc test. Oxidative parameters, water content and survival percentiles assessed during hydrated storage at each temperature were tested using a Spearman's Rank correlation (see Appendix B for correlation matrices). In all cases, Spearman's rho values were subjected to square root transformation to represent the proportion of shared variance between the two ranked variables. Where data did not meet parametric assumptions of normality (Kolmogorov-Smirnov test) and equal variance (Levene's test), even after transformation, a non-parametric alternative (Kruskal-Wallis) test was applied. All differences were considered significant at the 0.05 level.

CHAPTER 3: RESULTS

3.1 Preliminary studies evaluating axis shedding water content, maturity status and differences in harvesting locations.

Shedding water content did not differ significantly between different years of collection, but was significantly different when compared between species ($p < 0.0001$) (Figure 3.1.1). Embryonic axes of *T. dregeana* and *C. sinensis* were shed at a significantly higher water content than other species from the same provenance (*A. heterophyllus* and *Q. robur*, respectively) over two different years of collection (Figure 3.1.1).
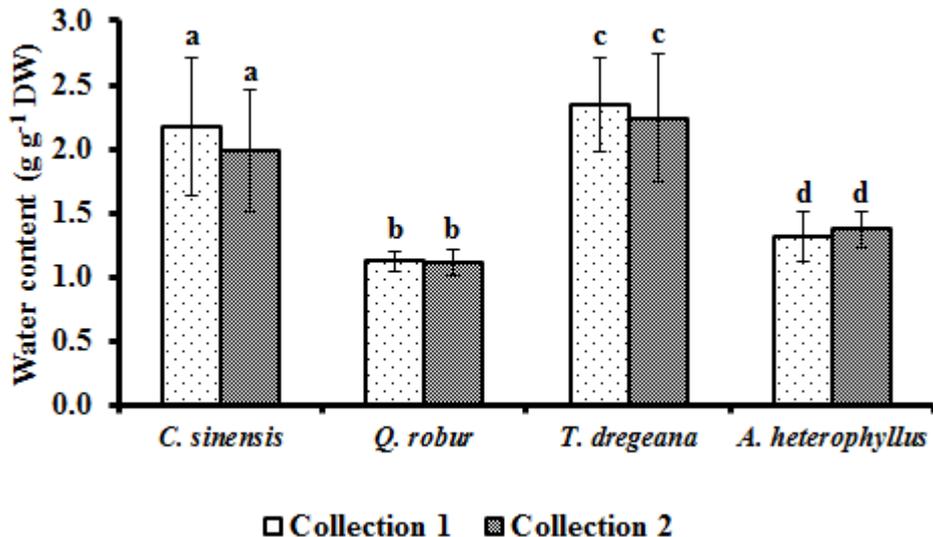
Figure 3.1.1: Shedding water content of embryonic axes of *C. sinensis*, *Q. robur*, *T. dregeana* and *A. heterophyllus* collected twice over a three year period (2013-2015). For each species, values represent the average (±SD) of n=10 axes, repeated twice. Columns labelled with different letters are significantly different, amongst species ($p < 0.05$; Kruskal-Wallis).

The effect of harvesting *C. sinensis* seeds earlier (December 2013) resulted in a significantly higher shedding water content ($p < 0.0001$) when compared with seeds harvested in February 2014 (Figure 3.1.2). Seeds harvested in December were considered to be immature, indicated by varying degrees in the physical consistency of the cotyledons. This ranged between a gel-like texture and opaque colour, to a semi-solid to solid texture and

cream to yellow colour. Seeds harvested in February were mature, cotyledons were uniform in colour and consistency.
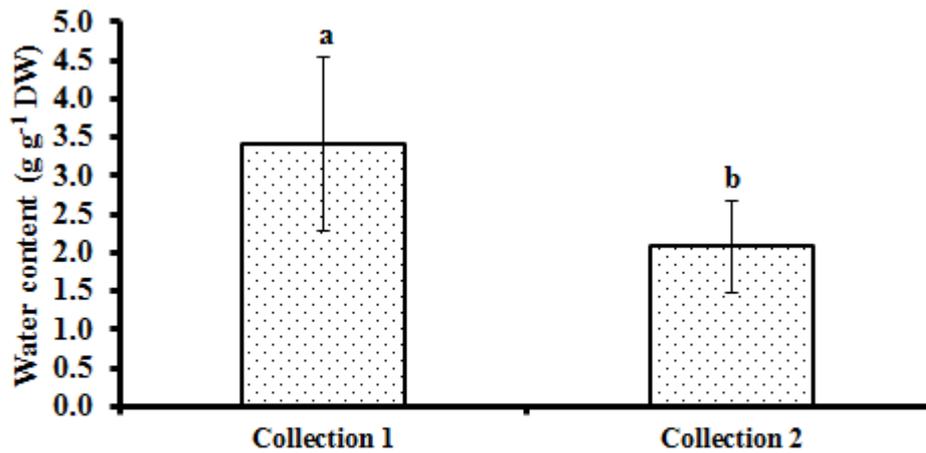
Figure 3.1.2: Effect of maturity status on water content of embryonic axes of *C. sinensis*, seeds harvested in February 2013 (collection 1) were considered mature, whilst those harvested in December 2013 (collection 2) were considered immature. Values represent the average (±SD) of n=10 axes. Columns labelled with different letters are significantly different between collections (p<0.05; t-test).

Q. robur seeds are shed in a variety of sizes and from great heights, which often result in breaks or cracks in the seed coat. Seed size and the presence/absence of air pockets had a significant impact on water content (p<0.05), although seeds that were small (~2 cm) did not differ in water content irrespective of the presence/absence of air pockets (Figure 3.1.3). Large seeds without an air pocket had a significantly higher water content than large seed with an air pocket, possibly indicating that those seeds were newly shed (Figure 3.1.3). Hence, seeds which were selected for storage experiments were large and did not contain air pockets.
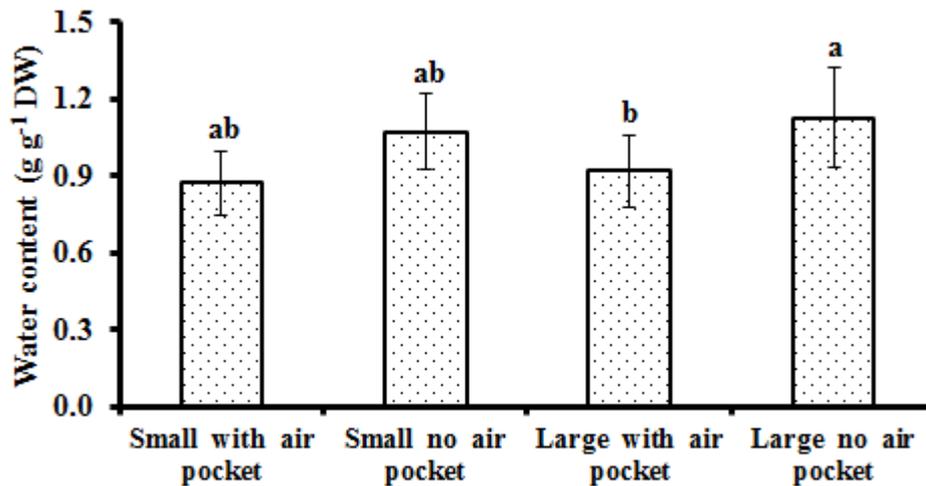
Figure 3.1.3: Variability of seed size and the presence/absence of air pockets as indicators of time elapsed after shedding of *Q. robur* seeds. Values represent the average (±SD) of n=10 axes. Columns labelled with different letters are significantly different between seed characteristics (p<0.05; One-way ANOVA).

Seeds of the same species harvested in different geographical locations may result in different shedding water contents. *Q. robur* seeds collected from the Drakensberg region in South Africa had a significantly lower water content than seeds collected in Poland (p<0.05) (Figure 3.1.4).
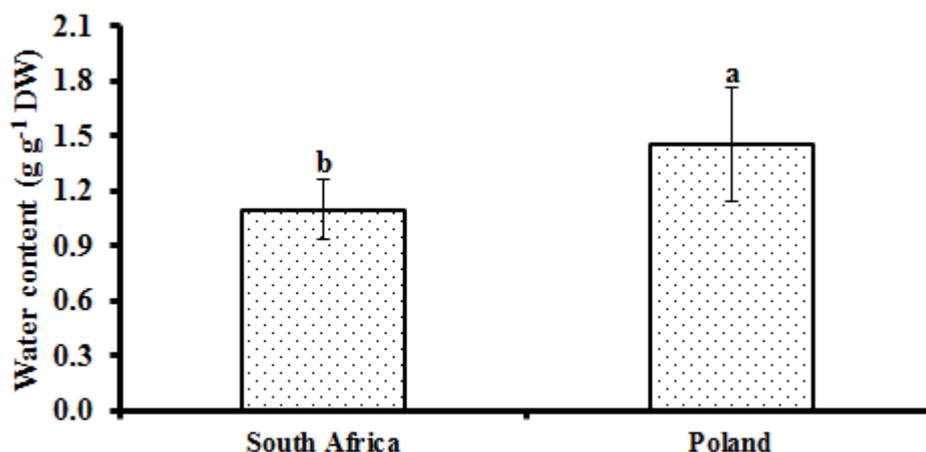
Figure 3.1.4: Effect of differing provenance on shedding water content of embryonic axes of *Q. robur* seeds harvested from South Africa (2014) and Poland (2015). Values represent the average (±SD) of n=10 axes. Columns labelled with different letters are significantly different between harvesting locations (p<0.05; t-test).

A. heterophyllus seeds are shed in an aggregate fruit and may have differing maturity and hence differing water content despite the fruit being ripe. Large seeds exhibited a significantly lower water content than smaller seeds harvested from the same ripe fruit ($p<0.05$) (Figure 3.1.5). Seeds of smaller size harvested from a green fruit, exhibited a significantly higher water content ($p<0.0001$) when compared with small seeds from a large ripe fruit (Figure 3.1.5). Also, TTZ staining showed axes from a green jackfruit to have a darker and denser staining compared with axes from a ripe fruit (data not shown). Hence, seeds which were selected for storage and desiccation experiments were large in size and harvested from large ripe fruit.
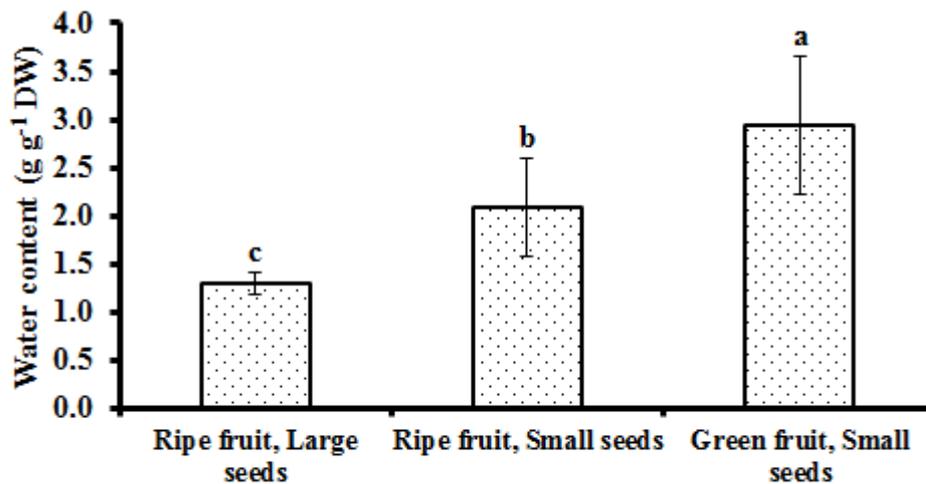
Figure 3.1.5: Effect of maturity status and seed size on water content of embryonic axes of *A. heterophyllus* seeds of different size within a ripe fruit compared with those within an immature (green) fruit harvested in June 2014. Values represent the average (±SD) of n=10 axes. Columns labelled with different letters are significantly different between maturity status (p<0.05; One-way ANOVA).

3.2 Initial germination of whole seeds and embryonic axes

After estimating maturity status and shedding water contents, whole seed germination and *in vitro* germination tests were performed. Apart from *Q. robur* seeds, all other species displayed 100% germination between different years of collection (Figure 3.2.1) and *in vitro* culture (Figure 3.2.2). *Q. robur* seeds were collected a month later in 2015 than the seeds collected in 2014. This extended period may have contributed to lower germinability as well

as higher incidences of fungal infection in seeds stored non-hydrated. Furthermore, when cultured *in vitro,* fungal proliferation ensued. As a result, seeds harvested in 2015 were not used, and a batch was received from Poland.
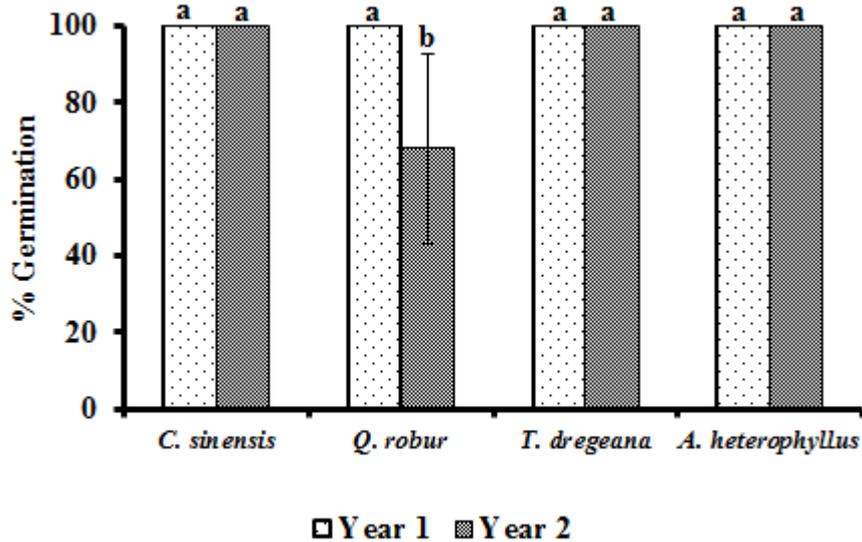
Figure 3.2.1: Initial whole seed germination (radicle protrusion > 1 cm) of *C. sinensis,* *Q. robur, T. dregeana* **and** *A. heterophyllus* **collected twice over a three year period (2013-2015). For each species, values represent the average (±SD) of n=15 seeds, repeated twice.**
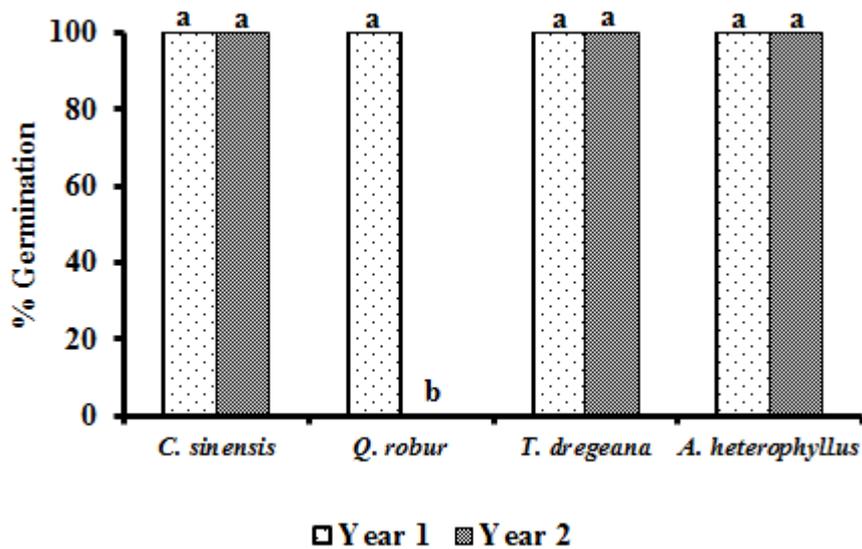
Figure 3.2.2: Initial *in vitro* **germination (radicle elongation > 1 cm) of** *C. sinensis,* *Q. robur, T. dregeana* **and** *A. heterophyllus* **collected twice over a three year period (2013-2015). For each species, values represent the average (±SD) of n=15 axes, repeated twice.**

3.3 Responses to rapid dehydration

Drying time had a significant effect on water content ($p<0.0001$) and root production ($p<0.0001$) in embryonic axes of *C. sinensis* (Figure 3.3.1a). Initially, the rapid decline in water content (2.0-0.5 g g^{-1} DW) resulted in no loss of root production when axes were dried for up to 60 min. Thereafter, root production declined linearly even though there was little change in water content (0.4-0.1 g g^{-1} DW). Extended periods of drying (180 min) resulted in the lowest root production ($20 \pm 20\%$). Despite losing a significant amount of water, shoot production in rapidly dehydrated *C. sinensis* axes was not significantly affected by extended drying periods ($p>0.05$) (Figure 3.3.1b). Although a large variability is noted, shoot production was maintained at around 80% throughout the drying time course (Figure 3.3.1b).

Drying time had a significant effect on water content ($p<0.0001$), root ($p<0.0001$) and shoot production ($p<0.0001$) in embryonic axes of *Q. robur* (Figure 3.3.2a and b). Initially, the rapid decline in water content (1.1-0.4 g g^{-1} DW) resulted in 20% loss of root production when axes were dried for up to 180 min, and root production was maintained at 80% (Figure 3.3.2a). Axes dried for 180-360 min resulted in a linear decline in root production congruent with a significant decline water content (0.4-0.2 g g^{-1} DW). Extending periods of drying (360-540 min) of *Q. robur* axes resulted in no further change in root production (40%), although callus production increased for the same drying period (data not shown). Shoot production was more sensitive to dehydration stress, after 90 min of drying, shoot production declined linearly until no shoots were produced after 450 min of drying (Figure 3.3.2b).
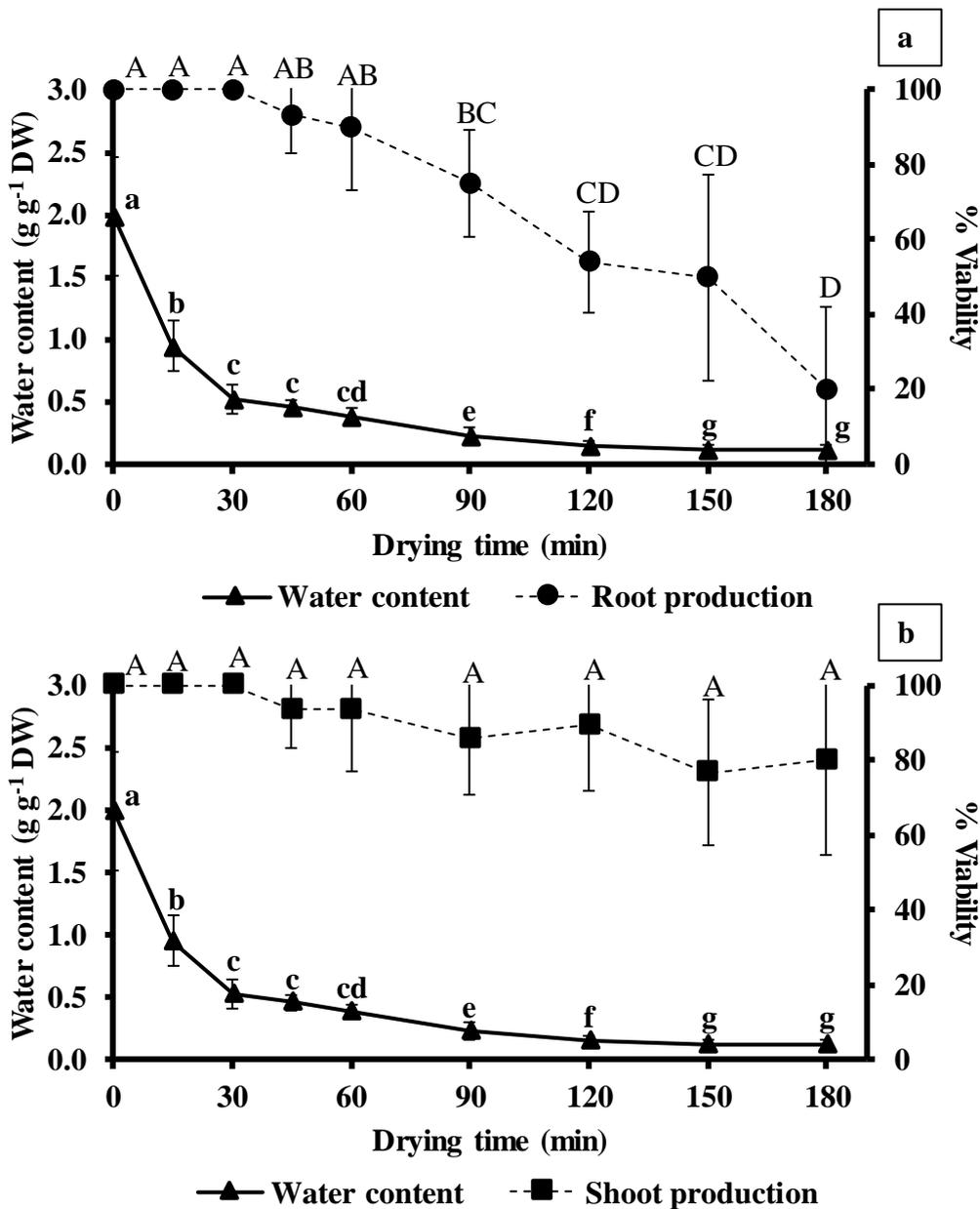
Figure 3.3.1: The relationship between drying time, water content and the associated root (a) and shoot (b) production of embryonic axes of *C. sinensis*. Values represent the average (±SD) of n=10 axes each for water content, repeated twice and 3 replicates of n=5 axes, repeated twice for root and shoot production. Data points labelled with different letters are significantly different between root or shoot production (uppercase), and between each water content (lowercase, bold) (p<0.05; Kruskal-Wallis).
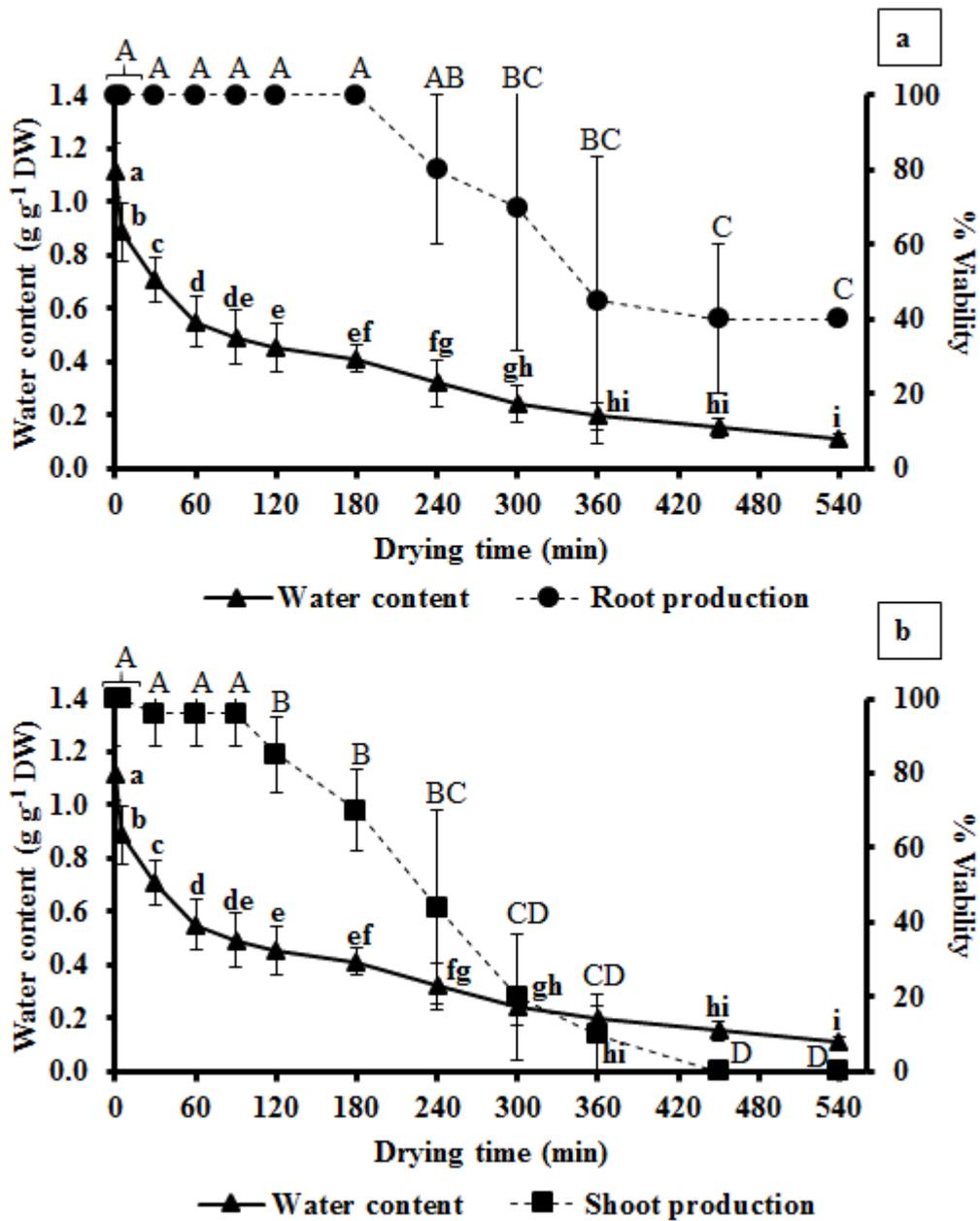
Figure 3.3.2: The relationship between drying time, water content and the associated root (a) and shoot (b) production of embryonic axes of *Q. robur*. Values represent the average (±SD) of n=10 axes each for water content, repeated twice and 3 replicates of n=5 axes, repeated twice for root and shoot production. Data points labelled with different letters are significantly different between root or shoot production (uppercase), and between each water content (lowercase, bold) (p<0.05; Kruskal-Wallis).

Drying time had a significant effect on water content ($p<0.0001$) and root ($p=0.003$) and shoot production ($p=0.002$) in embryonic axes of *T. dregeana* (Figure 3.3.3a and b). Initially, the rapid decline in water content (2.1-0.6 g g^{-1} DW) resulted in just 20% loss of root production when axes were dried for up to 90 min (Figure 3.3.2a). Axes dried for 90-360 min resulted in a linear decline in root production even though there was little change in water content (0.5-0.2 g g^{-1} DW), until no roots were produced. During the initial water loss phase, shoot production rapidly declined as water content decreased. Shoot production was more sensitive to dehydration stress; after 30 min of drying, shoot production in *T. dregeana* axes declined linearly until no shoots were produced after 270 min of drying (Figure 3.3.3b).

Drying time had a significant effect on water content ($p<0.0001$) and root ($p<0.0001$) and shoot production ($p<0.0001$) in embryonic axes of *A. heterophyllus* (Figure 3.3.4a and b). Initially, the rapid decline in water content (1.4-0.3 g g^{-1} DW) resulted in no loss of root production when axes were dried for up to 15 min (Figure 3.3.2a). Axes dried for 30-90 min resulted in a linear decline in root production although there was significant reduction in water content (0.3-0.1 g g^{-1} DW), until no roots were produced. Shoot production was maintained at 100% after 10 min of drying, corresponding to a water content of 0.5 g g^{-1} DW. Shoot production linearly declined as water content decreased after 30 min of drying, until 17 ± 16% shoots were produced after 90 min of drying (Figure 3.3.4b).
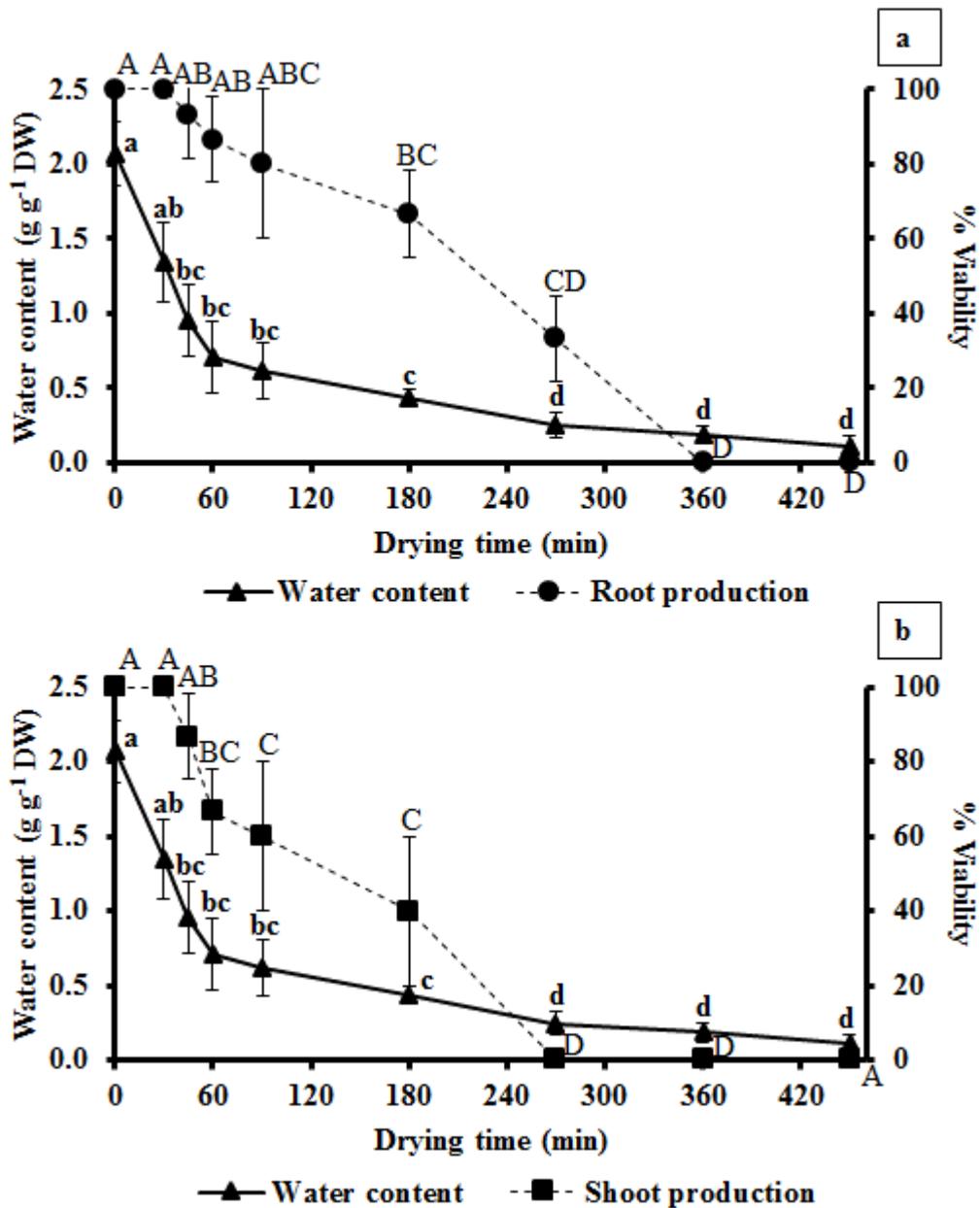
Figure 3.3.3: The relationship between drying time, water content and associated root (a) and shoot (b) production of embryonic axes of *T. dregeana*. Values represent the average (±SD) of n=10 axes each for water content, repeated twice and 3 replicates of n=5 axes, repeated twice for root and shoot production. Data points labelled with different letters are significantly different between root or shoot production (uppercase), and between each water content (lowercase, bold) (p<0.05; Kruskal-Wallis).
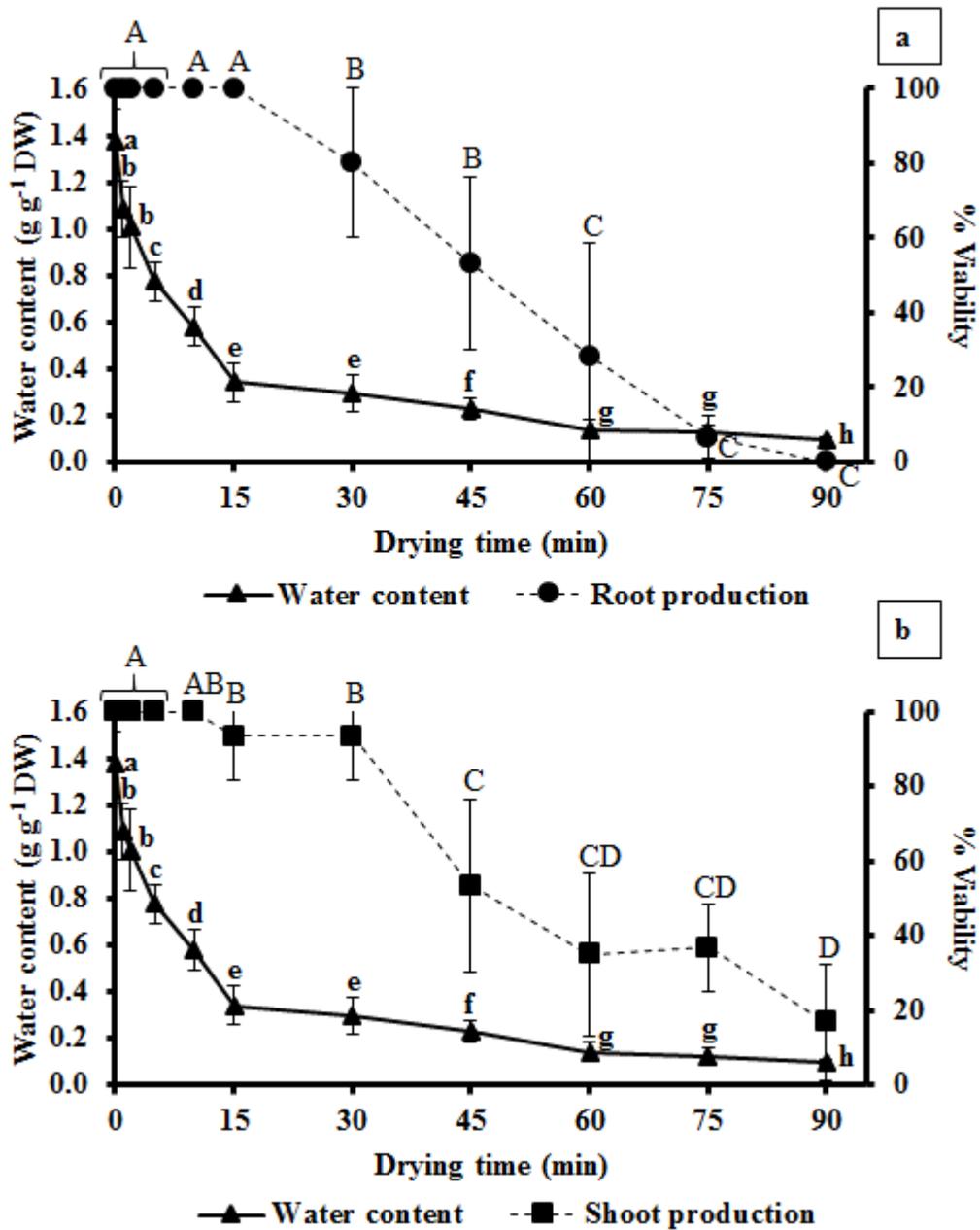
Figure 3.3.4: The relationship between drying time, water content and the associated root (a) and shoot (b) production of embryonic axes of *A. heterophyllus*. Values represent the average (±SD) of n=10 axes each for water content, repeated twice and 3 replicates of n=5 axes, repeated twice for root and shoot production. Data points labelled with different letters are significantly different between root or shoot production (uppercase), and between each water content (lowercase, bold) (p<0.05; Kruskal-Wallis).

Assessment of drying kinetics of embryonic axes of *C. sinensis*, *Q. robur*, *T. dregeana* and *A. heterophyllus*, show no distinct pattern between species of tropical and temperate regions (Figure 3.3.5). *C. sinensis* seeds are shed at a significantly higher water content and loses more water in a shorter time period than *Q. robur*, whilst *A. heterophyllus* seeds which are shed at a significantly lower water content and loses more water in a shorter time period than *T. dregeana*. Drying rates were fastest for *A. heterophyllus*, followed by *C. sinensis, T. dregeana* and the slowest for *Q. robur* (see Table 3.1 for actual figures).
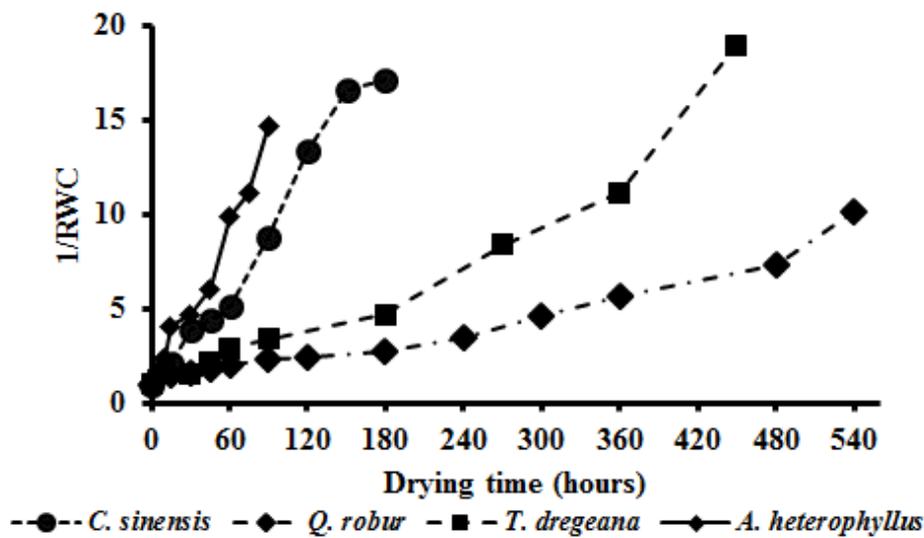
Figure 3.3.5: The relationship between drying time and the inverse of relative water content of embryonic axes of *C. sinensis*, *Q. robur*, *T. dregeana* and *A. heterophyllus* when rapidly dehydrated for various time intervals. The inverse function of RWC was calculated based on combined data of two replicates for each species.

Evaluation of the drying rate of embryonic axes of *C. sinensis*, *Q. robur*, *T. dregeana* and *A. heterophyllus*, show no distinct pattern between tropical and temperate provenances. The rate of water loss and the time taken to reduce water content to 50% (another indicator of drying rate) was fastest in *A. heterophyllus*, followed by *C. sinensis*, *T. dregeana* and the slowest for *Q. robur*. The slopes calculated from the fitted inverse equation also show a decrease in r^2 from the fastest drying rate to the slowest; however, *T. dregeana* had the slowest drying rate of all species, preceded by *Q. robur* (Table 3.1).

Table 3.1: Comparison of drying characteristics and the r^2 values of excised embryonic axes of *C. sinensis*, *Q. robur*, *T. dregeana* and *A. heterophyllus* when rapidly dehydrated. Drying rate and r^2 were calculated from the slopes of the fitted inverse equation.

Species	Temperate		Tropical	
	C. sinensis	*Q. robur*	*T. dregeana*	*A. heterophyllus*
Drying rate (g H_2O lost per min)	0.0982	0.0147	0.0355	0.1419
Time taken to reduce WC by 50% (min)	15	60	40	7.5
r^2	0.9789	0.9507	0.9358	0.9806

Based on drying curves generated for each species, three water contents were selected based on different states of water. Since drying rates were unrelated between provenances, a similar pattern emerged when drying time and % water loss were compared at the same water content. Species exhibiting the fastest drying rate (*A. heterophyllus* and *C. sinensis*) (Table 3.1) maintained a higher survival despite losing a significant amount of water (75 and 81%, respectively) (Table 3.2). *Q. robur* and *T. dregeana* axes maintained a higher survival at a higher water content, at 1.0 g g^{-1} DW, but thereafter lost viability at lower water contents (Table 3.2). This decline was more pronounced in axes of *Q. robur* than *T. dregeana*, despite losing a similar amount of water, which may be related to the extended drying period (240 min vs. 180 min, respectively). Axes of *A. heterophyllus*, *Q. robur* and *T. dregeana* lost viability completely when dehydrated to 0.1 g g^{-1} DW, and only axes of *C. sinensis* were able to maintain a low viability despite losing 94% water (Table 3.2).

Table 3.2: Differences between drying times, % water loss when partially dehydrated and associated survival of *C. sinensis*, *Q. robur*, *T. dregeana* and *A. heterophyllus* at three selected water contents or ranges when compared with embryonic axes excised from freshly harvested seeds. Data represents the average of 30 embryonic axes (survival: root and shoot development), % water loss calculated based on shedding water content. Numbers labelled with different letters are significantly different within various species across water contents ($p < 0.05$; One-way ANOVA).

	Temperate						Tropical					
Species	*C. sinensis*			*Q. robur*			*T. dregeana*			*A. heterophyllus*		
	Drying time (min)	Water loss (%)	Survival (%)	Drying time (min)	Water loss (%)	Survival (%)	Drying time (min)	Water loss (%)	Survival (%)	Drying time (min)	Water loss (%)	Survival (%)
Freshly harvested	0	0	100 ± 0^a	0	0	100 ± 0^a	0	0	100 ± 0^a	0	0	100 ± 0^a
1.0 g g^{-1} DW	15	52	100 ± 0^a	5	21	100 ± 0^a	45	54	87 ± 12^a	2	27	100 ± 0^a
0.3-0.4 g g^{-1} DW	60	81	90 ± 16^{ab}	240	71	13 ± 24^b	180	79	40 ± 20^b	15	75	93 ± 12^a
0.1 g g^{-1} DW	180	94	20 ± 22^c	510	90	0 ± 0^c	450	95	0 ± 0^c	90	93	0 ± 0^b

3.4 Oxidative metabolism at selected water contents

Extracellular superoxide production was significantly elevated when embryonic axes of *C. sinensis* were dehydrated to 0.1 g g^{-1} DW (p<0.0001), when compared with freshly excised axes, 1.0 and 0.3-0.4 g g^{-1} DW water contents (Figure 3.4.1). There was a significant increase in superoxide production at each water content for axes of *Q. robur* (p≤0.002), whilst a loss in water content resulted in a statistically similar superoxide production at 1.0 and 0.3-0.4 g g^{-1} DW for axes of *T. dregeana* and *A. heterophyllus*. Superoxide production was significantly elevated at 0.1 g g^{-1} DW in axes of *T. dregeana* and *A. heterophyllus* (p≤0.01) (Figure 3.4.1). Comparison of superoxide production between species, when dried to the same water content, showed that *T. dregeana* axes produce a significantly higher amount at each water content (p<0.0001), and *Q. robur* axes produce the lowest amount of superoxide at each water content (p≤0.004). Axes of *C. sinensis* and *A. heterophyllus* differ significantly in superoxide production at shedding, and at 0.1 g g^{-1} DW (p<0.0001) (Figure 3.4.1).
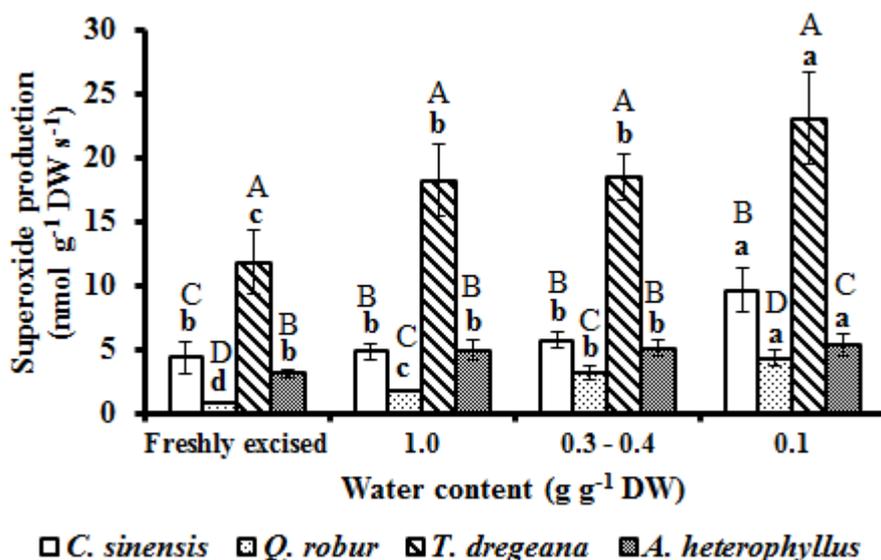
Figure 3.4.1: Extracellular superoxide production from embryonic axes of *C. sinensis*, *Q. robur*, *T. dregeana* and *A. heterophyllus* when partially dehydrated to selected water contents compared with freshly harvested axes (control). Values represent the average (±SD) of 4 replicates of n=5 axes each, repeated twice. Columns labelled with different letters are significantly different between various species at the same water content (uppercase) and across different water contents for the same species (lowercase) (p<0.05; One-way ANOVA).

Extracellular hydrogen peroxide production was significantly elevated when embryonic axes of *C. sinensis* were dehydrated to 0.1 g g^{-1} DW (p=0.048), when compared with axes from freshly harvested seeds (Figure 3.4.2). There was a significant increase in hydrogen peroxide production at 0.3-0.4 g g^{-1} DW for axes of *Q. robur* (p=0.008), relative to axes from freshly harvested seeds. Further dehydrating these axes to 0.1 g g^{-1} DW resulted in a non-significant decline hydrogen peroxide levels. Hydrogen peroxide production peaked significantly at 1.0 and 0.3-0.4 g g^{-1} DW in axes of *T. dregeana* (p<0.0001) and *A. heterophyllus* (p<0.0001), relative to axes from freshly harvested seeds. (Figure 3.4.2). Comparison of hydrogen peroxide production between species, at the same water content, showed that *C. sinensis* axes produce a significantly higher amount at each water content and when freshly shed (p<0.0001), whilst *Q. robur* and *A. heterophyllus* axes produce similar amounts of hydrogen peroxide when freshly shed, at 1.0 and 0.1 g g^{-1} DW. The response to dehydration at 0.3-0.4 g g^{-1} DW resulted in significant differences between each species (p≤0.01), *C. sinensis* axes producing the highest levels of hydrogen peroxide, followed by *T. dregeana*, *A. heterophyllus* and *Q. robur* (Figure 3.4.2).
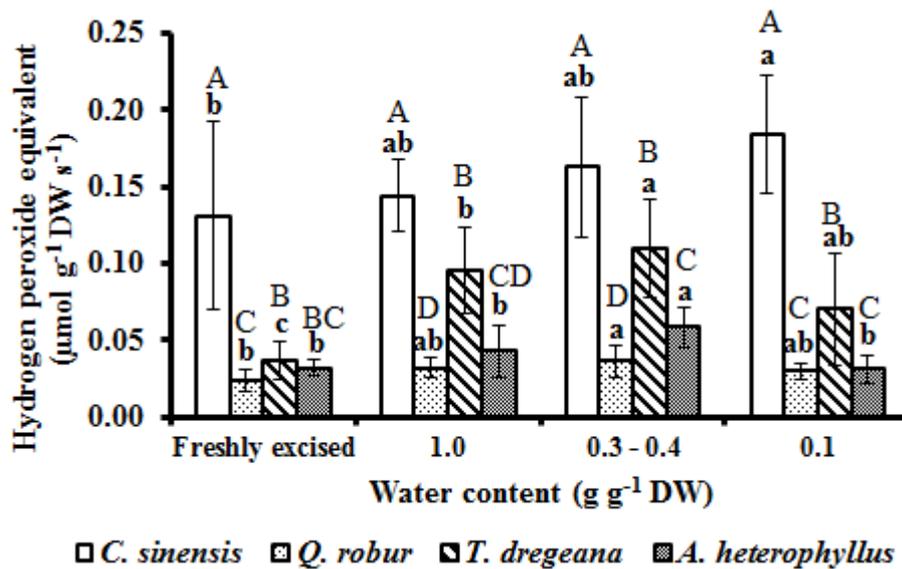
Figure 3.4.2: Extracellular hydrogen peroxide production from embryonic axes of *C. sinensis*, *Q. robur*, *T. dregeana* and *A. heterophyllus* when partially dehydrated to selected water contents compared with freshly harvested axes (control). Values represent the average (±SD) of 4 replicates of n=5 axes each, repeated twice. Columns labelled with different letters are significantly different between various species at the same water content (uppercase) and across different water contents for the same species (lowercase) (p<0.05; One-way ANOVA).

Total aqueous antioxidant activity was significantly elevated across all species when dehydrated to each water content compared with axes from freshly harvested seeds (p<0.0001), except for *A. heterophyllus* axes (Figure 3.4.3). Comparison of TAA activity between species, at the same water content, showed that *C. sinensis* and *Q. robur* (temperate provenance) axes produce a significantly higher amount at each water content and when freshly shed (p<0.0001), compared with *T. dregeana* and *A. heterophyllus* axes (tropical provenance). Furthermore, TAA activity was significantly lower in axes of *T. dregeana* than *A. heterophyllus* (p<0.0001) upon dehydration. (Figure 3.4.3).
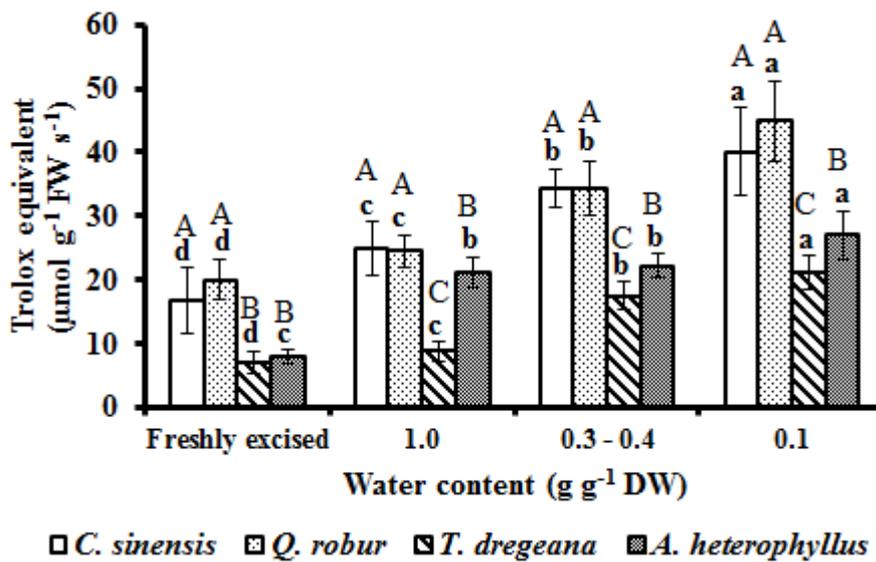
Figure 3.4.3: TAA activity from embryonic axes of *C. sinensis*, *Q. robur*, *T. dregeana* and *A. heterophyllus* when partially dehydrated to selected water contents compared with freshly harvested axes (control). For each species, values represent the average (±SD) of 5 replicates of n=5-8 axes each, repeated twice. Columns labelled with different letters are significantly different between various species at the same water content (uppercase) and across different water contents for the same species (lowercase) (p<0.05; One-way ANOVA).

3.5 Water content and viability loss during hydrated storage

An increase in storage time was significantly correlated with a loss of viability for each species when stored at 16°C, irrespective of the provenance of collection. Patterns of viability reduction were similar for embryonic axes of *C. sinensis*, *T. dregeana* and *A. heterophyllus* ($p<0.0001$) (Figure 3.5.1a, c and d), whilst the decline in viability was more rapid in axes from *Q. robur* ($p<0.05$) (Fig 3.5.1b). Storage at 3°C resulted in complete loss of viability, indicative of chilling sensitivity, after 4 months (*A. heterophyllus*) and 6 months (*T. dregeana*) ($p<0.0001$) (Fig. 3.5.1d), whilst *C. sinensis* axes maintained a higher viability over 8 months than *Q. robur* (Figure 3.5.1c). The optimum hydrated storage temperature for tropical recalcitrant-seeded species was 16°C; both *T. dregeana* and *A. heterophyllus* seeds maintained high viability (>80%) for 6 months. The optimum hydrated storage temperature for temperate recalcitrant-seeded species was 3°C; seeds of *C. sinensis* and *Q. robur* seeds exhibited >80% viability after 8 months and 4 months, respectively (Figure 3.5.1c).
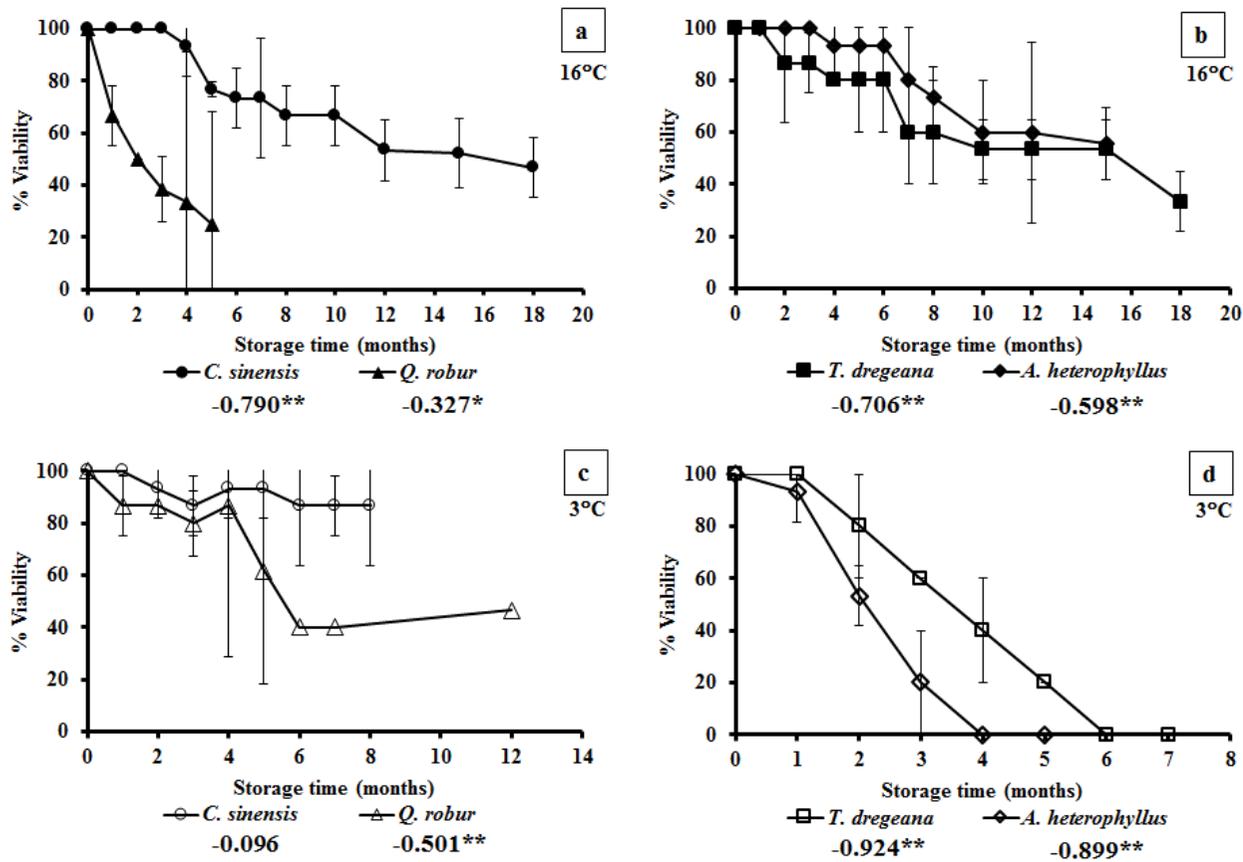
Figure 3.5.1: *In vitro* **viability (root and shoot production) of temperate (a, c) and tropical (b, d) recalcitrant-seeded species when stored hydrated at 3 and 16°C. For each species, values represent the average (±SD) of 3 replicates of n=5 axes each. Values displayed beneath each legend represent the correlation coefficient of storage time at each temperature (*p<0.05, **p<0.0001; Spearman's correlation)**

Although axes of *C. sinensis* were shed at a higher water content (1.9 ± 0.6 g g^{-1}), the effect of storage time on water content and survival at 16°C was variable since water content differed per harvest collection (2-8 months, collection two; 1, 10-18, collection one), also there was no significant correlation between viability and storage time, nor between viability and water content (Figure 3.5.2.1a). An increase in storage time at 16°C resulted in significant (p<0.0001) increase in water content for species shed < 1.5 g g^{-1} DW (*Q. robur* and *A. heterophyllus*) (Figure 3.5.2.1b and d). Furthermore, an increase in water content resulted in significant correlation associated with a decline in viability for axes from *Q. robur* after five months (Figure 3.5.2.1b). *T. dregeana* axes were shed at 2.5 ± 0.2 g g^{-1} DW and exhibited a general decline in water content as storage time increased up to 18 months (Figure 3.5.2.1c). There was a significant correlation between viability and storage time and viability and water content for axes of *T. dregeana*, indicating that a change in water content contributed to a loss in viability over 18 months (Figure 3.5.2.1).
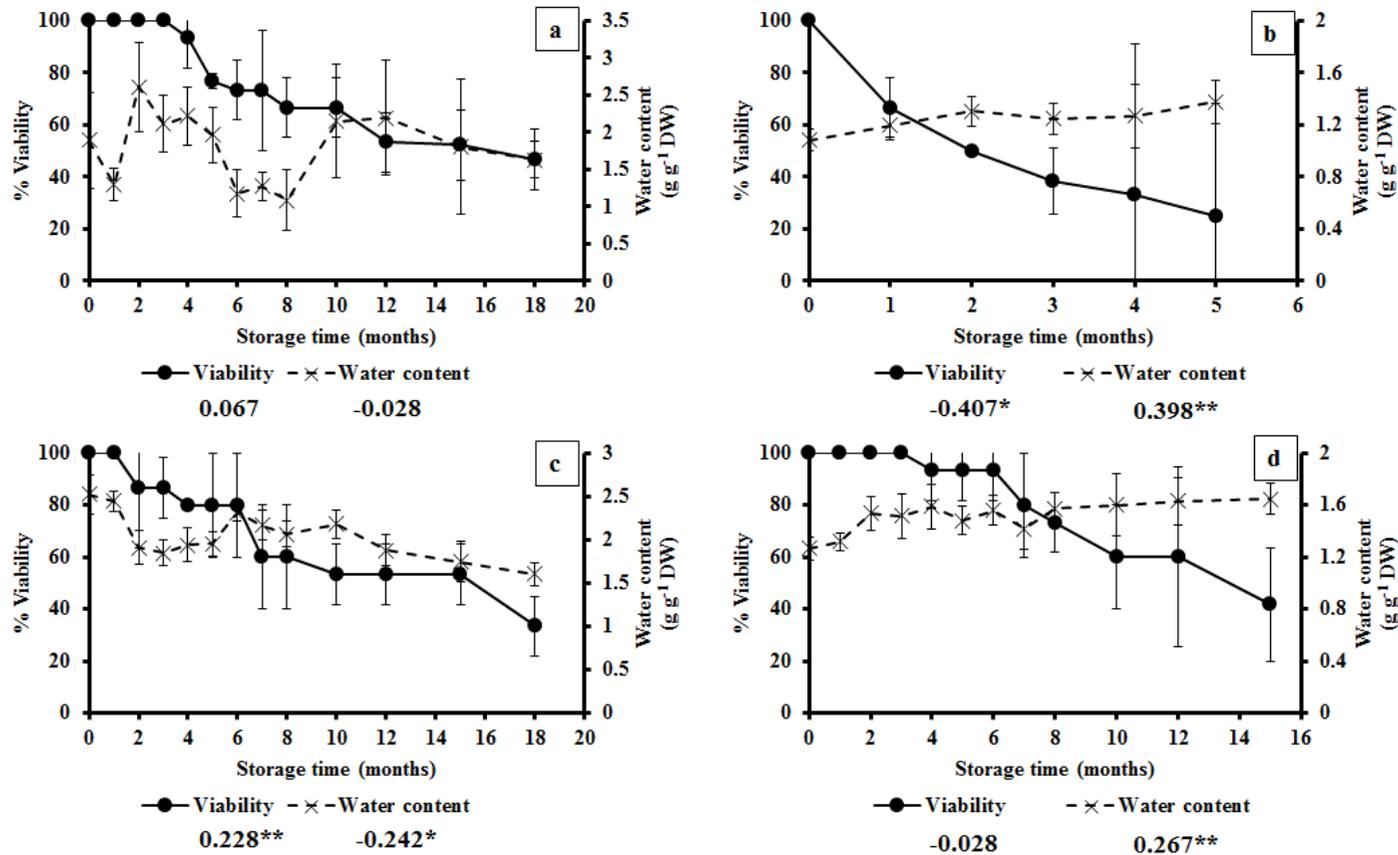
Figure 3.5.2.1: Relationship between *in vitro* **viability (root and shoot production) and water content in embryonic axes from (a)** *C. sinensis***, (b)** *Q. robur***, (c)** *T. dregeana* **and (d)** *A. heterophyllus* **seeds when stored hydrated 16°C. For each species, values represent the average (±SD) of n=10 axes. Values displayed beneath legend title 'viability' represent the correlation coefficient between water content and viability, and those beneath 'water content' represent the correlation coefficient between water content and time (*p<0.05, **p<0.0001; Spearman's correlation).**

Water content was significantly affected by storage time at 3°C for *C. sinensis*, *Q. robur* and *T. dregeana* axes (p=0.015) (Figure 3.5.2.2a, b and c). Although the water content of axes from *C. sinensis* decreased with an increase in storage time, this did not impact viability retention (Figure 3.5.2.2a). As also seen in storage at 16°C (in Figure 3.5.2.1b), water content of *Q. robur* axes significantly increased with an increase in storage time at 3°C, however, viability was not significantly affected by this uptake of water (Figure 3.5.2.2b). Both species of tropical provenance, *T. dregeana* and *A. heterophyllus* axes, were chilling sensitive and lost viability after six and four months, respectively. However, water content was not affected by storage time, nor did water content impact viability of *A. heterophyllus* axes from seeds stored at 3°C (Figure 3.5.2.2d). Conversely, water content of axes from *T. dregeana* increased gradually as storage time increased, and was negatively correlated with viability retention, indicating that the increase in water content coupled with the effect of chilling may have contributed to a loss in viability over six months (Figure 3.5.2.2c).
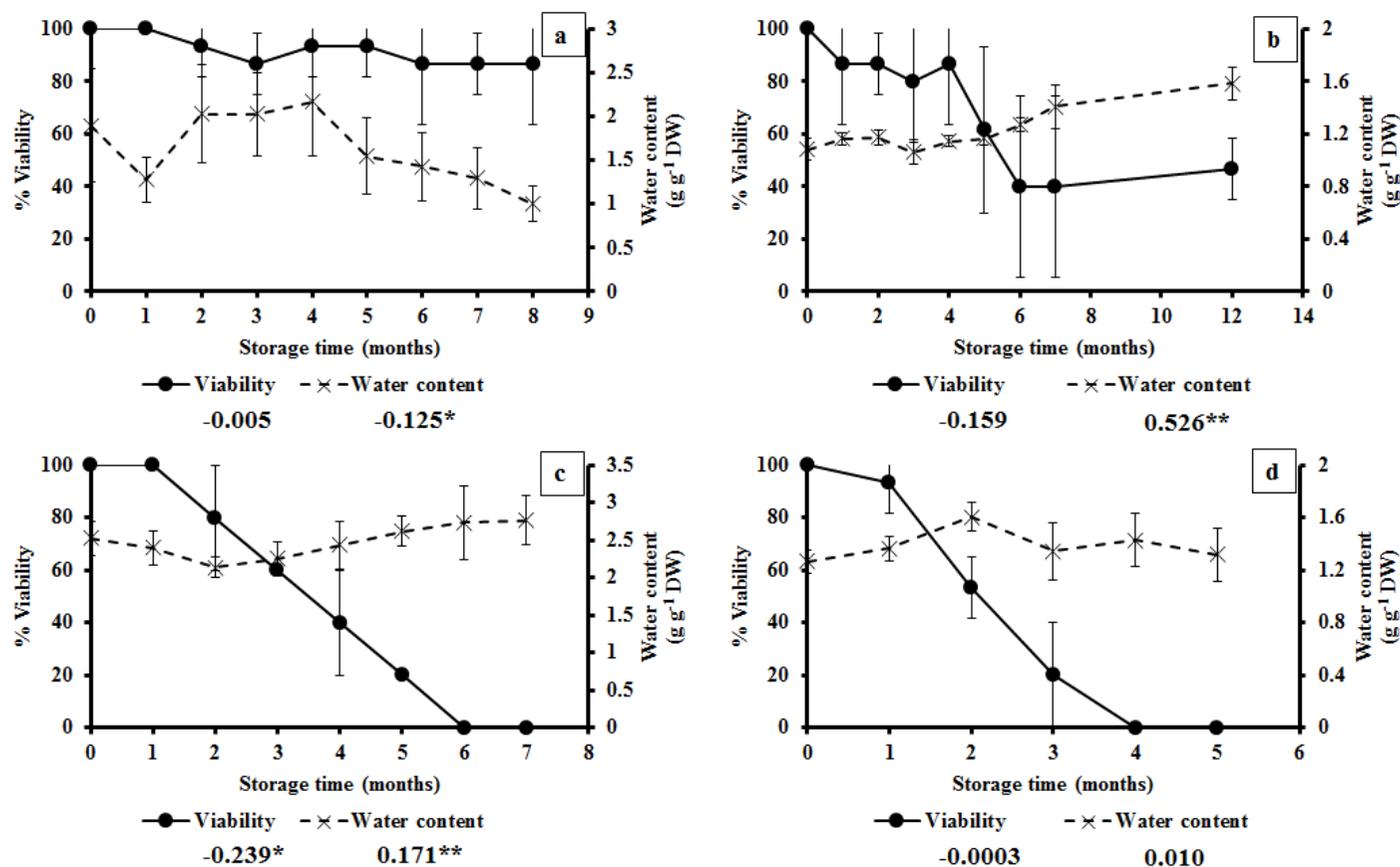
Figure 3.5.2.2: Relationship between *in vitro* viability (root and shoot production) and water content in embryonic axes from (a) *C. sinensis*, (b) *Q. robur*, (c) *T. dregeana* and (d) *A. heterophyllus* seeds when stored hydrated 3°C. For each species, values represent the average (±SD) of n=10 axes. Values displayed beneath legend title 'viability' represent the correlation coefficient between water content and viability, and those beneath 'water content' represent the correlation coefficient between water content and time (*p<0.05, **p<0.0001; Spearman's correlation).

3.6 Relationship between oxidative metabolism and viability loss during hydrated storage

A comparison of extracellular superoxide production from embryonic axes excised from seeds of temperate regions stored at 16°C revealed that despite having almost ten times higher superoxide levels throughout storage compared with *Q. robur*, viability of *C. sinensis* axes were not significantly affected by this, nor did an increase in storage time (up to 18 months) affect superoxide production (Figure 3.6.1.1a). Conversely, the significant accumulation of superoxide was positively correlated with a decline in viability in *Q. robur* seeds, although the storage duration was much shorter (five months) (Figure 3.6.1.1 b). Superoxide production in axes of *T. dregeana* followed a bell-shaped curve for the first eight months in storage, and thereafter declined with a decrease in viability for 18 months (Figure 3.6.1.1c). Superoxide levels were approximately ten times lower in axes of *A. heterophyllus* compared with *T. dregeana*, but an increase in superoxide production was significantly correlated with storage time and viability loss ($p < 0.0001$) (Figure 3.6.1.1d). Superoxide levels were comparable for the first six months in storage with little change in viability, thereafter, the increased accumulation appeared to be associated with the rapid decline in viability over the next 9 months (Figure 3.6.1.1d). The concentration of superoxide appears to be species-specific, although superoxide production was the lowest in axes of *Q. robur*, this level was sufficient to be associated with a decline in viability, whilst axes of *T. dregeana* exhibited 5-15x higher superoxide levels, this did not appear to affect viability retention (Figure 3.6.1.1).
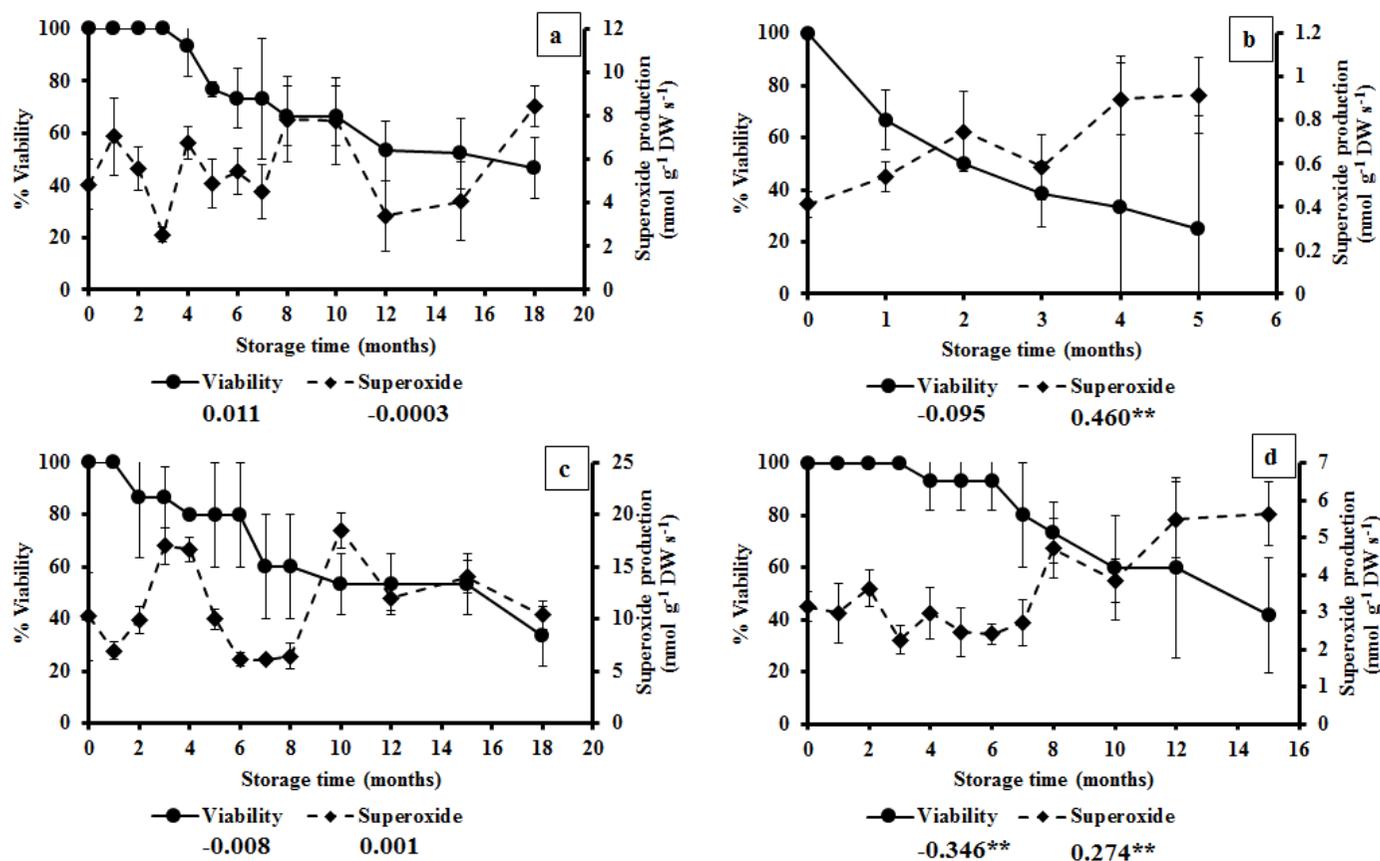
Figure 3.6.1.1: Relationship between *in vitro* **viability (root and shoot production) and extracellular superoxide production in embryonic axes from (a)** *C. sinensis*, **(b)** *Q. robur*, **(c)** *T. dregeana* **and (d)** *A. heterophyllus* **seeds when stored hydrated 16°C. For each species, superoxide values represent the average (±SD) of 4 replicates of n=5 axes. Values displayed beneath legend title 'viability' represent the correlation coefficient between superoxide and viability, and those beneath 'superoxide' represent the correlation coefficient between superoxide and time (**p<0.0001; Spearman's correlation).**

Axes of *C. sinensis* from seeds stored at 3°C fluctuated in extracellular superoxide production in a similar manner as 16°C, however these variations were not significantly associated with viability loss at either temperature (Figure 3.6.1.2a). Superoxide production significantly increased with storage time for axes of *Q. robur* (p=0.002) and *T. dregeana* (p=0.002), whilst an increase in superoxide correlated with a decrease in viability for *T. dregeana* axes (p=0.005) (Figure 3.6.1.2b and c). Superoxide levels in axes of *Q. robur* were similar (~0.96 µmol g^{-1} DW s^{-1}) when stored for the same period (five months) at 3 and 16°C, but increased drastically when storage time was extended to 12 months at 3°C (Figure 3.6.1.1b, Figure 3.6.1.2b). Irrespective of storage temperature, axes of *T. dregeana* exhibited a similar bell-shaped curve at 3°C similar to axes from seeds stored at 16°C for the same storage period (six months). Whilst this pattern in superoxide production was associated with a 20% reduction in viability at 16°C, at 3°C there was a complete loss in viability, indicating that superoxide production may be involved in different functions at different storage temperatures, despite similar concentrations (Figure 3.6.1.1c, Figure 3.6.1.2c). Superoxide production was slightly elevated as viability declined from 80 to 60% in axes of *A. heterophyllus* when stored at 3°C, however, the gradual decline in superoxide levels was not significantly related to the decline in viability (Figure 3.6.1.2. d). Viability of axes from temperate regions (*C. sinensis* and *Q. robur*) do not seem affected by superoxide production at 3 or 16°C, whereas viability loss in axes from tropical regions (*T. dregeana* and *A. heterophyllus*) may be related to the accumulation of superoxide in combination with chilling stress at 3°C (Figure 3.6.1.2).
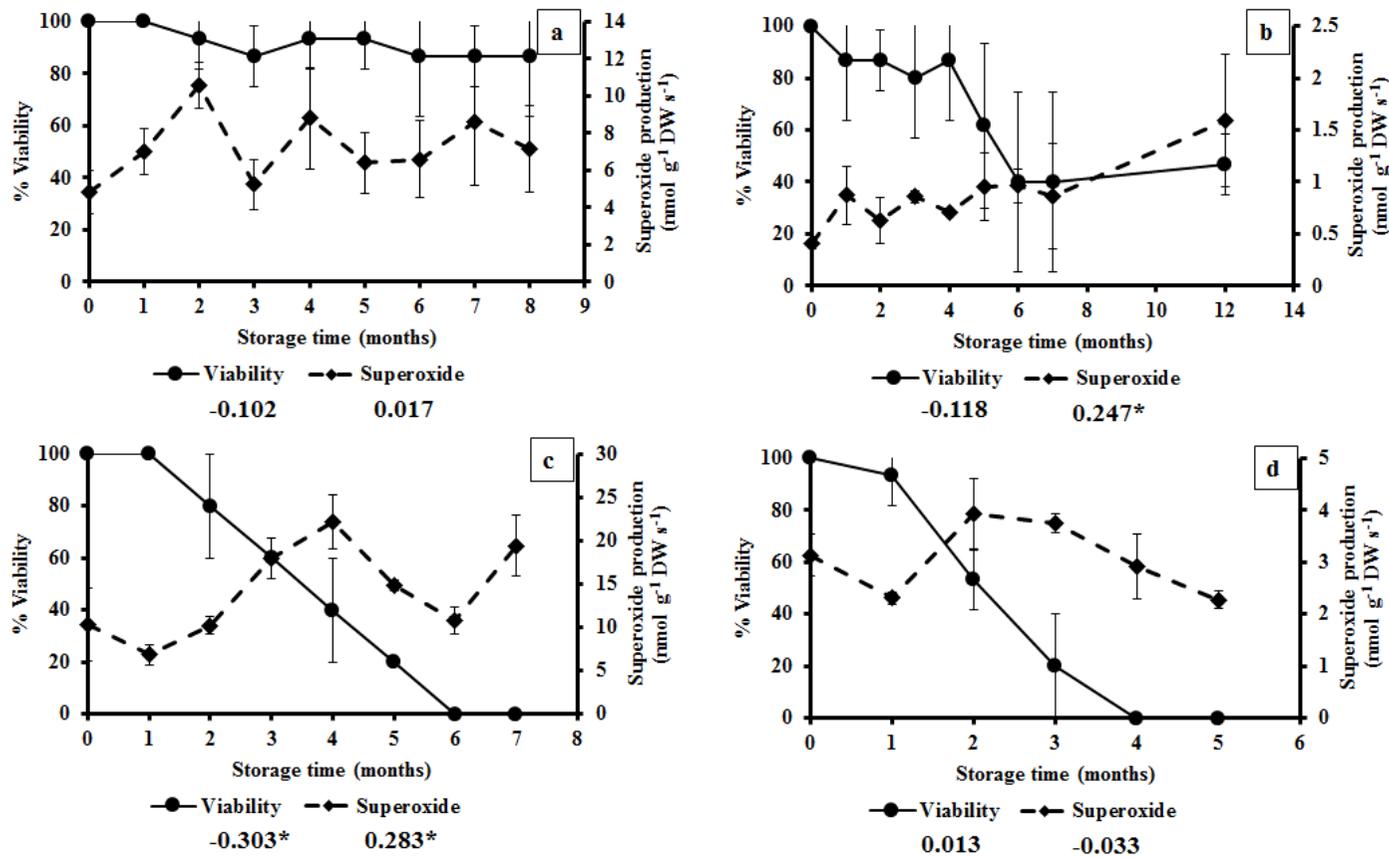
Figure 3.6.1.2: Relationship between *in vitro* **viability (root and shoot production) and extracellular superoxide production in embryonic axes from (a)** *C. sinensis*, **(b)** *Q. robur*, **(c)** *T. dregeana* **and (d)** *A. heterophyllus* **seeds when stored hydrated 3°C. For each species, superoxide values represent the average (±SD) of 4 replicates of n=5 axes. Values displayed beneath legend title 'viability' represent the correlation coefficient between superoxide and viability, and those beneath 'superoxide' represent the correlation coefficient between superoxide and time (****p<0.0001**; Spearman's correlation).**

A comparison of extracellular hydrogen peroxide production between embryonic axes from temperate recalcitrant-seeded species stored at 16°C revealed that *C. sinensis* axes produced 10x higher levels compared with *Q. robur* axes. Hydrogen peroxide production in axes of *C. sinensis* peaked after 1 month in storage, thereafter decreased to until three months and remained relatively unchanged for the following 9 months, and then increased up until 18 months, whereas hydrogen peroxide levels in *Q. robur* axes varied over time. Hydrogen peroxide production was not significantly related to storage time, nor viability loss in storage (Figure 3.6.2.1a and b). Hydrogen peroxide production in axes of *T. dregeana* followed a bell-shaped curve for the first seven months in storage, and thereafter increased slightly with a decrease in viability up to 18 months (Figure 3.6.2.1c). Hydrogen peroxide levels were lower in axes of *A. heterophyllus* compared with *T. dregeana*, but an increased hydrogen peroxide production was significantly correlated with storage time ($p=0.001$) and viability loss ($p=0.015$) (Figure 3.6.2.1d). Hydrogen peroxide levels were comparable for the first eight months in storage, was associated with a 30% decline in viability, thereafter remained high and appeared to be associated with the rapid decline in viability over the following seven months (Figure 3.6.2.1d). Similarly, axes of *T. dregeana* also produced and maintained high levels of hydrogen peroxide between 10-18 months. The concentration of hydrogen peroxide appears to vary within different provenances and between species, although axes of *C. sinensis* produced higher levels of hydrogen peroxide production throughout storage, compared with axes of *Q. robur*, *T. dregeana* and *A. heterophyllus* which produced similar values (Figure 3.6.2.1).
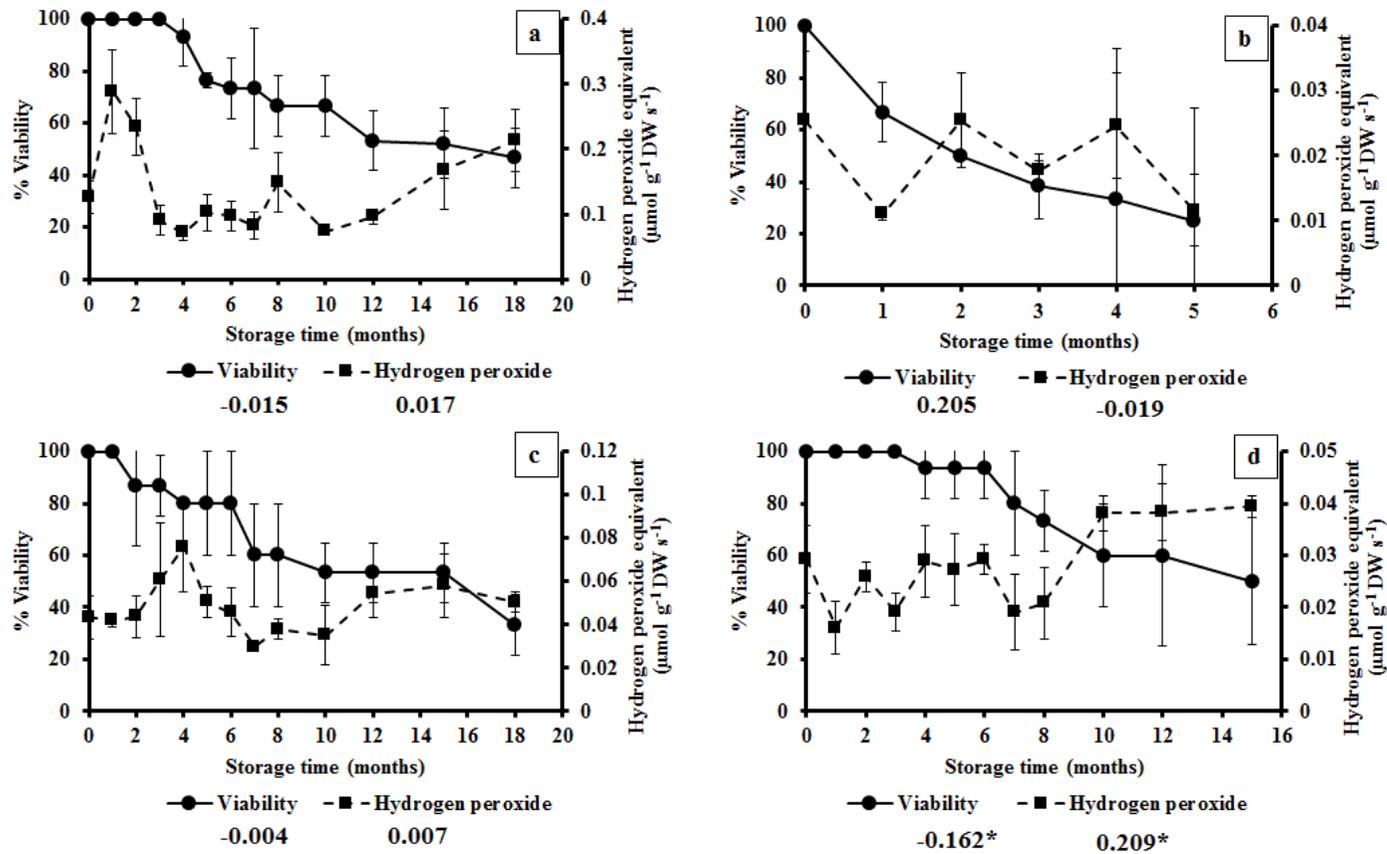
Figure 3.6.2.1: Relationship between *in vitro* viability (root and shoot production) and extracellular hydrogen peroxide production in embryonic axes from (a) *C. sinensis*, (b) *Q. robur*, (c) *T. dregeana* and (d) *A. heterophyllus* seeds when stored hydrated 16°C. For each species, hydrogen peroxide values represent the average (±SD) of 4 replicates of n=5 axes. Values displayed beneath legend title 'viability' represent the correlation coefficient between hydrogen peroxide and viability, and those beneath 'hydrogen peroxide' represent the correlation coefficient between hydrogen peroxide and time (*p<0.05; Spearman's correlation).

Axes of *C. sinensis* from seeds stored at 3°C increased in extracellular hydrogen peroxide production after one month, thereafter steadily decreased until six months followed by a significant increase for two months (Figure 3.6.2.2a). Hydrogen peroxide production remained comparable for six months in storage, thereafter rapidly increased when stored for 12 months in axes of *Q. robur* (Figure 3.6.2.2b). There was a significant accumulation of hydrogen peroxide exhibited by axes of *T. dregeana* when seeds were stored at 3°C (p=0.031), which was significantly correlated with the loss of viability (p=0.039) and these hydrogen peroxide levels may be related to a chilling response (Figure 3.6.2.2c). Axes of the tropical counterpart, *A. heterophyllus*, exhibited a similar pattern in hydrogen peroxide production accumulation, being highest after three months, however this was not significantly related to the loss of viability within four months of storage (Figure 3.6.2.2d). Hydrogen peroxide levels peak after one month in storage irrespective of storage temperature in axes of *C. sinensis*, however, levels were generally lower at 3°C (Figure 3.6.2.2a) than at 16°C (Figure 3.6.2.1a). Similarly, axes of *Q. robur* from seeds stored at 3 and 16°C produced comparable levels of hydrogen peroxide for the first five months of storage, whilst extended storage periods at 3°C resulted in increased hydrogen peroxide levels (Figure 3.6.2.2b). Irrespective of storage temperature, axes of *T. dregeana* exhibited a similar bell-shaped curve at 3°C similar to axes from seeds stored at 16°C for the same storage period (seven months). Whilst this pattern in hydrogen peroxide production was associated with a 40% reduction in viability at 16°C (Figure 3.6.2.1c), at 3°C there was a complete loss in viability (Figure 3.6.2.2c). Likewise, hydrogen peroxide levels were similar in axes of *A. heterophyllus* from seeds stored at 16 and 3°C (Figure 3.6.2.1d and Figure 3.6.2.2d respectively), indicating the hydrogen peroxide may not play a role in chilling sensitivity.
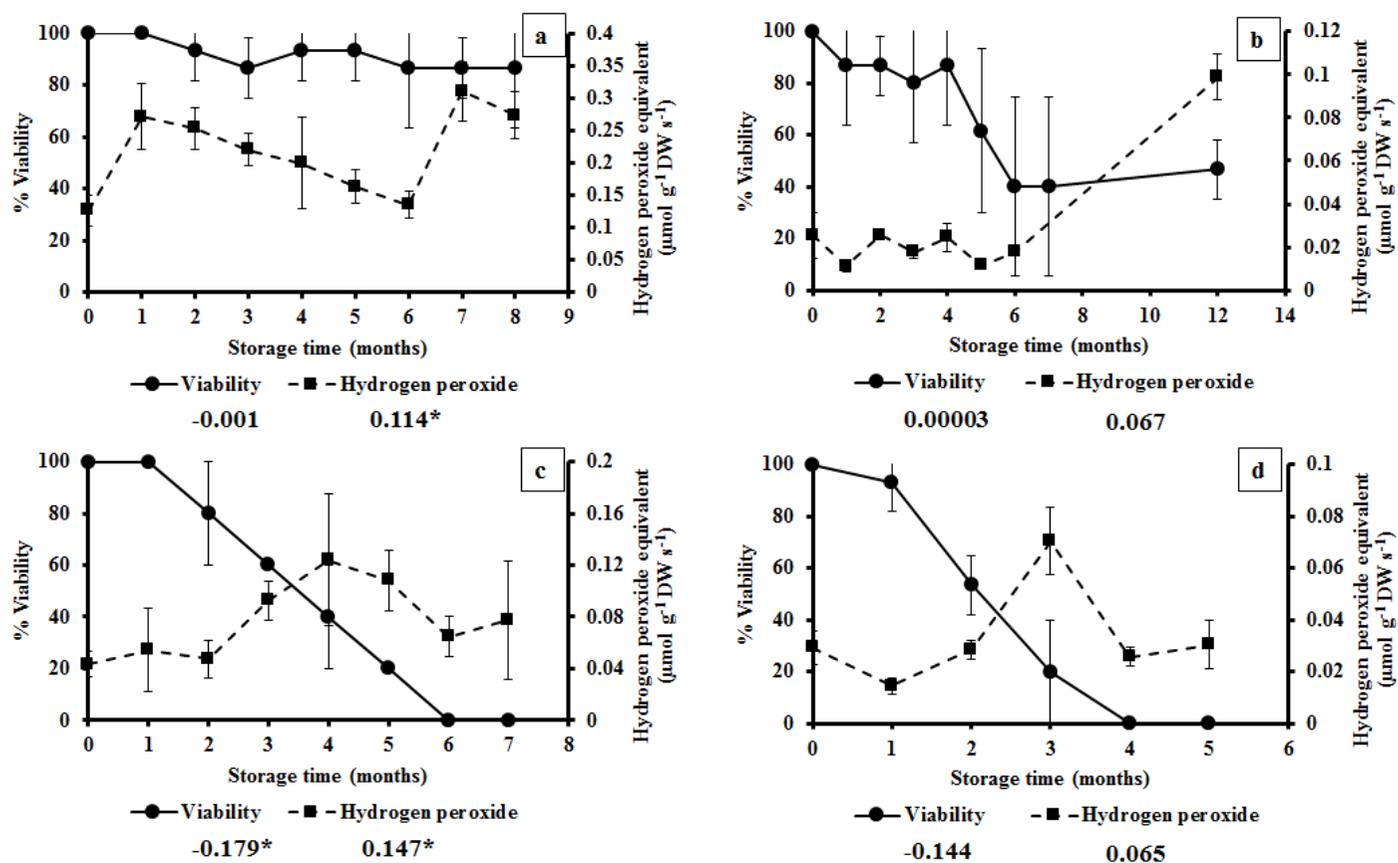
Figure 3.6.2.2: Relationship between *in vitro* **viability (root and shoot production) and extracellular hydrogen peroxide production in embryonic axes from (a)** *C. sinensis***, (b)** *Q. robur***, (c)** *T. dregeana* **and (d)** *A. heterophyllus* **seeds when stored hydrated 3°C. For each species, hydrogen peroxide values represent the average (±SD) of 4 replicates of n=5 axes. Values displayed beneath legend title 'viability' represent the correlation coefficient between hydrogen peroxide and viability, and those beneath 'hydrogen peroxide' represent the correlation coefficient between hydrogen peroxide and time (*p<0.05; Spearman's correlation).**

A comparison of TAA activity between embryonic axes from temperate recalcitrant-seeded species stored at 16°C revealed no significant relationship between TAA activity and storage time in embryonic axes of *C. sinensis*. The first peak in TAA activity at four months and a second smaller peak at 10 months showed no relation to the decline in viability (Figure 3.6.3.1a). In axes of *Q. robur,* as storage time increased, TAA activity decreased significantly (p=0.026). Antioxidant activity in this species remained high between 1-3 months in storage, despite a 30% reduction in viability. Thereafter, TAA activity decreased along with a further reduction in viability (Figure 3.6.3.b). Both tropical recalcitrant-seeded species exhibited a significant increase in TAA activity with increased storage time. *T. dregeana* axes displayed similar TAA activity over eight months in storage with an associated viability decline of 40%. Thereafter TAA activity increased significantly up to 18 months (p=0.004), whilst viability continually decreased to 35% (Figure 3.6.3.1c). There was a highly significant increase in TAA activity in axes of *A. heterophyllus* seeds (p<0.0001), with an increase in storage time. This significant increase in antioxidant production was negatively related to viability retention (p=0.023). A steady rise in TAA activity was associated with a high viability retention over six months in storage, thereafter declined to 10 months with an associated 30% loss in viability. Antioxidant capacity increased again until 15 months in storage but there was no significant change in viability for this period (Figure 3.6.3.1d). Apart from axes of *C. sinensis*, viability was negatively correlated with higher antioxidant expression in each of the other species. Although axes from temperate recalcitrant-seeded species showed variable responses in TAA activity to hydrated storage, these levels were much higher (~5-10 x) than tropical recalcitrant-seeded species. Axes of *Q. robur* displayed the highest antioxidant production, whilst *T. dregeana* axes produced the lowest antioxidant levels in storage (Figure 3.6.3.1).
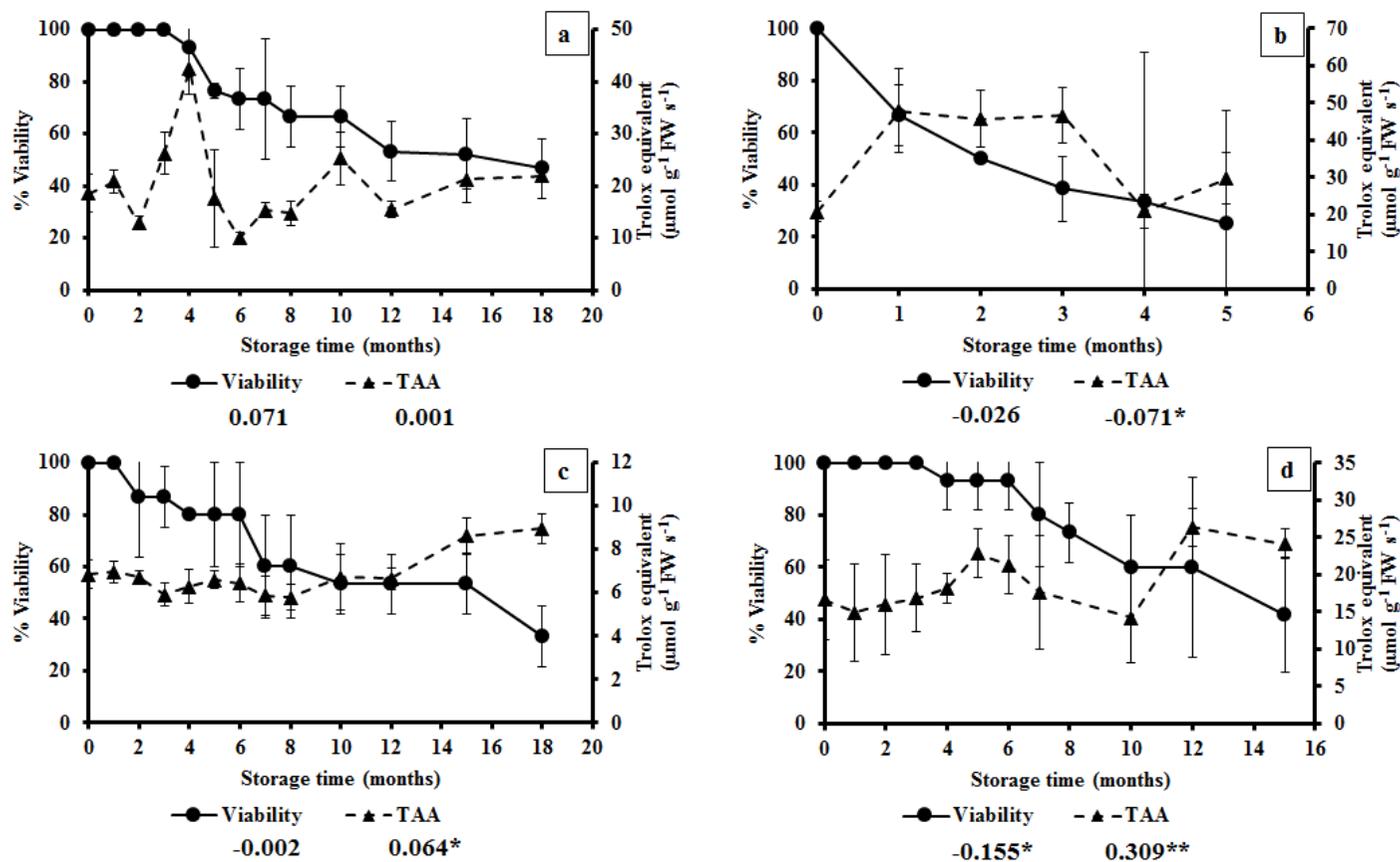
Figure 3.6.3.1: Relationship between *in vitro* viability (root and shoot production) and TAA activity in embryonic axes from (a) *C. sinensis*, (b) *Q. robur*, (c) *T. dregeana* and (d) *A. heterophyllus* seeds when stored hydrated 16°C. For each species, TAA values represent the average (±SD) of 5 replicates of n=7-10 axes, repeated twice. Values displayed beneath legend title 'viability' represent the correlation coefficient between TAA activity and viability, and those beneath 'superoxide' represent correlation coefficient between TAA activity and time (*p<0.05; Spearman's correlation).

Axes of *C. sinensis* from seeds stored at 3°C exhibited an increase in TAA activity after one month of hydrated storage, which decreased significantly after the second month in storage, and thereafter remained relatively unchanged up to eight months. Although there was a significant decline in TAA activity when correlated with an increase in storage time (p=0.027), this did not affect viability retention (Figure 3.6.3.2a). The antioxidant activity in axes of *Q. robur* displayed a similar pattern to viability retention (>80%) in storage corresponded to high antioxidant activity in axes. Once viability declined, there was a significant decline in TAA activity (p<0.0001) after four months in storage. Thereafter, TAA activity remained relatively low over 12 months in storage and was associated with 50% viability loss (Figure 3.6.3.2b). Chilling sensitive axes from tropical recalcitrant-seeded species varied in TAA activity when viability was lost. Axes of *T. dregeana* exhibited a significant decline in TAA activity when storage time at 3°C was increased (p<0.0001) and there was a significant effect of antioxidant capacity on viability loss (p=0.003), whilst axes of *A. heterophyllus* showed no significant relationship between TAA activity and viability, and storage time and viability (Figures 3.6.3.2c and d, respectively).

Antioxidant activity was much higher in temperate recalcitrant-seeded species than tropical recalcitrant-seeded species, even when viability was low. Axes of *Q. robur* displayed the highest antioxidant production, whilst *C. sinensis*, *T. dregeana* and *A. heterophyllus* axes produced similar antioxidant levels in storage (Figure 3.6.3.2). Antioxidant expression was higher in axes of *C. sinensis* seeds stored at 16 °C (Figure 3.6.3.a) than 3°C (Figure 3.6.3.2a), although viability loss was greater at 16°C. Axes of *Q. robur* seeds produced similar antioxidant values in response to differing storage temperatures (Figures 3.6.3.1b and 3.6.3.2b), although viability loss was associated with decrease in TAA activity at 3°C (Figure 3.6.3.2b). Tropical-seeded *T. dregeana* axes exhibited similar levels of antioxidants for the first six months of storage, although when viability was lost at 3°C antioxidant levels declined significantly, whilst there was an increase when storage time extended to 18 months (Figure 3.6.3.1c and Figure 3.6.3.c). *A. heterophyllus* seeds were more chilling sensitive than *T. dregeana*, particularly in antioxidant response. Axes produced similar values after one month in storage at 16 and 3°C (Figures 3.6.3.1d and 3.6.3.2d respectively), but were significantly lower at 3°C.
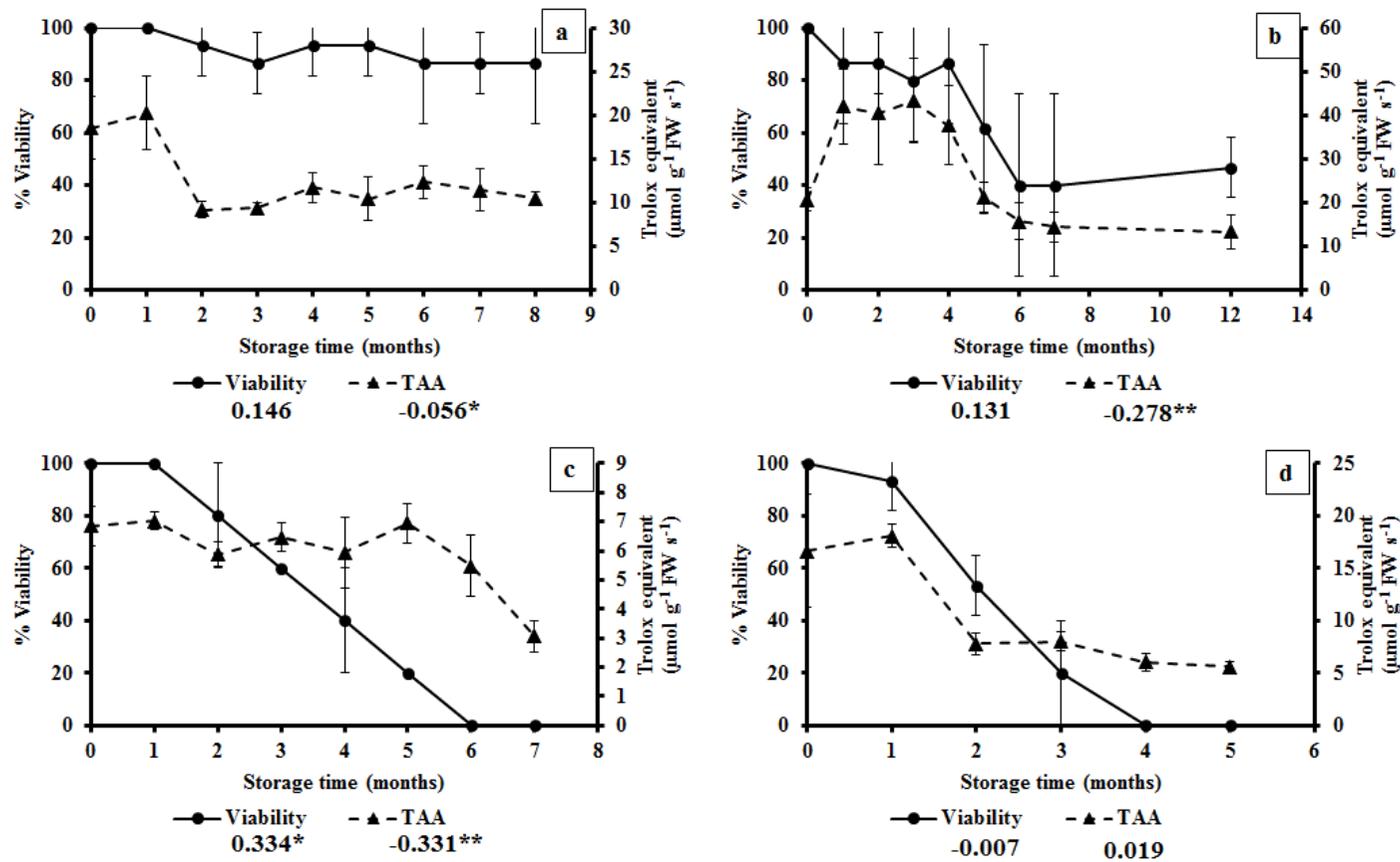
**Figure 3.6.3.2: Relationship between *in vitro* viability (root and shoot production) and TAA activity in embryonic axes from (a) *C. sinensis*, (b) *Q. robur*, (c) *T. dregeana* and (d) *A. heterophyllus* seeds when stored hydrated 3°C. For each species, TAA values represent the average (±SD) of 5 replicates of n=7-10 axes, repeated twice. Values displayed beneath legend title 'viability' represent the correlation coefficient between TAA activity and viability, and those beneath 'superoxide' represent the correlation coefficient between TAA activity and time (*$p<0.05$; Spearman's correlation).**

3.7   Relationship between ROS and TAA during hydrated storage

There was no significant correlation between superoxide production and TAA activity for each species stored hydrated at 16°C (Figure 3.7.1.1). Periods of heightened superoxide may be responsible for the initial loss of viability in storage (*C. sinensis*: four months, *Q. robur*: one month, *T. dregeana*: three months whilst *A. heterophyllus* axes exhibited lower superoxide levels after four months), relative to freshly harvested material (Figure 3.7.1.1). Reduction of viability to 50% may be a result of an accumulation of superoxide, particularly for *C. sinensis* and *T. dregeana* (Figure 3.7.1.1a and c, respectively). Viability decline to 50% occurred in a shorter period for *Q. robur* axes (five months), whilst *A. heterophyllus* axes lost 50% viability after 15 months in storage at 16°C (Figure 3.7.1.1b and d, respectively). The lowest viability obtained was observed in axes of *Q. robur* after five months, and may be related to the increase in superoxide and decrease in TAA activity, whereas this pattern was not observed in axes of *C. sinensis* and *A. heterophyllus* (periods of heightened superoxide was accompanied by an increase in TAA), but it was not as pronounced. Antioxidant capacity increased in axes of *T. dregeana* whilst superoxide decreased, however viability was reduced to 33% (Figure 3.7.1.1).
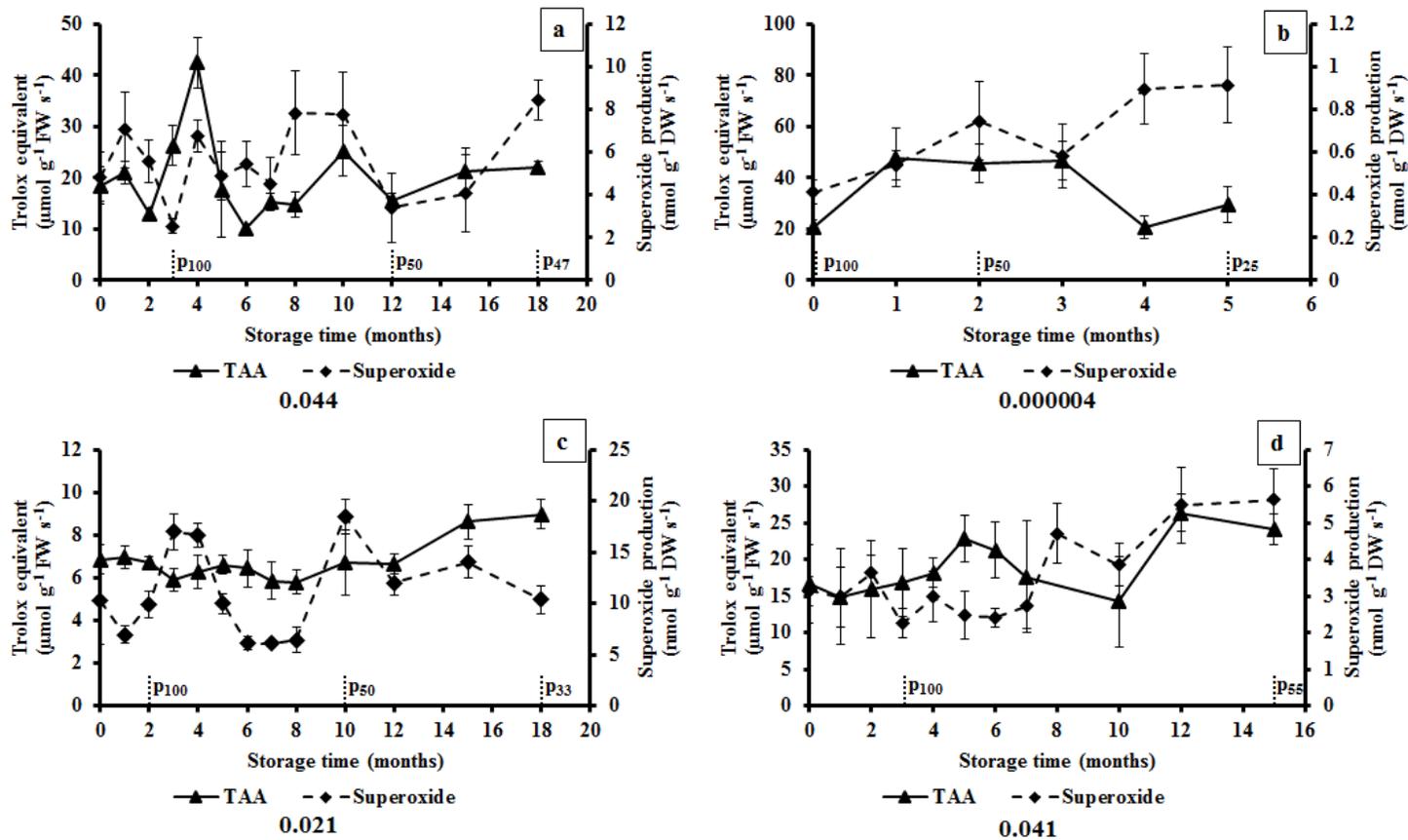
Figure 3.7.1.1: Relationship between extracellular superoxide and TAA in embryonic axes from (a) *C. sinensis*, **(b)** *Q. robur*, **(c)** *T. dregeana* **and (d)** *A. heterophyllus* **seeds when stored hydrated 16°C. For each species, values represent the average (±SD) of 4 replicates of n=5 axes for superoxide, and 5 replicates of 7-10 axes for TAA repeated twice. Values displayed next to a dotted line on the x-axes represent: P_{100} is the last point of 100% viability retention, P_{50} is 50% viability loss and a P with a variable number represents the lowest % viability obtained after maximum storage. Values displayed beneath legend title represent correlations between superoxide and TAA (*p<0.05, **p<0.0001; Spearman's correlation).**

The relationship between TAA activity and superoxide production was not significant nor did this relationship have a significant impact on viability retention in axes of *C. sinensis* and *T. dregeana* seeds stored at 3°C (Figure 3.7.1.2a and c). The TAA activity of the other species were, however, negatively correlated with superoxide production, and was more pronounced for *A. heterophyllus* (Figure 3.7.1.2b and d). This may be a result of oxidative imbalance induced by chilling stress, which contributed to the loss of viability. *A. heterophyllus* axes lost 50% viability in two months corresponding to an increase in superoxide production and diminished antioxidant sequestration (Figure 3.7.1.2 d). Complete loss of viability was associated with a reduction in both superoxide and TAA activities for tropical recalcitrant-seeded species, whilst viability in temperate recalcitrant-seeded species was species-specific (Figure 3.7.1.2). Axes of *Q. robur* increased in superoxide over six months, and subsequently caused a decrease in TAA activity (Figure 3.7.1.2b) whilst in *C. sinensis,* viability retention (>80%) was associated with constant TAA levels despite fluctuation in superoxide values in storage (Figure 3.7.1.b and a, respectively). Superoxide and TAA values were comparable between temperatures for each species. Storage of tropical recalcitrant-seeded species at 3 and 16°C resulted in similar patterns in superoxide production, however, TAA activity was reduced in axes of *A. heterophyllus*, when compared for the same period (Figure 3.7.1.1 and Figure 3.7.1.2). Storage of temperate recalcitrant-seeded species at 3 and 16°C resulted in similar patterns of superoxide production and TAA activity for *Q. robur* axes, whilst there was more variability in axes of *C. sinensis* which may explain the reduced viability (Figure 3.7.1.1 and Figure 3.7.1.2).
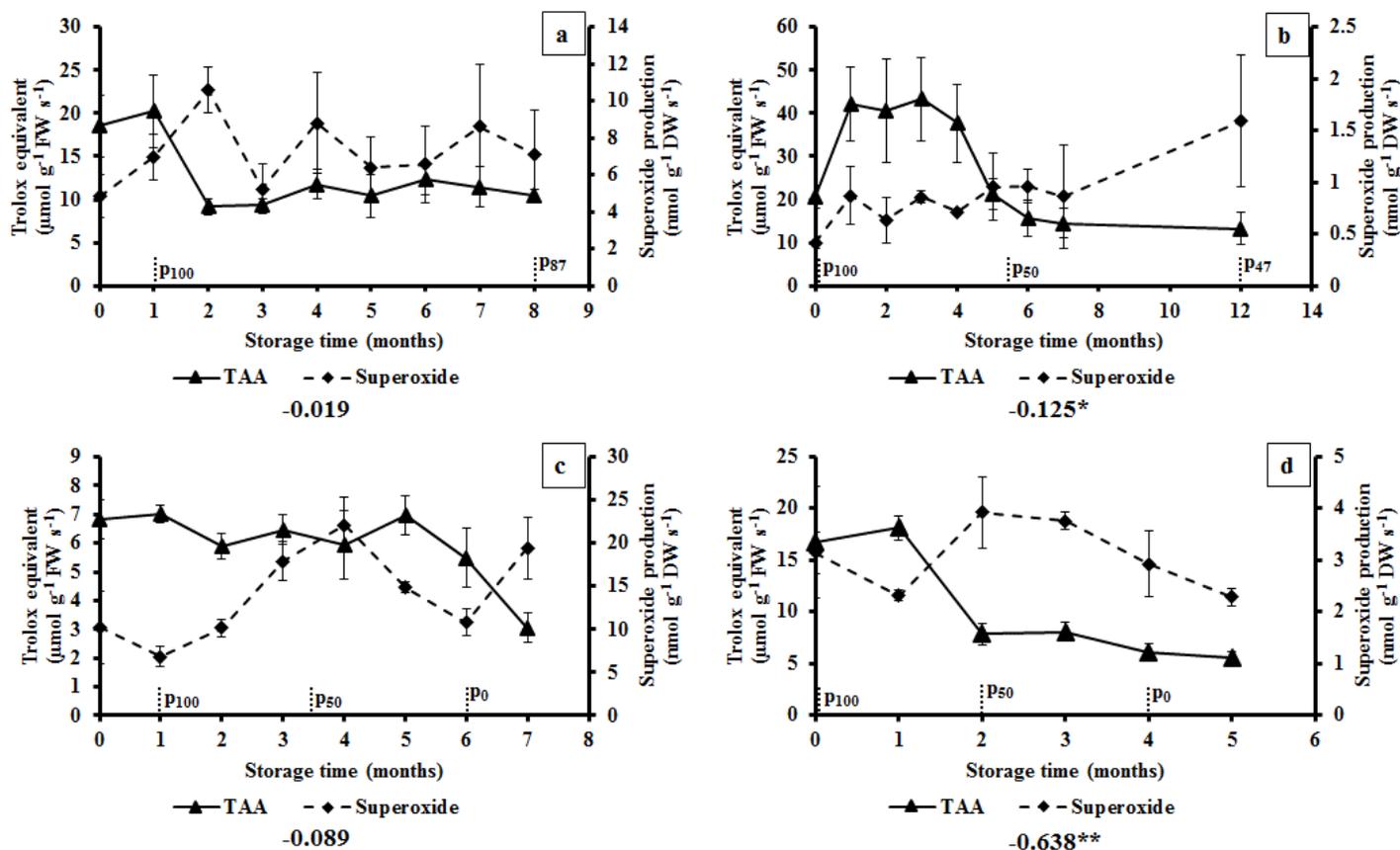
Figure 3.7.1.2: Relationship between extracellular superoxide and TAA in embryonic axes from (a) *C. sinensis*, **(b)** *Q. robur*, **(c)** *T. dregeana* **and (d)** *A. heterophyllus* **seeds when stored hydrated 3°C. For each species, values represent the average (±SD) of 4 replicates of n=5 axes for superoxide, and 5 replicates of n=7-10 axes for TAA repeated twice. Values displayed next to a dotted line on the x-axes represent: P_{100} is the last point of 100% viability retention, P_{50} is 50% viability loss, P_0 is 100% viability loss and a P with a variable number represents the lowest % viability obtained after maximum storage. Values displayed beneath legend title represent correlations between superoxide and TAA (*$p < 0.05$, **$p < 0.0001$; Spearman's correlation).**

Apart from axes of *C. sinensis* seeds, there was no significant correlation between hydrogen peroxide production and TAA activity for axes from seeds stored hydrated at 16°C (Figure 3.7.2.1). Periods of heightened hydrogen peroxide may be responsible for the initial loss of viability in storage (*C. sinensis*: three months, *Q. robur*: two months, *T. dregeana*: four months and *A. heterophyllus*: six months) (Figure 3.7.2.1). Temperate recalcitrant-seeded species showed a variable antioxidant response to fluctuating levels of hydrogen peroxide (Figure 3.7.2.1a and b). *C. sinensis* axes showed consistent quenching of hydrogen peroxide over time, particularly in the initial period of storage (<four months) (Figure 3.7.2.1a). Hydrogen peroxide production in axes of *A. heterophyllus* seeds was positively correlated (although not significantly) with TAA, however, for each of the other species, there was a negative, non-significant decrease in TAA response to heightened hydrogen peroxide levels in storage (Figure 3.7.2.1). Reduction of viability to 50% may be a result of an accumulation of hydrogen peroxide, particularly for tropical recalcitrant-seeded *T. dregeana* and *A. heterophyllus* (Figure 3.7.2.1c and d, respectively). Between 8-18 months there was a substantial increase in hydrogen peroxide production, congruent with an increase in TAA activity, although this response was not sufficient to retain viability in axes of *T. dregeana* (33% viability after 18 months; highest antioxidant expression) (Figure 3.7.2.1c). In axes of *A. heterophyllus*, antioxidant and hydrogen peroxide production were consistently matched throughout storage, and may be a contributing factor in viability retention (55% viability after 15 months; highest hydrogen peroxide and TAA activity) (Figure 3.7.2.1d). There was a significant negative correlation ($p<0.05$) between hydrogen peroxide and TAA activity in axes of *C. sinensis* (Figure 3.7.2.a). However, these levels fluctuated in storage and may not have played a role in viability loss in both temperate recalcitrant-seeded species (Figure 3.7.2.1).
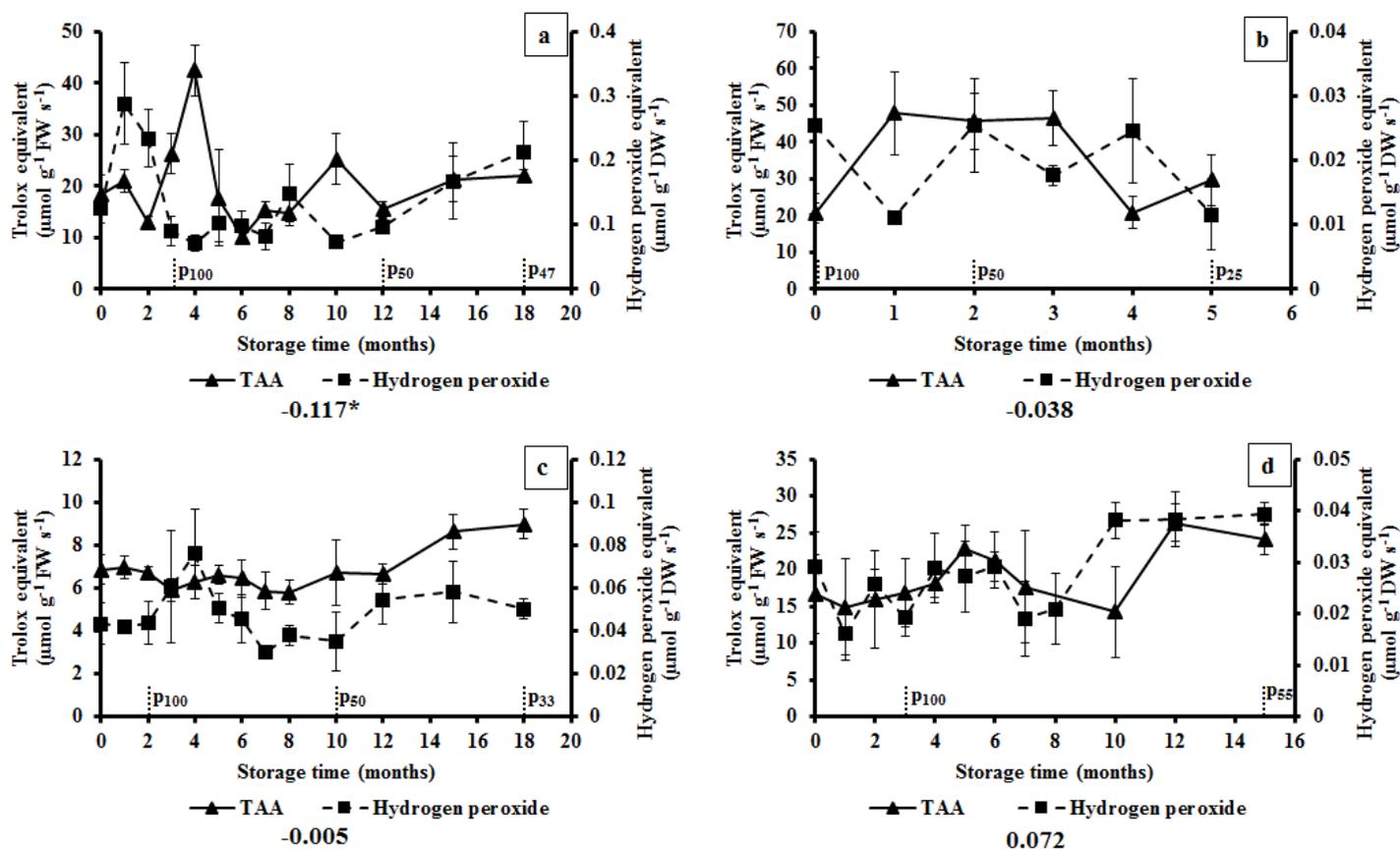
Figure 3.7.2.1: Relationship between hydrogen peroxide and TAA in embryonic axes from (a) *C. sinensis*, **(b)** *Q. robur*, **(c)** *T. dregeana* **and (d)** *A. heterophyllus* **seeds when stored hydrated 16°C. For each species, values represent the average (±SD) of 4 replicates of n=5 axes for hydrogen peroxide, and 5 replicates of 7-10 axes for TAA repeated twice. Values displayed next to a dotted line on the x-axes represent: P_{100} is the last point of 100% viability retention, P_{50} is 50% viability loss and a P with a variable number represents the lowest % viability obtained after maximum storage. Values displayed beneath legend title represent correlations between hydrogen peroxide and TAA (*p<0.05, **p<0.0001; Spearman's correlation).**

The relationship between TAA activity and hydrogen peroxide production was not significant in axes of seeds of each species stored at 3°C (Figure 3.7.2.2). The TAA activity was negatively correlated with hydrogen peroxide production for each species, and was more pronounced for *Q. robur* and *A. heterophyllus*, seen by the heightened levels in hydrogen peroxide, decreased levels of TAA and drastic decline in viability (Figure 3.7.2.2b and d, respectively). This may be a result of oxidative imbalance induced by chilling stress, which contributed to the loss of viability. Axes of *C. sinensis* seeds exhibited consistent high levels of hydrogen peroxide and TAA activity which may contribute to a high viability retention in storage over eight months (Figure 3.7.2.2a). *A. heterophyllus* axes lost 50% viability in two months corresponding to an increase in hydrogen peroxide production and diminished antioxidant sequestration (Figure 3.7.2.2d). Complete loss of viability was associated with a reduction in both hydrogen peroxide and TAA activities for tropical recalcitrant-seeded species, whilst *Q. robur* axes which maintained high levels of superoxide over six months, which may explain the subsequent decrease in TAA activity (Figure 3.7.2.2b). Hydrogen peroxide and TAA values were comparable between both temperatures for axes of *C. sinensis*, whilst hydrogen peroxide was produced in much higher quantities at 3°C than at 16°C for *Q. robur* (particularly at 12 months), *T. dregeana* (four months) and *A. heterophyllus* (three months) (Figures 3.7.2.1 and 3.7.2.2). Storage of tropical recalcitrant-seeded species at 16 and 3°C resulted in similar patterns in hydrogen peroxide production, however, TAA activity was reduced in axes of *A. heterophyllus*, when compared for the same period (Figures 3.7.2.1d and 3.7.2.2d, respectively). Storage of temperate recalcitrant-seeded species at 16 and 3°C resulted in varied patterns of hydrogen peroxide production and TAA activity (Figures 3.7.2.1a and b and Figure 3.7.2.2a and b, respectively).
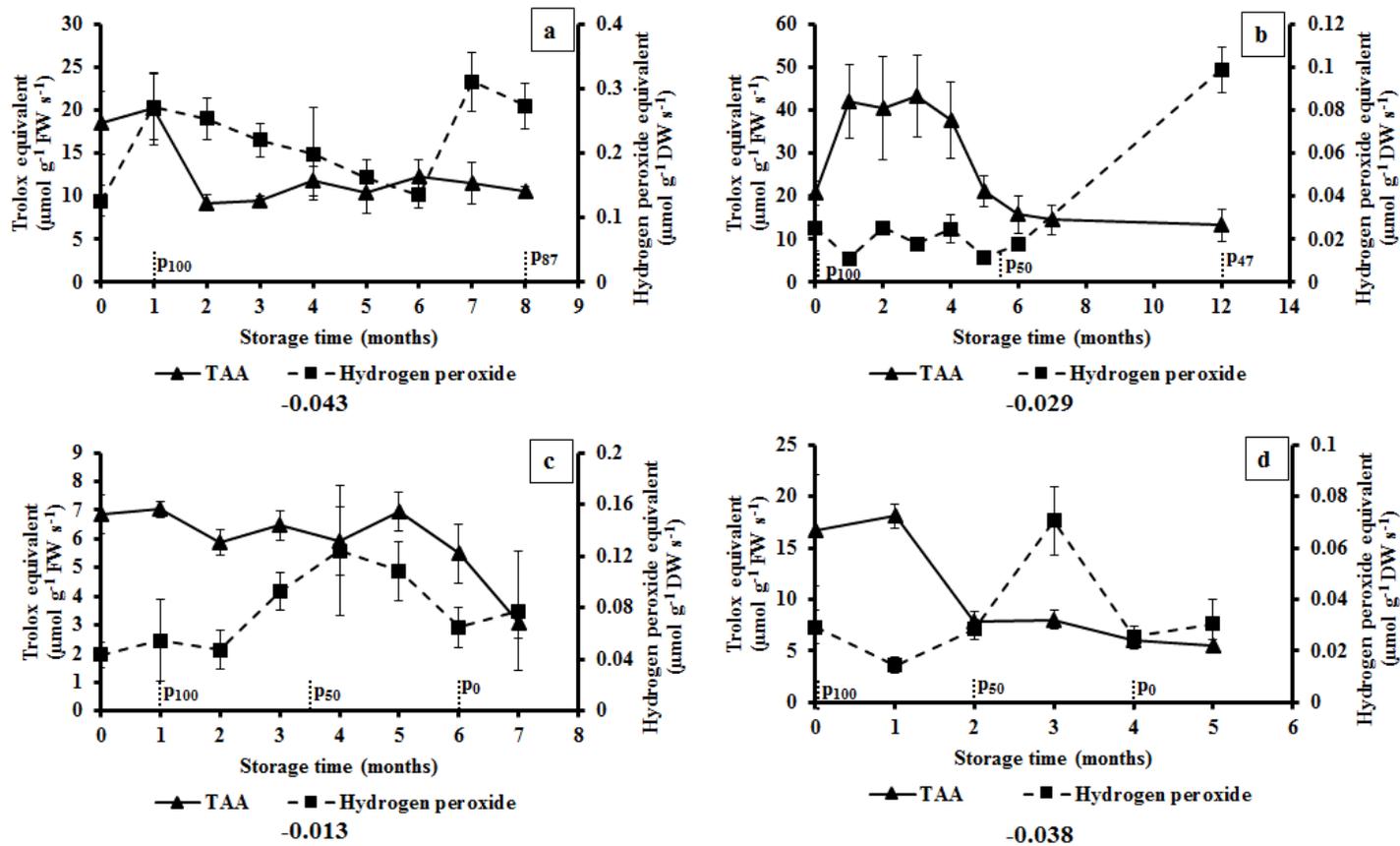
Figure 3.7.2.2: Relationship between extracellular hydrogen peroxide and TAA in embryonic axes from (a) *C. sinensis*, **(b)** *Q. robur*, **(c)** *T. dregeana* **and (d)** *A. heterophyllus* **seeds when stored hydrated 3°C. For each species, values represent the average (±SD) of 4 replicates of n=5 axes for hydrogen peroxide, and 5 replicates of 7-10 axes for TAA repeated twice, repeated twice. Values displayed next to a dotted line on the x-axes represent: P_{100} is the last point of 100% viability retention, P_{50} is 50% viability loss, P_0 is 100% viability loss and a P with a variable number represents the lowest % viability obtained after maximum storage. Values displayed beneath legend title represent correlations between hydrogen peroxide and TAA (*p<0.05, **p<0.0001; Spearman's correlation).**

3.8 Germination in storage

T. dregeana and *Q. robur* seeds germinated in a short time in storage (eight and three months, respectively). Axes of *T. dregeana* seeds that were freshly shed exhibited a significantly higher antioxidant activity when compared with seeds that germinated (p=0.003, mean difference 1.95 µmol g^{-1} FW s^{-1}). Similarly, axes of ungerminated *Q. robur* seeds showed a significantly higher antioxidant activity when compared with germinated seeds (p<0.0001, mean difference 17.8 µmol g^{-1} FW s^{-1}) (Figure 3.8.1).
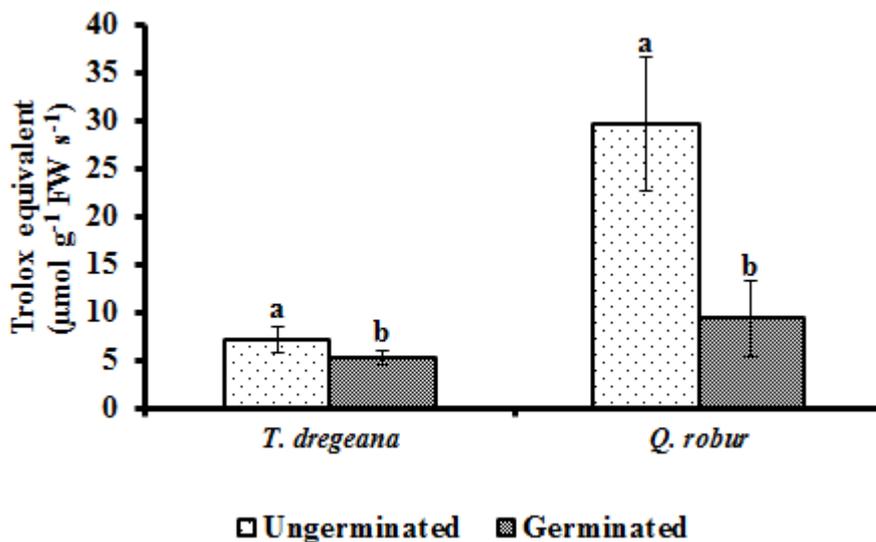
Figure 3.8.1: Differences in TAA in embryonic axes of *Q. robur* (5 months) and *T. dregeana* (8 months) that germinated and those that did not, at the same time period when stored hydrated at 16°C. For each species, values represent the average (±SD) of 3 replicates of n=3-5 axes. Columns labelled with different letters are significantly different within each species (p<0.05; t-test).

Storage at 3 and 16°C inevitably resulted in germination in hydrated storage-except for *A. heterophyllus* seeds, which did not germinate in storage nor were the seeds severely contaminated in storage (Table 3.3). Seeds of *T. dregeana* stored at 16°C, and *Q. robur* and *C. sinensis* seeds stored at 3 and 16°C, germinated in storage although variably in numbers and time periods. Of the three species, germination of *C. sinensis* seeds occurred in the lowest numbers at both temperatures, whilst it was highest in *Q. robur* seeds stored at 16°C

(Table 3.3). Seeds of *Q. robur* exhibited fungal proliferation in storage at both temperatures, which limited both seed numbers in storage and storage longevity. Seeds of *C. sinensis* stored at 3 and 16°C, and *T. dregeana* stored at 16°C were also contaminated by fungal or bacterial infection, but this did not reduce storage time drastically. Tropical recalcitrant-seeded species were not contaminated by fungal or bacterial infection when stored at 3°C, and as shown earlier, lost viability completely in a short period of time (Figure 3.5.1). The major limitation of storage time appears to be fungal contamination for *Q. robur* seeds, storage of *T. dregeana* seeds may be affected by a combination of germination and fungal proliferation, and oxidative stress may possibly result in loss of viability of *A. heterophyllus* seeds stored at 16°C (Table 3.3). Storage longevity of *C. sinensis* seeds may be further increased since viability retention was high (>80%) after eight months when stored at 3°C (Table 3.3).

Table 3.3: Comparison of storage behaviour of whole seeds of *C. sinensis*, *Q. robur*, *T. dregeana* and *A. heterophyllus* when stored hydrated for maximum storage periods.

	Temperate				Tropical			
Species	*C. sinensis*		*Q. robur*		*T. dregeana*		*A. heterophyllus*	
Temperature (°C)	3	16	3	16	3	16	3	16
Germination in storage	Yes	Yes	Yes	Yes	No	Yes	No	No
Complete loss of viability in storage[*]	No	No	No	No	Yes	No	Yes	No
Ideal hydrated storage time (months)[**]	8	6	4	<1	1	6	1	6
Limitation of storage time	Both, fungal contamination		Both, fungal contamination. 16°C: germination		16°C: germination, 3°C: chilling sensitivity		16°C: oxidative stress, 3°C: chilling sensitivity	

[*] 0% viability in storage; [**]storage time in which viability was maintained >80%.

CHAPTER 4: DISCUSSION

4.1   Relationship between shedding water content, maturity status and provenance

The range of shedding water contents of embryonic axes of recalcitrant seeds differs between species and provenances, and appears to be inter-seasonally variable within the same species (Berjak *et al.*, 1989; Berjak *et al.*, 2004a; Berjak and Pammenter, 2008). When compared, *T. dregeana* axes were shed at the highest water content, followed by *C. sinensis*, *A. heterophyllus* and *Q. robur* (Figure 3.1.1). Provenance did not appear to significantly differentiate shedding water content between tropical and temperate recalcitrant-seeded species, it appears to be species-specific in the present study. Also, seeds were shed with comparable axis water content between different years of collection over the course of this study (Figure 3.1.1). Although shedding water content of recalcitrant seeds can vary between 0.8-4.0 g g^{-1} DW, it is possible to incorrectly classify seeds as recalcitrant due to early harvesting resulting in immature seeds (Hong and Ellis, 1996). *C. sinensis* seeds harvested early (December 2013) exhibited a significantly higher water content than seeds harvested in February 2013 in the same year of collection (Figure 3.1.2). The embryonic axes lost 67% water after 15 min of rapid dehydration, freshly shed axes stained deep red via the TTZ test indicating high metabolic activity, these axes were prone to fungal contamination *in vitro* and seeds did not survive for more than two months in hydrated storage (data not shown). This clearly shows the importance of harvesting of recalcitrant seeds at the correct maturity status. Immature *Coffea arabica* and *C. robusta* seeds extracted from fruits of intermediate maturity (yellow fruits) were more desiccation tolerant than those from either ripe (red fruits) or immature (green fruits) (Ellis *et al.*, 1991; Hong and Ellis, 1995), although this may be related to seed-processing methods. Berjak *et al.* (1992) showed that recalcitrant-seeded *Landolphia kirkii* increased in desiccation tolerance from immature to mature embryonic axes, which may be explained by a decrease in lipid content during development. However, orthodox-seeded *Acer platanoides* harvested one month prior to natural senescence from the parent plant was more desiccation sensitive and did not tolerate dehydration to low water contents as the mature harvested seeds (Hong and Ellis, 1992a).

Furthermore, the variability of developmental status, even at the point of natural senescence in temperate recalcitrant seeds of *Q. robur* have been shown to exhibit varying levels in desiccation tolerance when harvested from the same tree in different years (Finch-Savage and Blake, 1994). Seeds shed at lower water contents were more desiccation tolerant. The variability in water content of *Q. robur* axes were observed to be an effect of drying time

on the ground after natural shedding (Figure 3.1.3), rather than developmental maturity. Large seeds shed with an air pocket had a significantly higher axis water content, possibly indicating that those seeds were newly shed, whilst smaller seeds did not differ in water content irrespective of the presence/absence of air pockets (Figure 3.1.3). Natural drying after maturity on the parent plant may prove advantageous for *Q. robur* seeds since these are shed from a great height and may break upon impact on the ground, creating a break in the testa which would facilitate water uptake and germination. This phenomenon was observed during seed collection; however, this may prove difficult to quantify as hundreds of seeds were being shed simultaneously. Although *Q. robur* acorns were collected in South Africa in the Drakensburg, an area of temperate climate, seeds collected in Poland were of higher water content (Figure 3.1.4), which may be due to condensation in transit, however, seeds from both regions germinated readily, and the only disparity observed were the lower fungal contamination in seeds from Poland. Studies performed on *Acer pseudoplatanus* seeds have shown that provenance and environmental conditions have a marked effect on both desiccation and germination (Daws *et al.*, 2006b). Colder conditions induced a higher shedding water content in *A. pseudoplatanus* seeds, resulting in under-developed seeds which were consequently more desiccation sensitive.

There are many developmental processes accompanying seed maturation, but for recalcitrant seeds there appears to be no distinct end point to development (Kermode and Finch-Savage, 2002). Whilst some authors have suggested that recalcitrance is the result of premature termination of seed development (Finch-Savage, 1992; Finch-Savage and Blake, 1994), others have questioned the existence of recalcitrance and hypothesised that amplifying the maturation period of these seeds may increase desiccation tolerance (Barbedo *et al.*, 2013). Farrant *et al.* (1993) showed that once *A. marina* seeds had become germinable, there was no change in water content nor increase in desiccation tolerance during seed development. Hong and Ellis (1996) cautioned that development of desiccation tolerance may occur at different developmental stages in different species and may not be consistent across all recalcitrant species. *A. heterophyllus* is cross-pollinated which results in innumerable variations in size, quality and maturity period both within the species and in individual fruits (Jagadeesh *et al.*, 2007; Khan, 2004). Previous work on *A. heterophyllus* seeds in our lab was performed on immature seeds harvested from green fruit which showed axes to be highly desiccation sensitive and shed at 3.85 g g^{-1} DW (Govender, 2009). For the current study, seeds harvested from green fruit exhibited a higher water content 2.94 ± 0.7 g g^{-1} DW and very dark TTZ staining, indicating high respiratory activity associated with ongoing

development (Figure 3.1.5). Water content of axes from ripe fruit was significantly lower than green fruit, large seeds having the lowest water content whilst smaller seeds were intermediate (Figure 3.1.5). Conversely, small seeds of *Q. robur* exhibited a lower water content than larger seeds (Figure 3.1.3), this was also observed in *Vitellaria paradoxa* seeds (Daws *et al.*, 2004). da Silva *et al.* (2014) found that seeds extracted from ripe *Pouteria ramiflora* fruits were more desiccation tolerant, shed at a lower water content with higher respiratory activity than seeds harvested from unripe fruit, and that desiccation sensitivity was most pronounced prior to physiological maturity. Chaudhury and Malik (2004) showed that *A. heterophyllus* axes were more desiccation and freezing tolerant at the most ripe and mature stage. Chandel *et al.* (1995) also found that desiccation tolerance increased with developmental maturity for both *C. sinensis* and *A. heterophyllus*. This validates the use of ripe *A. heterophyllus* fruit for further analyses in the present study, particularly in determining desiccation sensitivity as seeds are of physiological maturity. Berjak *et al.* (1993) advised that prior to undertaking dehydration studies on recalcitrant seeds, factors such as developmental status, provenance, climatic conditions of harvest as well as seed collection should be determined.

Recalcitrant seeds are generally very large but can vary in size (Pammenter and Berjak, 1999). An analysis of 16 tropical recalcitrant-seeded species in Cairns, Queensland (Australia) showed that species producing larger seeds are shed at higher water content and are able to withstand greater water loss (Hill *et al.*, 2012). Although seed size may be a poor predictor of desiccation tolerance in some species, the evolutionary advantage of producing large seeds is associated with larger nutrient reserves and thus results in large seedlings (Pammenter and Berjak, 2000b; Primack, 1987; Pritchard *et al.*, 2004). Large seeds of *A. heterophyllus* (weighing 12-14 g) were found to produce larger seedlings under varying light regimes when compared with seeds of smaller size (Khan, 2004). In this study, seeds also initiated germination within 10 days, which corresponded to the rapid germination of whole seeds observed in this study (5-8 d). Seeds of all species displayed 100% germination (Figures 3.2.1 and 3.2.2), although germination time varied between species, the exception being *Q. robur* seeds in 2015 which were collected later in that year, harboured a high fungal load, and cultures were overridden by fungal contamination (Figure 3.2.1 and 3.2.2). *A. heterophyllus* seeds germinated (radicle protrusion > 1cm) in the shortest time (3-4 days), followed by *T. dregeana* (10-16 d), *Q. robur* (12-15 d) and *C. sinensis* (18-24 d). Tropical recalcitrant-seeded species germinated faster than temperate-recalcitrant seeded species. However, *in vitro* germination (radicle elongation >1 cm) was fastest for *A. heterophyllus* (8-

10 d), followed by *Q. robur* (10-12 d), *C. sinensis* (15-18 d) and lastly *T. dregeana* (16-18 d) (Figure 3.2.2).

According to Berjak *et al.* (1989), recalcitrant seeds may be classified into four groups based on the rate of germination in storage: (1) very slow germinators, (2) relatively rapid (slow) germinators, (3) rapid germinators and (4) very rapid germinators. In very slow germinators, the period of axis differentiation is quite extended resulting in reduced germinative processes whereas in relatively rapid (slow) germinators seeds are shed with relatively undifferentiated axes which must be completed prior to germination. In rapid germinators, the seeds are shed containing a fully differentiated embryonic axis, following a brief period of cellular organisation and division (1-3 d), after which the seeds will initiate germination, and finally, when very rapid germinators are shed there is no acquisition of desiccation tolerance, seeds rather proceed directly to germination upon shedding. Although whole seed germination may have been hindered by a hard seed coat (*C. sinensis* and *Q. robur*), upon scarification, this greatly improved germination rate. *In vitro* germination rate may have been reduced due to a variety of stresses induced by *in vitro* environment, particularly for axes of *T. dregeana* (Gaspar *et al.*, 2002). Faster recalcitrant seed germination is associated with greater desiccation sensitivity and shorter seed longevity (Marcos-Filho, 2014).

4.2   Response to desiccation

Water plays a plethora of roles in cells which ensures survival. Dehydration removes water, causing cells to shrink, and thus alters the physical, mechanical and chemical function of cells (Pammenter and Berjak, 1999; Vertucci and Farrant, 1995; Walters *et al.*, 2002). Due to their inability to tolerate desiccation, recalcitrant seeds lose viability at high water contents, although the degree of water loss tolerated, developmental status, seed quality, type of explant and rate of drying varies per species (Berjak *et al.*, 1993; Mycock *et al.*, 1995; Pammenter and Berjak, 1999, 2014; Pammenter *et al.*, 2000; Pammenter *et al.*, 1991). It has been well documented that rapid drying of recalcitrant germplasm outperforms slow drying, possibly due to the rapid transition to low water content resulting in less time spent at intermediate water contents which limits the progression and accumulation of deleterious metabolism-linked damage (Berjak *et al.*, 1993; Farrant *et al.*, 1989; Kioko *et al.*, 2006; Mycock *et al.*, 2000; Pammenter and Berjak, 1999, 2014; Pammenter *et al.*, 2000; Pammenter *et al.*, 1991; Perán *et al.*, 2004; Pritchard and Prendergast, 1986; Varghese *et al.*, 2011; Wesley-Smith *et al.*, 2001a; Wesley-Smith *et al.*, 1992). It has already been shown that

rapid drying outperforms slow drying in the species investigated in this study- *C. sinensis* (Berjak *et al.*, 1993; Wesley-Smith *et al.*, 1992), *Q. robur* (Ntuli *et al.*, 2011), *T. dregeana* (Varghese *et al.*, 2011) and *A. heterophyllus* (Wesley-Smith *et al.*, 2001a).

Drying time had a significant impact on viability retention (both root and shoot production) of each species and shown a similar pattern of water loss, irrespective of provenance (Figures 3.3.1-3.3.4). Initially, there was a rapid decline in water content (loss of free or bulk water) associated with very little change in viability, thereafter, any further loss in water resulted in a rapid decline in viability. The lowest safe water content at which viability is retained at 100% varied per species; both *C. sinensis*: 0.52 ± 0.11 g g^{-1} DW, 30 min of drying, 74% water loss (Figure 3.3.1) and *A. heterophyllus*: 0.58 ± 0.09 g g^{-1} DW, 10 min of drying, 58% water loss (Figure 3.3.4) axes could tolerate a greater loss of water in a short time and maintain 100% viability, whilst axes of *Q. robur*: 0.88 ± 0.11 g g^{-1} DW, 5 min of drying, 21% water loss (Figure 3.3.2) and *T. dregeana* 1.34 ± 0.26 g g^{-1} DW, 30 min of drying, 35% water loss (Figure 3.3.3) tolerated less water loss to maintain 100% viability in a short drying period. Furthermore, both *C. sinensis* and *A. heterophyllus* axes exhibited faster drying rates (based on calculations derived from Ntuli and Pammenter (2009)) than *Q. robur* and *T. dregeana*. *A. heterophyllus* axes exhibited the shortest drying course (90 min) and fastest rate (0.1419 g H$_2$O lost min^{-1}) whilst *Q. robur* axes exhibited the longest drying course (540 min) and slowest drying rate (0.0143 g H$_2$O lost min^{-1}) (Table 3.1). Additionally, *T. dregeana* and *Q. robur* axes required extended periods of drying to obtain a low water content of 0.1 g g^{-1} DW, which may be related to the cotyledonary segments attached which could have indirectly affected the drying rate by providing an additional source of water from which axis tissue could withdraw upon dehydration (Table 3.2). These cotyledonary segments were necessary to ensure shoot formation (Goveia, 2007), and were removed prior to water content estimation. Particularly in axes of *T. dregeana*, drying embryonic axes only without a segment of cotyledon attached resulted in no shoot production (data not shown). Ntuli and Pammenter (2009) found that axes of *A. marina* had a slower drying rate due to a greater dry mass. This may explain the extended drying time for *Q. robur*, since axes were the heaviest. Drying rate of *T. dregeana* axes appeared to be related to transfer resistances (interaction of water and/or air/tissues) within the axes, axis surface and boundary layer, rather than weight (Ntuli and Pammenter, 2009). Wesley-Smith *et al.* (2001a) showed that *A. heterophyllus* axes exposed to short, rapid drying resulted in preferential withdrawal of water from the outermost tissues, while inner layers of the axes, particularly cells in the core of the axis (stele) remained relatively unaffected by drying and thus were able to retain

viability. Similarly, this may also occur in axes of *C. sinensis*, whereby uneven drying confers greater survival.

A distinct difference in shoot production was noted in axes of *C. sinensis*, whilst root production was reduced, though not to zero, high shoot production was maintained at 80 ± 25% (Figure 3.3.1), even when 94% water was lost (Table 3.2). Axes of *A. heterophyllus* exhibited 93 ± 12% viability when water content was reduced to 0.3 g g^{-1} DW, whilst at the same water content, the tropical counterpart, *T. dregeana* showed only 40 ± 20% root and shoot production (Figures 3.3.4b and 3.3.3b, respectively) (Table 3.2). These findings are in agreement with many authors who found that root and shoot tissue within isolated embryos of *Araucaria hunsteinii* (Pritchard and Prendergast, 1986), *Castanea sativa* (Pence, 1992), *Q. robur* (Poulsen, 1992), *Theobroma cacao* (Chandel *et al.*, 1995) and *T. emetica* (Kioko *et al.*, 2006), exhibited different responses and tolerances to dehydration. Years of research on recalcitrant seeds in our lab has shown that embryonic axes may often produce roots first and be scored as germinated, but damage to the shoot meristematic tissue due to excision (Berjak *et al.*, 2011b; Goveia, 2007; Naidoo *et al.*, 2011) or drying (Ballesteros *et al.*, 2014) may be so severe that there is no subsequent shoot development and seedling establishment (Berjak *et al.*, 2004b; Berjak *et al.*, 1999). This commonly occurs in tropical and sub-tropical species where embryonic axes produce roots and/or callus, but no shoots (Pammenter *et al.*, 2009). This may be explained by the tissue surrounding the root meristem conferring better protection than the tissue layers covering the shoot meristem (Berjak *et al.*, 2004b) or even the proximity of shoot meristems to the point of excision of the embryonic axis (Ballesteros *et al.*, 2014). Pammenter *et al.* (2002) warned that assessing embryonic axes based on swelling and/or greening may indicate that the tissue is not dead, however, it does not necessarily imply that a functioning seedling may be produced.

It is unclear whether the observed changes in growth, morphology, or metabolic activity was a result of desiccation-induced damage, or a protective strategy in response to dehydration (Walters *et al.*, 2002). Desiccation damage is not quantified based on differences between fully hydrated material and embryonic axes that have been dried, but rather on the resumption of normal metabolic activity upon rehydration (Walters *et al.*, 2002). In this study, direct immersion in cathodic water, which has been shown to ameliorate oxidative damage in recalcitrant axes (Berjak *et al.*, 2011b), was used to rehydrate all embryonic axes prior to culturing. Although rehydration technique plays an important role in survival (Perán *et al.*, 2004), if cells do not acclimatise to the loss of water, they rupture when rehydrated, irrespective of the medium (Walters *et al.*, 2002). In addition to imbibition injury, axes may

also experience osmotic stress from the media as well as a host of other stresses induced by the tissue culture environment which may lead to further reduction in viability possibly mediated via ROS production (Benson, 2000; Gaspar *et al.*, 2002). Dehydration is a crucial step to remove cellular water in order to obtain a glassy state and prevent lethal ice crystal formation upon liquid nitrogen exposure and storage at low temperatures (Benson, 2008a; Berjak *et al.*, 2011b; Pammenter and Berjak, 1999, 2014; Vertucci *et al.*, 1991; Wesley-Smith *et al.*, 2014). Many procedural steps involved in cryopreservation result in oxidative stresses which are accumulative (Berjak *et al.*, 2011b); assessment of drying rates and their subsequent effects may assist in improving post-cryo survival (Naidoo *et al.*, 2016; Pammenter and Berjak, 2014; Pammenter *et al.*, 2003; Sershen *et al.*, 2016; Varghese *et al.*, 2011).

Based on the drying time courses, three water contents were selected to assess the effect of dehydration on ROS and antioxidant production. Superoxide and hydrogen peroxide are synthesized at very high rates even under fully hydrated conditions in recalcitrant seeds. They are involved in many aerobic biochemical reactions and are produced by several interrelated enzyme systems (Bowler *et al.*, 1992; Noctor and Foyer, 1998). ROS themselves may not necessarily be toxic, but the accumulation and their ability to initiate and propagate cascade reactions which generate toxic by-products such as hydroxyl radicals and lipid peroxides pose the greatest threat to survival, particularly in desiccation-sensitive systems. (Foyer and Noctor, 2005a, b; Kranner and Birtić, 2005; Noctor and Foyer, 1998; Pammenter and Berjak, 1999). The presence and operation of an antioxidant network is essential for quenching ROS to appropriate levels, however, this process may generate other toxic ROS, e.g. superoxide is dismutated to hydrogen peroxide and water via SOD, whilst hydrogen peroxide may be detoxified via CAT or a variety of POXs (Bowler *et al.*, 1992; Dietz *et al.*, 2016; Neill *et al.*, 2002; Noctor and Foyer, 1998; Scandalios *et al.*, 1997; Willekens *et al.*, 1995). An increase in ROS production in response to desiccation will elicit an increase in antioxidant activities, however, the result of over-accumulated ROS to levels that are not sufficiently quenched often results in oxidative stress and eventually cell death (Foyer and Noctor, 2005a; Kranner and Birtić, 2005; Mittler, 2016).

Extracellular superoxide production appeared to be related to shedding water content and not provenance; embryonic axes shed at high water content exhibited higher superoxide production (Figure 3.4.1). Extracellular hydrogen peroxide production also did not appear to be related to provenance at shedding (Figure 3.4.2); however, TAA activity was significantly higher in embryonic axes from the temperate provenance than the tropical species

(Figure 3.4.3). Even mild dehydration to 1.0 g g^{-1} DW elicited a response in both ROS and antioxidants. A decrease in water content significantly increased superoxide, hydrogen peroxide and TAA activity, relative to the initial values at harvest for each species (Figures 3.4.1-3.4.3). However, at the lowest water content (0.1 g g^{-1} DW), there was a slight decrease in hydrogen peroxide production (Figure 3.4.2). *T. dregeana* axes produced significantly higher superoxide levels (Figure 3.4.1), *C. sinensis* produced significantly higher hydrogen peroxide levels (Figure 3.4.2), whilst *Q. robur* axes produced the lowest levels of both ROS, but had the highest antioxidant expression (Figures 3.4.1-3.4.3).

Many enzymatic antioxidant systems, which are fully operational in the hydrated state, may not function efficiently when water is removed, or only the molecular or non-enzymatic antioxidants (e.g. glutathione, ascorbate, and tocopherol) may be functional (Vertucci and Farrant, 1995). Embryonic axes of *Q. robur* that were rapidly dried to 0.66 g g^{-1} DW produced significantly higher α-tocopherol levels, heightened activities of the enzymatic scavengers APX and GR as well as POX and SOD when compared with slow dried axes (Pukacka *et al.*, 2011). Those authors reported that rapid dehydration resulted in greater mobilization of antioxidant systems in embryonic axes, which resulted in greater viability retention after drying to lower water content levels. Ntuli *et al.* (2011) reported that at low water contents (0.27-0.08 g g^{-1} DW), viability loss during rapid dehydration was associated with an increase in hydroperoxide levels due to free radical-mediated lipid peroxidation. These authors also found that dehydration to low water contents impaired the efficiency of CAT and GR activities, whilst increased SOD activity (Ntuli *et al.*, 2011). It is possible that the longer desiccation period experienced by *Q. robur* axes (8.5 hours) may have caused irreversible damage to embryonic axes due to increased accumulation of ROS, which was not efficiently removed despite increased levels of antioxidants (Figure 3.4.3). However, it should be noted that both superoxide and hydrogen peroxide production in *Q. robur* were the lowest of all species investigated, and although the antioxidant system may have regulated ROS production at higher water contents, at very low water contents there may be a severe reduction in water availability. At very low water contents, the only water present is tightly bound to organelles, free radical production occurs via auto-oxidation since cells continue to respire but cannot scavenge ROS or their toxic by-products (Vertucci and Farrant, 1995; Walters *et al.*, 2005). Similarly, *T. dregeana* axes rapidly dried for 5 hours to 0.13 g g^{-1} DW in a study by Varghese *et al.* (2011) showed a decline in superoxide and hydroxyl production, an increase in CAT activity and a decrease in SOD, GR, APX, GPX axes relative to axes of freshly shed seeds. Those authors state that the desiccation damage in recalcitrant seeds may

be a function of two interrelated factors: the intensity and duration of desiccation stress, which may be difficult to distinguish and integrate between due to the complex nature and activity of tissue water content and the rate of drying (Pammenter *et al.*, 2003; Pammenter *et al.*, 2002; Varghese *et al.*, 2011).

Contrastingly, axes of *C. sinensis* and *A. heterophyllus* that were dried to low water contents in a much shorter time, could tolerate drying to low water contents (0.3-0.4 g g^{-1} DW) and exhibited much higher % survival than their tropical and temperate counterparts (Table 3.2). Whilst superoxide production was only significantly elevated upon drying to 0.1 g g^{-1} DW in axes of *C. sinensis* relative to the control and other drying times, superoxide levels increased upon drying and remained relatively unchanged in axes of *A. heterophyllus* (Figure 3.4.1). These levels only differed from each other in freshly harvested material, and when dried to 0.1 g g^{-1} DW. Hydrogen peroxide levels were significantly higher in axes of *C. sinensis* at each water content when compared with other species, as well as markedly higher in TAA activity (Figures 3.4.2-3.4.3). Whole seeds of *C. sinensis* were dried for 96 hr (embryonic axis water content of 0.25-0.30 g g^{-1} DW) also showed an increase in hydrogen peroxide levels and antioxidant enzymes such SOD, POX, APX, CAT, DHAR and GR despite a loss in viability (Chen *et al.*, 2011). Similarly, Jamalomidi and Gholami (2013) also showed that in five cultivars of *C. sinensis*, whole seeds dried for 80 hrs exhibited an increase in hydrogen peroxide, SOD, CAT, APX and POX relative to undried material, and that maximum enzyme activities were observed after 40 hours of drying. It is well established that hydrogen peroxide functions as an essential signalling molecule in a variety of molecular, biochemical and physiological processes due to the relatively long half-life >1ms and ability to travel long distances (>1 µm) (Baxter *et al.*, 2014; Mittler, 2016; Neill *et al.*, 2002). Hydrogen peroxide is also produced in response to such a variety of stimuli, that it is likely to facilitate cross-talk between signalling pathways, and contribute to the phenomenon of 'cross-tolerance' (Baxter *et al.*, 2014; Foyer and Noctor, 2005a; Neill *et al.*, 2002). Efficient signalling and the ability to tolerate high levels of hydrogen peroxide congruent with an increase in antioxidant activity when dehydrated, may explain why axes of *C. sinensis* are able to tolerate desiccation stress and survive upon rehydration.

ROS and antioxidants did not seem to play a major role in viability retention of *A. heterophyllus* axes, possibly due to the short drying times which did not elicit an oxidative response (Figures 3.4.1-3.4.3; Table 3.2). Although ROS levels did not change drastically, and antioxidants were significantly elevated, relative to the control, the rapid rate of drying appeared to influence survival (Table 3.1). As discussed earlier, Wesley-Smith *et al.* (2001a)

found that selective removal of water conferred protection on meristematic cells, and at low water contents, electrolyte leakage was not severe. Axes of *A. heterophyllus* which were dried under a laminar flow hood to 0.17 g g^{-1} DW after 4 hours, showed a slight increase in electrolyte leakage and minimal change in lipid peroxidation (Chandel *et al.*, 1995). Although there is little information published on ROS and antioxidant activities of axes that were rapidly dried, the literature shows some disparity linked to varied maturity levels (Chaudhury and Malik, 2004; Jagadeesh *et al.*, 2007). It is likely that survival of axes is directly related to mechanical and physical changes that result from the rapid transition to low water content (0.3 g g^{-1} DW) rather than oxidative stress (Cheng and Song, 2008). In desiccation-tolerant organisms, sugars such as sucrose and raffinose stabilise membrane phospholipids either by replacing water with hydroxyl groups or form glasses upon dehydration thereby maintaining structural integrity in the absence of water (Crowe *et al.*, 1992; Hoekstra *et al.*, 2001; Kermode, 1990). Vitrification slows down molecular mobility and thus limits biochemical reactions which may explain the slight changes in ROS and antioxidants levels (Vertucci and Farrant, 1995). Cherussery *et al.* (2015) showed that *T. cacao* seeds desiccated over six days at 28°C resulted in enhanced electrolyte leakage and a significant increase in sucrose content. These authors state that if sucrose: raffinose exceeds a ratio of 8:1, loss of viability ensues. Furthermore, Greggains *et al.* (2000) showed that sucrose, raffinose and stachyose production were elevated in orthodox-seeded *Acer platanoides* when compared with recalcitrant-seeded *A. pseudoplatanus* after dehydration. These authors also found that during drying, there was no evidence of oxidative stress; free radical production did not accumulate, lipid peroxidation levels remained comparable and free radical scavenging enzymes did not decrease in either species. However, in mature seeds of *A. platanoides* and *A. pseudoplatanus* which were desiccated at 18-20°C at 60-70% RH to 0.15 g g^{-1} DW, there was in an increase in superoxide and hydrogen peroxide in whole seeds as well as an increase in APX, MDHAR, DHAR, GR and GPX in embryonic axes in both species but was much higher in orthodox *A. platanoides* than recalcitrant *A. pseudoplatanus* (Pukacka and Ratajczak, 2007b). Interestingly, these heightened levels of ROS and antioxidant enzymes did not correspond to complete loss of survival, *A. platanoides* retained 100% viability, whilst *A. pseudoplatanus* exhibited 65% survival.

Quite often, oxidative stress is equated to changes in ROS and the subsequent antioxidant levels/measures of damage and thereafter related to viability. The heightened production of ROS was previously considered to be detrimental due to oxidant accumulation leading to oxidative stress (Noctor and Foyer, 1998). Although cell death may be

accompanied by an increase in ROS production and many by-products of their reactivity (lipid peroxidation, protein denaturation and damage to DNA), the question of what kills the cell is most important (Colville *et al.*, 2012; Finch-Savage *et al.*, 1996; Mittler, 2016; Noctor and Foyer, 1998). Death by oxidative stress may not be a direct consequence of high levels of ROS, but rather by a series of triggered reactions leading to programmed cell death, for example, Roach *et al.* (2008) showed that excision injury resulted in a burst of superoxide which lead to increased hydrogen peroxide levels in freshly excised *Castanea sativa* axes. Upon stress perception, ROS signalling activates an autopropagating wave of ROS in response to a specific stress (Mittler *et al.*, 2011; Noctor *et al.*, 2016). Noctor *et al.* (2016) stated that ROS may also accumulate in cellular compartments that have low antioxidative function. Furthermore, this accumulation may not increase uniformly in a cell, but rather at sites requiring oxidation (Noctor and Foyer, 2016). Therefore, ROS accumulation may not be stressful, but rather specific to functional requirements particularly in redox signalling (Noctor *et al.*, 2016).

4.3   Response to hydrated storage

Hydrated storage of recalcitrant seeds is strictly a short- to medium-term strategy, possibly for a few days to weeks for seeds of many tropical species, and up to a year for seeds of chilling-tolerant temperate species (Pammenter and Berjak, 2014; Pammenter *et al.*, 1994). A characteristic of recalcitrant seeds is that when placed in hydrated storage for prolonged periods, they eventually lose viability (Pammenter *et al.*, 1994). In the present study, this is evident in seeds stored at 16°C, viability significantly decreased with an increase in storage time, irrespective of provenance (Figure 3.5.1a, b). Axes of *C. sinensis* seeds did not significantly lose viability at 3°C, although *Q. robur* seeds were sensitive to hydrated storage, losing viability faster at 16°C (5 months) than 3°C (12 months) (Figure 3.5.1a, c). Similarly, *Coffea arabica* seeds desiccated under high RH (81%) and stored at 20°C, showed 60% loss in viability, whilst storage at 5°C maintained 85% viability after 3 months of storage (Dussert *et al.*, 2006). Beech seeds (*Fagus sylvatica*) stored at 4°C lost 50% viability after nine weeks, whilst storage at 20 and 30°C resulted in 90% loss in viability under the 75% RH (Pukacka and Ratajczak, 2005). Tropical recalcitrant seeds, as expected, were chilling sensitive and lost viability in a short period when stored at 3°C (Figure 3.5.1d). Similarly, Kioko *et al.* (2006) found that seeds of *T. emetica* stored at 6°C lost complete viability after 20 d in hydrated storage. *T. dregeana* seeds lost 90% viability retention after 14 d and *T. emetica* lost 95% viability retention after 7 d in hydrated storage at 6°C (Eggers *et al.*, 2007). There are many

species that produce recalcitrant seeds, (see Farnsworth (2000) of which each have varying longevity, chilling tolerance and storage temperature between both species and provenance (Bharuth *et al.*, 2007; Ellis *et al.*, 1991; Pammenter and Berjak, 2000a; Suszka *et al.*, 2014). Although hydrated storage is a suitable method for recalcitrant seed short-term storage, many studies choose not to store seeds in the hydrated state, usually in perforated polyethylene bags, but rather perform experiments within a short period of time (Pasquini *et al.*, 2012; Patui *et al.*, 2014; Sershen *et al.*, 2008; Wesley-Smith *et al.*, 2001a). If seeds have been stored hydrated, the time period is only for a few weeks to months since germinative metabolism commences (Kioko *et al.*, 2006; Moothoo-Padayachie *et al.*, 2016; Motete *et al.*, 1997; Ntuli *et al.*, 2011; Sershen *et al.*, 2008). Kioko *et al.* (2006) showed that *T. emetic*a seeds stored at 16 and 25°C resulted in 100% germination in storage after 60 d. In another study, recalcitrant *A. marina* seeds were reported to have maintained high viability for 25 d in hydrated storage, but viability decreased drastically until 40 d, whereas *T. dregeana* seeds maintained high viability up to one year in hydrated storage at 16°C (Moothoo-Padayachie *et al.*, 2016). Nonetheless, those authors also reported substantial germination in hydrated storage conditions. Eggers *et al.* (2007) found that *T. dregeana* seeds were able to store well for five and a half months at 16 and 25°C, exhibiting low viability loss, *T. emetica* seeds for up to three weeks at 16°C, and *Syzygium cuminii* for two weeks at 16°C and four weeks at 25°C. Furthermore, some researchers do not store seeds under high relative humidity and may have measured an interactive stress of drying and storage (Connor and Sowa, 2002; Lan *et al.*, 2012; Masetto *et al.*, 2016).

Hydrated storage maintains seeds at high relative humidity, thus preventing water loss; however, due to the inherent requirement for water to complete germination, this results in a mild, but increasingly severe water stress (Pammenter *et al.*, 1994). In this study, seeds which were shed at low water contents (Figure 3.1.1) significantly increased in water content as storage time increased at 16°C (Figure 3.5.2.1b, d). For example, *Q. robur* axes significantly increased in water content at 3°C, but this may have been due to fungal proliferation in storage. *Q. robur* trees, leaves and seeds are prone to a myriad of microflora, in seeds these may be inherent and develop with storage time (Murray, 1974). Fungal proliferation results in an increase in water produced by respiratory metabolism, which is then absorbed and enhances germination in storage (Mycock and Berjak, 1990). A study on *A. marina* seeds in hydrated storage showed that if fungal activity is curtailed, then the hydrated storage lifespan can be extended (Calistru *et al.*, 2000). Furthermore, upon inoculation with *Fusarium moniliforme*, freshly harvested seeds were more sensitive to the

effects of the fungus than seeds inoculated after 4 d in storage. This resilience was lost after 14 d in storage (Calistru *et al.*, 2000). In the present study, fungal proliferation became increasingly severe with storage time and contributed to a reduction in storage lifespan in *Q. robur* seeds due to fungal-induced deterioration as well as increased germination in storage (Table 3.3). Seeds which germinated in storage in this study were not used for any experiments, although whole seeds may have germinated despite the fungal proliferation. *In vitro* cultures were extremely contaminated by inherent fungus and may explain the large variability in viability estimations (Figure 3.5.1b, d). When seeds were stored non-hydrated (observations based on *Q. robur* seeds from Poland), sterilised seeds dusted with Benomyl were placed in large polyethylene containers (~50-75 l), fewer seeds germinated in storage and fungal contamination was almost negligible (physical observations). This indicates that hydrated storage may not be an appropriate storage method for *Q. robur* seeds.

Seeds of *C. sinensis* were also affected by fungal contamination, however this was not as severe as *Q. robur*, the source of the fungal inoculum appeared to be inherent and contained within the hard pericarp and small micropylar region. Water loss was restricted by the hard seed coat, and varied possibly due to different years of collection (Figure 3.5.2.1a; 0-8 months in collection 2 and 10-18 months in collection 1). At 3°C, water content and fungal contamination both reduced, although viability remained unchanged (Figure 3.5.2.2a). Water content of tropical recalcitrant-seeded *T. dregeana* seeds steadily increased in storage at 3°C, but it was more likely caused due to chilling injuries such as membrane alterations resulting in increased permeability and solute leakage, changes in respiratory activity, and the generation of free radicals which eventually lead to increased decay and seed death in both topical species (Figure 3.5.2.2c, d) (Lyons, 1973; Saltveit and Morris, 1990).

4.4   Redox status in storage

As mentioned earlier, the regulation of redox reactions is directly related to ROS production and the ability of antioxidant systems to sufficiently quench excess levels of ROS (Mittler, 2016). Although desiccation induces an oxidative response due to the removal of water, maintenance at shedding water content appeared to elicit a different oxidative response in seeds stored hydrated at 3 and 16°C. Viability retention in storage declined irrespective of temperature and oxidative status, and was species-specific. Axes of *C. sinensis* seeds stored hydrated at 16°C showed similar fluctuations in superoxide levels as seeds stored at 3°C, and viability remained high (>80%) (Figure 3.6.1.1a and 3.6.1.2a, respectively), indicating that superoxide may not play a role in short-term storage viability loss. Axes of *Q. robur*,

however, produced an increase in superoxide levels at both temperatures as storage time proceeded, (Figure 3.6.1.1b and 3.6.1.2b), and this may be linked to an increase in water uptake (Figure 3.5.2.1 and 3.5.2.2) although these levels were the lowest of all species. Moothoo-Padayachie *et al.* (2016) showed that in axes of both *A. marina* and *T. dregeana* seeds, superoxide levels increased with an increase in water uptake congruent with the onset of germination. This phenomenon also occurs in *Q. robur* as seeds germinated in storage (Table 3.3).

Axes of *T. dregeana* produced a similar bell-shaped peak in superoxide production at 16 and 3°C, (Figure 3.6.1.1c and 3.6.1.2c, respectively) which was the highest levels in all species. At 3°C, this accumulation may be related to the loss of viability, whilst at 16°C this may indicate ongoing germinative metabolism (Table 3.3). This further supports literature that ROS plays a dual role in germinative processes in *Pisum sativum* (Kranner *et al.*, 2010) and *Lepidium sativum* seeds (Müller *et al.*, 2009), enhancing germination via cold stratification of *Hedysarum scoparium* seeds (Su *et al.*, 2016), as well as stress perception in desiccated and stored *Coffea arabica* L. seeds (Dussert *et al.*, 2006), apoptosis-like events in cryopreserved embryogenic callus in *Agapanthus praecox* (Zhang *et al.*, 2015), and wounding in *Castanea sativa* (Roach *et al.*, 2008). In axes of *A. heterophyllus* seeds, an increase in superoxide production was significantly related to both storage time, and a loss of viability at 16°C (Figure 3.6.1.1d). As seen in *Q. robur* axes, superoxide production increased with an increase in water content, although no signs of germination were recorded (Table 3.3). At 3°C, superoxide production peaked after one month in storage and gradually decreased (Figure 3.6.1.2d).

Hydrogen peroxide production was not affected by storage temperature, and did not appear to be related to viability retention or storage time for temperate recalcitrant-seeded species (Figures 3.6.2.1a, b and 3.6.2.2a, b). *C. sinensis* axes produced the highest levels of hydrogen peroxide at both 16 and 3°C (Figures 3.6.2.1a and 3.6.2.2a). In tropical recalcitrant-seeded species, hydrogen peroxide was elevated in axes of both species, and produced a similar bell-shaped curve prior to a loss of viability (Figures 3.6.2.1c, d and 3.6.2.2c, d). Hydrogen peroxide functions as a potent signalling molecule (Neill *et al.*, 2002; Petrov and Van Breusegem, 2012), capable of transducing signals over a large distance in a variety of cell compartments (Dietz *et al.*, 2016; Mittler, 2016; Noctor and Foyer, 2016). Apart from activating signalling cascades which further increase ROS production and accumulation within a cell, hydrogen peroxide may generate the highly reactive hydroxyl radical via the Fenton Reaction which non-specifically attacks biomolecules (Apel and Hirt, 2004; Benson

and Bremner, 2004; Foyer and Noctor, 2005b; Kremer, 1999; Mittler *et al.*, 2011). Dat *et al.* (2003) found that transgenic tobacco (reduced catalase activity) leaves accumulated high levels of hydrogen peroxide which induced cell death. In barley roots treated with aluminium, heightened levels of hydrogen peroxide were produced 48 and 72 h after exposure relative to the control (Tamás *et al.*, 2005). However, in maize seedlings pre-treated with exogenous hydrogen peroxide, the induction of antioxidant enzymes conferred protection in mitochondrial cells which was not observed in non-acclimated seedlings (Prasad *et al.*, 1994). Germination of *Zinnia elegans* seeds was enhanced by hydrogen peroxide at different stages after imbibition, suggesting that hydrogen peroxide is continuously involved in the process of germination (Ogawa and Iwabuchi, 2001). Although accumulation of high intracellular concentrations of hydrogen peroxide were detrimental in axes of tropical recalcitrant seeds stored at 3°C in the present study, it may be worth investigating the application of exogenously applied hydrogen peroxide on germination of recalcitrant seeds during hydrated storage.

Mittler (2016) stated that low levels of ROS are cytostatic and may not be sufficient for signalling and control of cellular processes, whilst very high levels are cytotoxic. The optimum range exists where ROS levels are strictly controlled by the antioxidant networks and cellular responses are regulated. Once basal levels are exceeded, there is a period in which increased ROS will be tolerated, but thereafter, oxidative stress ensues. In axes of *C. sinensis* seeds stored at 16°C, TAA activity fluctuated whilst TAA activity in axes from seeds stored at 3°C was initially high, thereafter decreased and remained constant, as did viability (Figures 3.6.3.1a and 3.6.3.2a). In axes of *Q. robur* seeds stored at 16 and 3°C, antioxidant values were the highest of the species investigated, and in particular, appeared to follow the same pattern in viability loss when stored at 3°C (Figures 3.6.3.1b and 3.6.3.2b). Ntuli *et al.* (2011) found that embryonic axes stored hydrated at 20°C exhibited decreased production of enzymatic antioxidants such as SOD, CAT and GR after 30 d. Furthermore, as storage time increased, the level of hydroperoxides decreased following viability loss, possibly due to the breakdown of unstable free radicals in dead tissue. In the present study, this may also occur in axes of tropical recalcitrant-seeded species upon viability loss at 3°C, since antioxidant activity was impaired as storage time increased (Figures 3.6.3.2 c, d). At 16°C, there was a significant increase in antioxidant activity over time for both tropical recalcitrant-seeded species, and an increase in antioxidant activity was negatively related to viability in *A. heterophyllus* axes (Figures 3.6.3.1c, d). Pukacka and Ratajczak (2005) found that *F. sylvatica* seeds stored at 20°C under 75% RH for 9 weeks was correlated with

increased ROS levels and despite the increase in ascorbate content, and antioxidant enzymes such as APX, DHAR, GR, SOD and CAT loss of seed viability ensued.

The relationship between superoxide, hydrogen peroxide and TAA activity did not appear to be consistently significant between species and temperature. At 16°C, all species exhibited a weak positive correlation between superoxide and TAA activity (Figure 3.7.1.1), whilst at 3°C the relationships were negative (Figure 3.7.1.2). Axes of *Q. robur* and *A. heterophyllus* exhibited a significant increase in superoxide congruent with a decline in TAA activity when seeds were stored at 3°C (Figures 3.7.1.2 b, d). Hydrogen peroxide and TAA were significantly related, and an increase in hydrogen peroxide associated with a decline in TAA, particularly after four months in storage when viability began to decline (Figure 3.7.2.1a). All other species did not exhibit significant relationships, although *A. heterophyllus* was the only species that exhibited a positive relationship between hydrogen peroxide and TAA. At 3°C, all species exhibited a weak negative correlation between hydrogen peroxide and TAA activity (Figure 3.7.2.2). Axes of *A. heterophyllus* seeds appeared to be most sensitive to redox changes throughout storage, and did not germinate when stored at 16°C, whilst axes of *T. dregeana* showed very little fluctuation in TAA activity despite the variations in ROS production. This may indicate ongoing developmental processes, which require high levels of ROS to facilitate germinative metabolism (Kranner *et al.*, 2010; Müller *et al.*, 2009; Shaban *et al.*, 2013). Interestingly, ROS levels displayed a similar bell-shaped curve irrespective of storage temperature in axes of *T. dregeana* seeds, further supporting the notion of the dual role of ROS, where high concentrations may be implicated in germinative processes at 16°C as well as chilling stress at 3°C (Figures 3.7.1.1c, 3.7.1.2c, 3.7.2.1c, 3.7.2.2c).

The origin and sequence of events following the production of ROS may be related to increased storage time at lethal temperatures, proliferation of storage fungi (Goodman, 1994), slow uptake of water in storage, accumulation and further propagation of ROS signals which interact both with each other and an antioxidant network during seed deterioration or after the cells have died (Finch-Savage *et al.*, 1996; Sharma *et al.*, 2012).

4.5   Constraints on hydrated storage

Germination in hydrated storage results in the end of useful storage periods. Excluding *A. heterophyllus* seeds, seeds of each species germinated in storage (Table 3.3). Fewer *C. sinensis* seeds germinated at 3 and 16°C after an extended time in storage, whilst

seeds of *T. dregeana* and *Q. robur* germinated in a much shorter time (Table 3.3). Germination was associated with an increase in TAA activity, but was more elevated in axes of *Q. robur* than *T. dregeana* (Figure 3.8.1). Germination involves many processes to physically accommodate growth such as cell expansion, breakdown of storage products and mobilisation of nutrient reserves (Bewley and Black, 1994; Pammenter and Berjak, 1999). This process is tightly regulated and requires ROS and antioxidant systems to be strictly controlled. Pammenter and Berjak (1999) suggested that the higher the metabolic activity of a recalcitrant seed when freshly shed, the less desiccation tolerant it will be and the shorter the storage lifespan. Some recalcitrant seeds are shed in autumn and over winter and thus are more chilling tolerant and have lower respiratory and germinative rates (Pammenter and Berjak, 2000b). Although this was the case for *Q. robur* seeds in this study, upon uptake of water in hydrated storage, these seeds began germinating relatively quickly. In a study by Pasquini *et al.* (2012), *Quercus ilex* seeds stored in peat or moistened sand exhibited reduced vigour after 12 months stored at 3°C compared with seeds stored in polyethylene bags. Furthermore, Noland *et al.* (2013) found that *Q. rubra* acorns stored in polyethylene bags at -1°C for 42 months retained 80% germination, whilst those stored at +2°C decreased to 40%. Acorns stored at -2°C, did not germinate in storage and was the preferred long-term storage temperature, maintaining high viability after 18 months. It can be concluded that hydrated storage may not be a suitable method to store *Q. robur* acorns since high humidity and storage above freezing temperatures favours germination. *Q. robur* seeds were successfully cold-stored for up to 2-3 years (Chin and Roberts, 1980), however, this is dependent on the initial quality at harvest and if seeds harbour any active, systemic fungi.

Low temperature storage also reduces proliferation of microorganisms in storage (Berjak, 1996; Berjak *et al.*, 2004b; Calistru *et al.*, 2000; Mycock and Berjak, 1990). Some recalcitrant seeds are shed containing fungal inoculum which, upon proliferation, accelerates the post-harvest deterioration in hydrated storage. In the present study, a wide range of antibacterial and antifungal cocktails were used to surface sterilise whole seeds, as well as the application of an antifungal powder prior to storage to prevent contamination. However, contamination reduced the storage longevity of temperate recalcitrant-seeded species, particularly since the testa was not removed and fungal development was inherent. Most surface applications of fungicides do not inhibit the activity of mycelium below the pericarp/testa, and are only effective on particular groups of fungal species, therefore it may be necessary to investigate the efficacy of systemic fungicides on storage fungi at non-lethal concentrations to prevent damage to the seeds (Berjak *et al.*, 2004b). Although elimination of

fungal species may increase storage longevity, due to the ongoing germinative metabolism and the absence of additional water, seeds will ultimately lose viability in storage (Berjak *et al.*, 2004b; Pammenter *et al.*, 1994).

In vitro viability was also compromised by both fungal and bacterial growth. Although radicle extension was easily determined since most species develop roots first, some cultures were overridden with fungus prior to shoot development despite proper sterilisation and maintenance of tissue cultures. Processing axes for tissue culture may pose an additional accumulative stress on embryonic axes (Benson, 2000). Excision injury (Roach *et al.*, 2008), rehydration and imbibitional damage (Hong and Ellis, 1996; Perán *et al.*, 2004), sterilisation (Berjak *et al.*, 2004b) and osmotic stress (Gaspar *et al.*, 2002) can collectively act to bring about a reduction in viability that may not be attributed to storage. Although many studies assess germination in whole seeds, and this may have been the preferred method, the analyses were performed on embryonic axes only, negating the effects of breakdown products from cotyledons which may have interfered with the results.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER RESEARCH

It is imperative that good quality, mature seeds are used for any type of seed science research. Often, immature seeds may be classified as recalcitrant, therefore harvest timing and developmental status are key factors in both desiccation tolerance and storage longevity (Hong and Ellis, 1996). Response to desiccation appeared to be species-specific and not related to provenance. This may be explained by the morphology of the embryonic axis, being of different sizes, the axis itself composed of heterogeneous tissue, as well as cotyledonary vestiges attached. These may collectively affect drying rate, viability as well as alter redox state. Drying rate was fastest in *A. heterophyllus* axes, followed by *C. sinensis* axes, *T. dregeana* and *Q. robur* axes. Cotyledonary pieces may have acted as an additional water source for axes to draw on upon dehydration, and may explain the longer drying times required by the latter two species. As water content decreased, viability decreased, but was dependent on the rate of drying more than the actual extent of dehydration. For example, *C. sinensis* axes were the only species capable of surviving low water contents, and maintained high shoot production throughout drying whilst *A. heterophyllus* axes maintained high viability when dried to 0.3 g g^{-1} DW in 15 min. ROS production remained comparable for each species at different water contents, whilst superoxide was produced in higher quantities in *T. dregeana*, hydrogen peroxide production was significantly higher in *C. sinensis* axes. The TAA activity was markedly higher in temperate recalcitrant-seeded species, and continuously increased despite drying to very low water contents when compared with tropical recalcitrant-seeded species at the same water content. High levels of free radicals have been detected in desiccation-sensitive seeds/axes upon dehydration (Hendry *et al.*, 1992; Leprince *et al.*, 1999; Pukacka *et al.*, 2011; Roach *et al.*, 2010; Varghese *et al.*, 2011; Whitaker *et al.*, 2010). The origin and sequence of the events following the appearance of these toxic compounds appears to be related directly to water-stressed cells (Leprince *et al.*, 1999). Improving drying rates via vacuum flash-drying may assist in decreasing desiccation-induced damage.

Since ROS reacts with various biomolecules such as proteins, lipids and nucleic acids, damage incurred by elevated concentrations may not be isolated at the primary site, but trigger a cascade of events at other locations resulting in loss of viability (Bailey-Serres and Mittler, 2006; Mittler, 2016; Mittler *et al.*, 2011; Noctor and Foyer, 2016). In tropical recalcitrant seeds stored at 3°C, chilling-induced injuries may have occurred due to the

accumulation of ROS, and not necessarily sustained heightened levels. Storage at 3°C was the optimum temperature for seeds of *C. sinensis*, which exhibited a slight reduction in viability over 8 months, whilst the temperate counterpart, *Q. robur* did not store well hydrated at both 3 and 16°C. Non-hydrated storage in polyethylene bags may represent a better storage method for these seeds. Storage at 16°C resulted in germination of *T. dregeana* seeds in storage, whilst *A. heterophyllus* seeds did not show any signs of germination. ROS production may be linked to ongoing development and germinative metabolism in axes of *T. dregeana* seeds, whilst *A. heterophyllus* axes may be in a state of oxidative stress, seen by the significant accumulation of superoxide, hydrogen peroxide and TAA activity as storage time increased. It is possible that storage at 16°C may elicit a mild chilling response, in *A. heterophyllus* seeds, since a reduction in metabolic rates may explain why seeds did not germinate in storage. However, viability was retained above 50% throughout the 15-month storage period. Seed storage at 3°C resulted in complete loss of viability. Since each recalcitrant-seeded species exhibits its own threshold before desiccation or chilling injury is incurred, storage temperature should be optimised and individually tested (Berjak and Pammenter, 1994). Viability of tropical recalcitrant-seeded species stored hydrated at 16°C gradually declined, whilst at 3°C viability was completely lost. Seeds of *Q. robur* temperate origin appear to be more tolerant of storage at 3°C, however, hydrated storage may not represent a suitable method for *Q. robur* seeds. Seeds of *C. sinensis* stored well at both temperatures for up to 8 months, however, there were more fluctuations in ROS levels when stored at 16°C than at 3°C.

Fluctuations in ROS production is often matched by antioxidant activity in storage for species stored at 16°C, whilst at 3°C there was a reduction in antioxidant activity congruent with a loss of viability for *Q. robur*, *T. dregeana* and *A. heterophyllus*. Recalcitrant seeds from tropical regions may be stored hydrated for 4-6 months at 16°C without loss of viability, whilst seeds from temperate regions may be stored for much longer at 3°C. This is the first study examining ROS and TAA activity in hydrated storage of *C. sinensis* and *A. heterophyllus* seeds, whilst other studies have researched storability of *Q. robur* (Noland et al., 2013) and *T. dregeana* (Moothoo-Padayachie *et al.*, 2016) seeds, the storage time was reduced or storage may not have under high relative humidity conditions. Recalcitrant seeds germinate in storage which necessitates high respiratory rates, therefore ROS levels will vary depending on the stage of germination and the duration of storage. Whilst germination is viewed as a positive outcome in many studies, this result effectively curtails useful storage periods. Oxidative stress alone may not be responsible for loss of viability, but may be the

principle cause of secondary deleterious reactions. Also, the effects of fungal proliferation may further reduce storage longevity.

Future studies pertaining to both desiccation and chilling sensitivity in seeds from tropical and temperate origin should investigate specific enzymes such as SOD, CAT, peroxidases and the ascorbate-glutathione cycle to determine the specificity of damage to enzymes and antioxidants. Furthermore, investigations into membrane systems could also be undertaken since ROS target lipid membranes and peroxidation results in a decrease in the fluidity of membranes which interferes with the selective permeability, measuring electrolyte leakage may indicate the degree of damage to membranes (Bryant *et al.*, 2001; Colville *et al.*, 2012). *In situ* visualisation via ROS-sensitive exogenous fluorescence probes to detect superoxide anions and hydrogen peroxide (Ratajczak *et al.*, 2015), as well as electron paramagnetic resonance (EPR) spectroscopy of superoxide and hydrogen peroxide may assist in determining the sites of ROS accumulation, particularly in short-lived species (Davies, 2002; Schmitt *et al.*, 2014).

Since recalcitrant seeds are shed metabolically active with high respiratory rates, reducing respiratory activity with nitrous oxide (an anaesthetic which reduces respiratory rates in seeds) may increase seed longevity in storage (Edwards and Mumford, 1985). Bhargava *et al.* (1999) used halogen and chlorine vapour treatments to enhance the storability of *Shorea robusta* seeds and to decrease their sensitivity to lower storage temperatures. The application of a specially-prepared alginate gel extracted from the seaweed, *Ecklonia maxima,* has been shown to increase storage longevity of *A. marina* seeds by reducing germinative metabolism and fungal proliferation in storage (Motete *et al.*, 1997; Pammenter *et al.*, 1997). Furthermore, Wahid *et al.* (2007) showed that wheat seeds pre-treated with hydrogen peroxide improved salt tolerance in seedlings by reducing oxidative damage. These methods may be used prior to, or during hydrated storage to reduce oxidative stress and increase storage lifespan, however, long-term storage of recalcitrant material may only be achieved via cryopreservation techniques, since hydrated storage is strictly an interim measure for research purposes and seeds become increasingly desiccation sensitive or germinate in storage.

Prasad *et al.* (1994) states that chilling reduces the respiratory activity, impairs the cytochrome pathway of the electron transport chain and decreases ATPase activity. These authors state that acclimation tolerance may induce a greater tolerance towards higher levels of the same stress. Based on this, it may be useful to undertake a study to determine if low levels of chilling stress (~1 month for tropical recalcitrant-seeded species) may elicit greater

tolerance to cryogen exposure. This type of priming may not induce a full range of cellular responses, but may reduce respiratory activity sufficiently whilst maintaining high antioxidant potential. The main reason behind failure of shoot production in *T. dregeana* axes is due to the superoxide burst upon excision, when cotyledonary pieces are attached, shoot production occurs, however, exudation of inhibitory substances from the cotyledonary pieces reduces root growth (Goveia *et al.*, 2004). These authors suggest that allowing the shoot apex to elongate sufficiently in hydrated storage may negate the need to excise embryonic axes with cotyledonary pieces. Based on the results from this study, both extracellular superoxide and hydrogen peroxide displayed a bell-shaped curve over the first seven months of storage. Cryopreservation of *T. dregeana* axes may be attempted after four months in storage, when viability is at 80%, ROS levels are high and may assist in signalling processes for germination, or at seven months when ROS levels are low and antioxidant levels are slightly elevated which may reduce the chances of accumulative oxidative stress, although viability was reduced to 60% and axes may be more desiccation sensitive. In cases where embryonic axes cannot be successfully cryostored, alternative explants such as shoot meristems or apices, axillary buds, somatic embryos or embryogenic callus may be utilised, although optimising and establishing *in vitro* protocols for normal plant development is a necessary prerequisite (Berjak *et al.*, 2004b).

Seed storage is an important, often overlooked aspect in recalcitrant seed biology. Proper categorisation of seed storage behaviour, optimisation of storage conditions (including temperature), correct harvest-timing of seeds and control of microbial contaminants may prolong storage longevity. ROS are present and produced in many reactions in aerobic organisms and function as potent signalling molecules for both germinative and deteriorative processes in seeds. Oxidative stress caused by heightened endogenous ROS levels may cause irreversible damage to cells, culminating in seed death, or may be implicated in developmental processes leading to germination in stored hydrated seeds. In both cases, the presence and operation of an effective antioxidant network to control ROS levels and their secondary reactions is imperative in maintaining redox homeostasis. The results of this study support the notion that axes of temperate recalcitrant-seeded *C. sinensis* are more desiccation and chilling tolerant than tropical recalcitrant-seeded species. Whilst short-term non-hydrated storage of *Q. robur* seeds was favourable; seeds of both species of tropical origin stored better under hydrated conditions. Cryopreservation represents the only long-term solution for recalcitrant germplasm conservation, however, the possible cumulative effects of oxidative

metabolism during hydrated storage, prior to cryogen exposure may contribute to reduced cryo-survival.

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APPENDIX A: CONFIRMATORY ASSAYS

Table 1: Confirmatory assays performed to test the effect of superoxide dismutase (SOD) on extracellular $O_2^{\bullet-}$ production (nmol g^{-1} DW s^{-1}) and catalase (CAT) on extracellular H_2O_2 production (μmol g^{-1} DW s^{-1}) in desiccated embryonic axes of *C. sinensis, Q. robur*, *T. dregeana* and *A. heterophyllus*. Data represents the average ($\pm$ SD) of 3 replicates (5 axes each) for each species.

	Rapid dehydration			
	C. sinensis	*Q. robur*	*T. dregeana*	*A. heterophyllus*
Drying time	60 min	240 min	180 min	35 min
Superoxide	6.3 ± 0.4	3.7 ± 0.4	20.6 ± 1.1	5.8 ± 0.2
SOD	4.2 ± 0.8	0.8 ± 0.1	7.4 ± 0.7	2.3 ± 0.1
% decline	**34**	**78**	**64**	**60**
Hydrogen peroxide	0.24 ± 0.04	0.10 ± 0.01	0.12 ± 0.02	0.08 ± 0.01
CAT	0.06 ± 0.02	0.05 ± 0.01	0.02 ± 0.01	0.01 ± 0.01
% decline	**74**	**49**	**86**	**83**

Table 2: Confirmatory assays performed to test the effect of superoxide dismutase (SOD) on extracellular $O_2^{\bullet-}$ production (nmol g^{-1} DW s^{-1}) and catalase (CAT) on extracellular H_2O_2 production (µmol g^{-1} DW s^{-1}) in embryonic axes of *C. sinensis*, *Q. robur*, *T. dregeana* and *A. heterophyllus* stored hydrated. Data represents the average (± SD) of 3 replicates (5 axes each) for each species.

	Hydrated storage								
	C. sinensis		*Q. robur*			*T. dregeana*		*A. heterophyllus*	
Temperature (°C)	3	16	3	3, NH	16	3	16	3	16
Storage time (months)	18	18	11	12	-	-	18	-	15
Superoxide	9.5 ± 1.8	8.6 ± 2.1	1.4 ± 0.3	1.2 ± 0.1	-	-	17.4 ± 1.2	-	3.3 ± 0.4
SOD	4.6 ± 0.5	4.0 ± 0.7	0.6 ± 0.2	0.4 ± 0.1	-	-	8.0 ± 0.5	-	1.1 ± 0.2
% decline	**51**	**53**	**54**	**67**	**-**	**-**	**54**	**-**	**68**
Hydrogen peroxide	0.22 ± 0.04	0.12 ± 0.03	0.03 ± 0.01	0.08 ± 0.01	-	-	0.10 ± 0.03	-	0.04 ± 0.02
CAT	0.06 ± 0.03	0.07 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	-	-	0.01 ± 0.01	-	0.02 ± 0.01
% decline	**71**	**40**	**67**	**96**	**-**	**-**	**86**	**-**	**61**

APPENDIX B: CORRELATION MATRICES

Table 1: Correlation coefficients obtained for various analyses of embryonic axes from seeds of *C. sinensis* when stored hydrated at 16°C for 18 months.

	Time	Superoxide	Hydrogen peroxide	TAA	WC	Survival
Time	1					
Superoxide	0.011	1				
Hydrogen peroxide	0.017	0.0003	1			
TAA	0.001	0.044	-0.117*	1		
WC	-0.028	-0.001	-0.099*	0.132**	1	
Survival	-0.790**	-0.0003	-0.015	0.071	0.067	1

* $p < 0.05$; ** $p < 0.0001$

Table 2: Correlation coefficients obtained for various analyses of embryonic axes from seeds of *C. sinensis* when stored hydrated at 3°C for 8 months.

	Time	Superoxide	Hydrogen peroxide	TAA	WC	Survival
Time	1					
Superoxide	0.017	1				
Hydrogen peroxide	0.114*	0.052	1			
TAA	-0.056*	-0.019	-0.043	1		
WC	-0.125*	0.066	-0.063	-0.081*	1	
Survival	-0.096	-0.102	-0.001	0.146	-0.005	1

* $p < 0.05$; ** $p < 0.0001$

Table 3: Correlation coefficients obtained for various analyses of embryonic axes from seeds of *Q. robur* when stored hydrated at 16°C for 5 months.

	Time	Superoxide	Hydrogen peroxide	TAA	WC	Survival
Time	1					
Superoxide	0.460**	1				
Hydrogen peroxide	-0.019	-0.052	1			
TAA	-0.071*	0.000004	-0.038	1		
WC	0.398**	0.119	0.0003	-0.003	1	
Survival	-0.327*	-0.095	0.205	-0.026	-0.407*	1

$*\ p < 0.05$; $**\ p < 0.0001$

Table 4: Correlation coefficients obtained for various analyses of embryonic axes from seeds of *Q. robur* when stored hydrated at 3°C for 12 months.

	Time	Superoxide	Hydrogen peroxide	TAA	WC	Survival
Time	1					
Superoxide	0.247*	1				
Hydrogen peroxide	0.067	0.052	1			
TAA	-0.278**	-0.125*	-0.029	1		
WC	0.526**	0.154*	0.146*	-0.140**	1	
Survival	-0.501**	-0.118	0.00003	0.131	-0.159	1

$*\ p < 0.05$; $**\ p < 0.0001$

Table 5: Correlation coefficients obtained for various analyses of embryonic axes from seeds of *T. dregeana* when stored hydrated at 16°C for 18 months.

	Time	Superoxide	Hydrogen peroxide	TAA	WC	Survival
Time	1					
Superoxide	0.001	1				
Hydrogen peroxide	0.007	0.065	1			
TAA	0.064*	0.021	-0.005	1		
WC	-0.242*	-0.032	-0.041	-0.05*	1	
Survival	-0.729**	-0.007	-0.00004	-0.002	0.228**	1

* $p < 0.05$; ** $p < 0.0001$

Table 6: Correlation coefficients obtained for various analyses of embryonic axes from seeds of *T. dregeana* when stored hydrated at 3°C for 7 months.

	Time	Superoxide	Hydrogen peroxide	TAA	WC	Survival
Time	1					
Superoxide	0.283*	1				
Hydrogen peroxide	0.147*	0.240*	1			
TAA	-0.331**	-0.089	-0.013	1		
WC	0.171**	0.028	0.080	-0.021	1	
Survival	-0.924**	-0.303*	-0.179*	0.334*	-0.239*	1

* $p < 0.05$; ** $p < 0.0001$

Table 7: Correlation coefficients obtained for various analyses of embryonic axes from seeds of _A. heterophyllus_ when stored hydrated at 16°C for 15 months.

	Time	Superoxide	Hydrogen peroxide	TAA	WC	Survival
Time	1					
Superoxide	0.274**	1				
Hydrogen peroxide	0.209*	0.143*	1			
TAA	0.309**	0.041	0.072	1		
WC	0.267**	0.011	0.153*	0.001	1	
Survival	-0.598**	-0.346**	-0.162*	-0.155*	-0.028	1

* $p < 0.05$; ** $p < 0.0001$

Table 8: Correlation coefficients obtained for various analyses of embryonic axes from seeds of _A. heterophyllus_ when stored hydrated at 3°C for 5 months.

	Time	Superoxide	Hydrogen peroxide	TAA	WC	Survival
Time	1					
Superoxide	-0.033	1				
Hydrogen peroxide	0.065	0.147	1			
TAA	0.019	-0.638**	-0.038	1		
WC	0.010	0.182*	0.031	-0.041	1	
Survival	-0.899**	0.013	-0.144	-0.007	-0.0003	1

* $p < 0.05$; ** $p < 0.0001$