Factors governing seed recalcitrance in two species of contrasting storage longevity

by

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ABSTRACT

Recalcitrant seeds unlike orthodox seeds are desiccation sensitive, chilling sensitive and initiate germination on shedding which precludes their storage using conventional seed storage methods. There are different mechanisms that confer protection against the consequences of water loss at different hydration levels in desiccation tolerant plant material. The present study aimed to characterise some of the desiccation tolerance mechanisms that may be either absent or poorly expressed in recalcitrant seeds, rendering them desiccation sensitive. The study involved a comparative analysis of some of the physiological, biochemical and proteomic changes associated with desiccation, hydrated storage and seed germination in two recalcitrant-seeded species of contrasting storage longevity, *viz. Avicennia. marina* (Forssk.) Vierh (short storage lifespan) and *Trichilia dregeana* Sond. (relatively longer storage lifespan). This thesis presents results on: (1) viability and redox metabolism between *A. marina* and *T. dregeana* seeds under conditions of partial dehydration and hydrated storage using biochemical assays for superoxide ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) determination; high pressure liquid chromatography (HPLC) was also employed to provide quantitative data of glutathione levels (2) the germination capacity, velocity and associated redox metabolism between *A. marina* and *T. dregeana* seeds using the same molecular techniques as described above (3) proteins in terms of their involvement in cellular pathways in *T. dregeana* seeds. This was done by identifying, quantifying, annotating and comparing proteins expressed in *T. dregeana* seeds exposed to partial dehydration and hydrated storage using iTRAQ (isobaric tags for relative and absolute quantification) in conjunction with liquid chromatography-tandem mass spectrometry on a Q-Exactive quadrupole-Orbitrap mass spectrometer, and (4) the proteomic responses of *A. marina* and *T. dregeana* seeds exposed to partial dehydration and hydrated storage via nLC-MS/MS (nano-scale liquid chromatographic tandem mass spectrometry) on a Thermo Scientific Fusion mass spectrometer equipped with a Nanospray Flex ionization source to obtain protein profiles of these seeds immediately after harvest, partial drying and hydrated storage using a label-free LC-MS/MS method. The research objectives for this study were derived from previous research which suggested that the hydrated storage lifespan of recalcitrant seeds is contingent on the rate at which these seeds germinate naturally as well as studies that have proposed that a decline in reactive oxygen species (ROS) production (referred to as a 'dampening of the biochemical trigger for germination') may be responsible for the reduced germinability in partially dehydrated recalcitrant seeds.

One of the major challenges in this work, in relation to comparing physiological and biochemical parameters between the two contrasting species investigated, was the fact that unlike orthodox seeds, recalcitrant seeds do not have a clear identifiable switch from developmental to germinative metabolism. Proteomic analysis of the recalcitrant embryonic axes investigated also presented challenges in terms of total protein extraction, peptide labelling and trypsin digestion due to the presence of interfering compounds which required considerable optimisation.

The principal findings of this research were:

- Increased ROS ($\cdot O_2^-$ and H_2O_2) production is necessary for the completion of germination in both species and differences in the timing of a ROS-based trigger for germination may account for differences in both germination velocity and storage longevity between *A. marina* and *T. dregeana*.
- In slower germinating *T. dregeana* seeds, GSH levels far exceeded those of GSSG levels at harvest and throughout the period leading up to germination which strongly suggested that sustained antioxidant protection present in *T. dregeana* seeds delayed germination-associated metabolism i.e. suppression of the ROS based trigger for germination provides reasons for its relatively longer storage longevity compared with *A. marina* seeds.
- *A. marina* seeds lost all viability after 40 days in hydrated storage while *T. dregeana* seeds could be stored for 12 months and showed 100% viability when seeds that had not germinated in storage were assessed.
- The ROS-based trigger for germination took place early during storage in *A. marina* seeds but appeared to be delayed in those of *T. dregeana* which may account for the longer storage longevity of the latter. These data suggest that the mechanisms of desiccation-induced seed viability loss may differ across recalcitrant-seeded species based on the rate and extent to which they lose water during partial drying and storage.
- Partial dehydration studies revealed that ROS play a deleterious role in *A. marina* embryonic axes as a spike in ROS production ($\cdot O_2^-$ and H_2O_2) coincided with 50% viability loss. Contrastingly, in partially dehydrated *T. dregeana* axes ROS production decreased with prolonged dehydration which supports previous findings that viability loss in this species is accompanied by a dampening of the ROS-based trigger for germination.
- A comparison of the protein profiles of embryonic axes of *A. marina* and *T. dregeana* seeds immediately after harvest, partial drying and hydrated storage revealed proteomic changes during storage and drying in both species. Proteins linked to key metabolic functions (e.g. cellular redox balance and cell energy demands) were compromised in both species after drying

and storage (which is in effect a mild dehydration stress), which may explain the stress and/or viability loss exhibited by the seeds of both species during these processes.

- A comparison of the proteomic changes during storage and drying in both species further support suggestions that higher metabolic activity and faster germinative development in *A. marina* relative to *T. dregeana* seeds, are responsible for the reduced seed storage lifespan of the former.
- The relatively higher abundance of proteins found in *T. dregeana* embryonic axes compared with *A.marina* embryonic axes such as superoxide dismutase (SOD), adenosylhomocysteinase and calmodulin proteins in *T. dregeana* embryonic axes may also account for why *T. dregeana* seeds are less desiccation sensitive relative to *A. marina* seeds.

Collectively, the results suggest that the syndrome of traits that render recalcitrant seeds sensitive to desiccation and unamenable to conventional storage methods appear to be largely common across species, irrespective of their storage longevity. However, the comparisons made here suggest that inter-species differences in storage longevity may be based on differential responses to water loss (during physical dehydration and storage) in terms of ROS production, ROS scavenging capacity and the expression of proteins related to key metabolic functions. These findings should be used to inform the design of short-, medium- and long- term seed germplasm conservation protocols for recalcitrant-seeded species.

PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Biochemistry and Plant Ecophysiology, 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 the NRF-DAAD.

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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DECLARATION 2: PUBLICATIONS

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

Chapter 3

1. Moothoo-Padayachie, A., Varghese, B., Pammenter, N. W., Govender, P. & Sershen* 2016. Germination associated ROS production and glutathione redox capacity in two recalcitrant-seeded species differing in seed longevity. *Botany*, 94**,** 1103-1114.

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Signed: Anushka Moothoo-Padayachie

Date: 10 January 2017

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APX	Ascorbate peroxidase
BGCI	Botanic Gardens Conservation International
CAT	Catalase
DW	Dry weight
DHAR	Dehydroascorbate reductase
FAO	Food and Agriculture Organization of the United Nations
GSSG	oxidised glutathione
GSH	reduced glutathione
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
HPLC	High pressure liquid chromatography
iTRAQ	Isobaric tags for relative and absolute quantitation
MDAR	Monodehydroascorbate reductase
nLC-MS/MS	Nano-scale liquid chromatographic tandem mass spectrometry
$\cdot O_2^-$	Superoxide
SOD	Superoxide dismutase

CHAPTER 1: INTRODUCTION

1.1 Biodiversity conservation

The unprecedented rate of extinction of biodiversity is currently a global crisis and one of the major challenges facing humanity. Biological diversity or biodiversity as defined by the International Convention on Biological Diversity (2003), refers to the variability among living organisms from all sources including, *inter alia*, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part of; this includes diversity within species, between species, and of ecosystems. Over the last four decades there has been a global decline in biodiversity, accompanied by notable declines in populations of vertebrates, habitat specialist birds, the extent of forest, mangrove and sea grass beds, and the condition of coral reefs (Butchart *et al.*, 2010). There are many threats to biodiversity including climate change (Walther *et al.*, 2002), the size and distribution pattern of human populations (McKee *et al.*, 2004), increased domestic animal populations, increased levels of resource consumption, land degradation and deforestation (Groom, 2006). Agricultural expansion, urbanization and grazing are regarded as the greatest contemporary threats to terrestrial species worldwide (Bellard *et al.*, 2014). In addition, through ever increasing local and global trade, invasive alien species have been introduced into most ecosystems across the world, to the severe detriment of ecological networks, biodiversity and ecosystem functioning (Bellard *et al.*, 2014).

Conservationists are unable to prioritise all species under threat, mainly due to limited funding (James *et al.*, 1999, Waldron *et al.*, 2013). Therefore conservationists have developed a promising approach that involves the identification of "hotspots", or areas featuring exceptional concentrations of endemic species and experiencing exceptional loss of habitat (Myers *et al.*, 2000). According to the criteria developed by Myers (2000), 34 biodiversity hotspots have been recognized around the world (Bellard *et al.*, 2014), which together hold 50% of the world's plant species and 42% of all terrestrial vertebrates. South Africa, the location for the present study, contains three of the 34 hotspots namely the Cape Floristic region, Maputaland Pondoland Albany and Succulent Karoo (Bellard *et al.*, 2014). However, many hotspots are severely threatened by climate change, land-use change, and biological invasions (Bellard *et al.*, 2014). It is estimated that up to 100000 plants, representing more than one third of all the world's species, are currently threatened or face extinction in the wild (Panis and Lambardi, 2006). The value of conserving plant species has never before been more paramount and this has been increasingly acknowledged by international organisations, in treaties and legislation, notably, the Convention on Biological Diversity (Balmford *et al.*, 2005), the Global Strategy for Plant Conservation (Jackson and Kennedy, 2009), the Convention on the Conservation of European Wildlife and Natural

Habitats (the Bern Convention) (Genovesi and Shine, 2004), and the Gran Canaria Declaration on Climate Change and Plant Conservation (BGCI, 2000, 2006).

Plant biodiversity not only facilitates ecosystem functioning but is extremely important for food and agriculture and forms the basis of global food security (Walters *et al.*, 2013, FAO, 2014). In the 1950's and 1960's the "Green revolution" brought about major advances in plant breeding which led to the development of high-yielding crop varieties but such breakthroughs were not without ecological consequences. Many of these high-yielding varieties are genetically uniform. The erosion of plant genetic resources reduces the adaptive abilities of plant species, making many of them vulnerable to disease (Engelmann and Engels, 2002). The need to conserve plant biodiversity is also motivated by the fact that 75%-80% of the world population, rely on herbal medicine as a mainstay, especially in developing countries (Pal and Shukla, 2003). It is estimated that 60% of anti-tumour and anti-infectious drugs already on the market or under clinical trial are of natural origin. The vast majority of these cannot yet be synthesized economically and are still obtained from wild or cultivated plants (Rates, 2001).

Conservation of plant genetic diversity is therefore critical to safeguard the present and future wellbeing of humankind (Noor *et al.*, 2011). The conservation of plant biodiversity can be achieved using two basic strategies, viz. *in situ* and *ex situ* conservation, each composed of various techniques. *In situ* conservation refers to the conservation of ecosystems and natural habitats and the maintenance and recovery of viable species populations in the area in which they developed their unique properties (Engelmann and Engels, 2002). *Ex situ* conservation in contrast, refers to the conservation of components of biological diversity outside their natural habitat. Seed storage is one of the most widespread and valuable approaches to the *ex situ* conservation of plant germplasm as it is both practical and economical (Phartyal *et al.*, 2002, Panis and Lambardi, 2006, Rajasekharan, 2015). The conservation of seeds *in situ*, in particular, has many advantages as it allows for the preservation of the species in its natural habitat and allows for natural selection to take place which cannot be recreated *ex situ*. However, factors such as habitat destruction may cause endangered species to become extinct, warranting the conservation of such species *ex situ*. *Ex situ* plant germplasm conservation affords an opportunity to study the biology of an endangered species and uncover the threats to its survival which can inform recovery programmes for its' restoration and even re-introduction (Engelmann and Engels, 2002, Rao, 2004). Therefore, seed storage is one of the most widespread and valuable approaches to the *ex situ* conservation of plant germplasm as it is both practical and economical (Phartyal *et al.*, 2002, Panis and Lambardi, 2006, Rajasekharan, 2015).

1.2 Rationale and motivation for this study

As mentioned above, one of the most effective and widely practiced method of *ex situ* conservation of plant germplasm is in the form of seed storage (Phartyal *et al.*, 2002, Panis and Lambardi, 2006, Rajasekharan, 2015). There are currently more than 1750 seed banks established worldwide for the *ex situ* conservation of plant biodiversity (Hay and Probert, 2013). Seed banks play a pivotal role in conservation by providing continued availability of genetic resources for crop improvement research, food security, breeding and in the case of wild species reintroduction and habitat restoration (Hay and Probert, 2013). Since the adoption of the Global Strategy for Plant Conservation (GSPC) in 2002, many thousands of seeds from wild species have been placed into long-term storage. However, seed banks are not without limitations as the successful long-term preservation of the seeds of any particular species depends on its post-harvest physiology, which is species-specific (Berjak and Pammenter, 2013). This variability in seed storage longevity is based on the fact that seeds can be desiccation tolerant (orthodox) or sensitive (recalcitrant) (Roberts, 1973).

Orthodox seeds acquire desiccation tolerance during development and thereafter enter a phase of maturation drying, characterized by metabolic shutdown before shedding (Vertucci and Farrant, 1995). Typically orthodox seeds are stored in seed banks in a dry state with low relative humidity, around 15%, and at sub-zero temperatures, generally around −18°C (FAO, 2014). In this dry state, metabolic events associated with germination are not triggered and seeds will germinate only upon imbibition, provided that environmental conditions are favourable (Bewley and Black, 1994). Therefore, orthodox seeds can be stored successfully for long periods of time while still retaining high vigour and viability, throughout the storage period (Pammenter *et al.*, 2000). Contrary to this, a large proportion of species from tropical and sub-tropical regions produce highly hydrated seeds that are intolerant to desiccation and often sensitive to low temperatures, which effectively precludes their storage for any useful period of time (Roberts, 1973, Chin and Roberts, 1980, Farrant *et al.*, 1993c, Pammenter *et al.*, 2000). These seeds are labelled ‘recalcitrant’ (Roberts, 1973). Recalcitrant seeds can only be stored for a short to medium period of time (days to months) under hydrated storage conditions that is at high relative humidity and slightly reduced temperatures that do not permit water loss (Pammenter and Berjak, 2014).

The categories orthodox and recalcitrant (Roberts, 1973) have been suggested to account for only those species that display the extremes of post-harvest behaviour: the ability or inability to tolerate desiccation (Farrant *et al.*, 1993c, Finch-Savage *et al.*, 1994, Berjak and Pammenter, 2013). This may explain why these categories were later augmented by a third seed category, described as being ‘intermediate’ between the extremes of recalcitrant and orthodox behaviour. Seeds so categorised are relatively desiccation tolerant (but not to the extent of orthodox seeds) and may be chilling sensitive in the dry

state, particularly if they are of tropical origin (Ellis *et al.*, 1990, Hong and Ellis, 1996). This wide range in post-harvest responses suggests an open-endedness to the three categories, such that post-harvest physiology may be considered as constituting an extended continuum of seed behaviour, which grades from extreme desiccation-sensitivity, through a range of responses, to seeds capable of extreme desiccation tolerance (Pammenter *et al.*, 1993, Finch-Savage *et al.*, 1994, Berjak and Pammenter, 2008)

The phenomenon of desiccation tolerance in orthodox seeds is acquired before or during maturation drying and this ability is believed to be dependent on the operation of a suite of interactive protective mechanisms (Berjak and Pammenter, 2008). In a review by Pammenter *et al*. (1999) these mechanisms were suggested to include the following: the ability to retain physical integrity on removal of water; intracellular dedifferentiation as suggested by Farrant *et al*. (1997); a decrease in respiratory rates with development resulting in metabolic shutdown (Farrant *et al.*, 1997); the balance between pro- and antioxidants, i.e. the activity of antioxidants to control the levels of reactive oxygen species (ROS) produced during drying, the ability to synthesise intracellular glasses as well as the presence of important protective molecules such as late embryogenic abundant proteins, sucrose and certain oligosaccharides.

The absence or poor expression of many of these protective mechanisms found in desiccation tolerant seeds may be the underlying reasons for desiccation sensitivity and hence the reduced storage lifespan of recalcitrant seeds (Pammenter and Berjak, 1999). For example, studies on desiccation sensitive seeds have revealed that oxidative stress is one of the major causes of viability loss in seeds exposed to drying (Pammenter and Berjak, 1999, Pukacka *et al.*, 2011). It has been suggested that directly after seed shedding ROS production in recalcitrant seeds is still controlled but that during dehydration metabolism becomes unbalanced and ROS production becomes uncontrolled, resulting in the inability of protective antioxidants to remove/quench ROS effectively (Leprince *et al.*, 1999). High concentrations of activated forms of oxygen particularly superoxide ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) (Berjak and Pammenter, 2008, Roach *et al.*, 2008) have been reported to be toxic and to lead to cellular death in desiccation sensitive seeds (Halliwell, 2006). However, it has also been suggested that ROS play a dual role in seeds (Bailly, 2004, Bailly *et al.*, 2008, Roach *et al.*, 2010). Interestingly, Varghese *et al*., (2011) found that during dehydration of *Trichilia dregeana* Sond. axes, $\cdot O_2^-$ production levels decreased and suggested that a decline in ROS levels during the later stages of dehydration could have resulted in poor germination due to the "dampening of this germination trigger". Roach *et al*. (2008), have also suggested that reduced ROS levels in dehydrated axes may be as a result of the inactivation of redox enzymes involved in the production of ROS. The specific mechanisms underlying desiccation sensitivity have yet to be fully elucidated though. A further challenge to understanding desiccation sensitivity in recalcitrant seeds is the fact that there is considerable variability among recalcitrant-seeded species

particularly in terms of the amount of water loss they can tolerate as well as the rate at which drying occurs (Ballesteros *et al.*, 2014) .

Due to their desiccation sensitivity recalcitrant seeds need to be stored at or close to their shedding water content; even a mild dehydration can adversely affect their viability (Eggers *et al.*, 2007). Therefore, recalcitrant seeds are stored in the short-to-medium term under hydrated storage conditions, which as described above involves maintaining seeds under saturated relative humidity conditions (Berjak and Pammenter, 2004, FAO, 2013). Recalcitrant seeds of all species will, however, eventually germinate or die in hydrated storage but the rate at which this occurs differs across species based on a combination of factors, e.g. storage conditions, maturity at collection, metabolic rates and developmental biology (Berjak and Pammenter, 2008, 2013). Ultrastructural studies have shown that during hydrated storage the embryonic axes of recalcitrant seeds undergo germination-associated changes such as extensive vacuolation, increase in cell size, and development of mitochondria which are changes that are very similar to those that occur in germinating orthodox seeds (Pammenter *et al.*, 1984, Farrant *et al.*, 1986b, Berjak and Pammenter, 2000). These findings suggest that if additional water is not supplied, the seeds are vulnerable to water stress and possible viability loss (Farrant *et al.*, 1986b). Recalcitrant-seeded species storage longevity can vary greatly across species; for example, *Avicennia marina* (Forssk.) Vierh. seeds are fully developed when shed and the period of time in which signs of germination can be observed in storage is very short, placing major constraints on its storage lifespan (16-21 d; Farrant *et al.*, 1997, Calistru *et al.*, 2000). In contrast, *T. dregeana*, which are shed relatively immature, display a lag between shedding and visible signs of germination and can be stored for months. Berjak *et al*. (1989) suggested that the hydrated storage lifespan of recalcitrant seeds was dependant on the rate at which seeds germinated naturally, however, the molecular mechanisms underlying recalcitrant seed storage longevity are still unclear.

At present the most promising method to conserve recalcitrant seed germplasm in the long-term is via cryopreservation (Berjak and Pammenter, 2014). Cryopreservation generally entails storage of the germplasm in liquid nitrogen at −196°C or in the vapour phase above liquid nitrogen at −160°C. It is not possible to cryopreserve whole seeds and thus often the embryonic axes/zygotic embryos represent the explants of choice (Berjak and Pammenter, 2014). Nevertheless, there are recalcitrant-seeded species such as *A. marina* that possess embryonic axes that are very large making them unamenable for cryopreservation. In other species, while sufficiently small zygotic explants can be obtained (e.g. *T. dregeana*), these do not survive cryopreservation irrespective of how they are partially dried (Pammenter *et al.*, 2000), which is a prerequisite for successful cryopreservation in all species studied to date.

At present, it is still unclear why orthodox seeds are able to tolerate the removal of considerable amounts of structure-associated water and recalcitrant seeds are unable to do so. Recalcitrant seeds are shed highly hydrated ($\geq$ 1.5 g g^{-1}), and the embryonic axes are damaged even after slight dehydration and especially during slow drying (Berjak and Pammenter, 2008). Therefore, during cryopreservation embryonic axes of recalcitrant seeds are rapidly dried from fully hydrated to 0.3-0.4 g g^{-1} which is somewhere above the level of non-freezable water to prevent ice-crystal formation during cryogenic cooling. Many of the sequential manipulations that have to be followed prior to and after cryostorage also have the potential to compromise post-cryo survival (Varghese *et al.*, 2011, Walters *et al.*, 2013) and there are hence, no generic protocols for the cryopreservation of zygotic germplasm from recalcitrant-seeded species (Berjak and Pammenter, 2014). This may explain why so many authors have recently suggested that the development of successful cryopreservation protocols for many recalcitrant-seeded species demands a more fundamental understanding of the factors governing seed recalcitrance and more broadly, desiccation sensitivity (Noor *et al.*, 2011, Berjak and Pammenter, 2014). This motivated the present study which investigates some of the factors governing seed recalcitrance in two species of contrasting storage longevity. The two species selected for investigation are indigenous to South Africa and have been shown to produce recalcitrant seeds that can be stored hydrated (Sershen *et al.*, 2010, Whitaker *et al.*, 2010); for 16-21 d in the case of *A. marina* (Farrant *et al.*, 1997, Calistru *et al.*, 2000) and several months in the case of *T. dregeana* (Goveia *et al.*, 2004).

1.3 Aims

The broad aim of the study was to characterise some of the physiological, biochemical and proteomic changes associated with seed germination, desiccation and hydrated storage in recalcitrant seeds. For comparative purposes, two species that differ in terms of the hydrated storage longevity of their seeds were selected for investigation: *A. marina* which has short storage lifespan (16-21 d; Farrant *et al*. 1997; Calistru *et al*. 2000), and *T. dregeana* which has relatively long storage lifespan (several months; Goveia *et al*. 2004).

1.4 Objectives

The specific objectives of the study included:

- Comparing the responses of *A. marina* and *T. dregeana* seeds to partial dehydration and storage in terms of viability, ROS production and glutathione redox capacity.
- Comparing germination capacity, velocity and redox metabolism between *A. marina* and *T. dregeana* seeds.

- Characterizing the proteomic response of *T. dregeana* seeds to partial dehydration and hydrated storage using isobaric Tags for Relative and Absolute Quantitation (iTRAQ)
- Comparing seed physiological and proteomic responses of *A. marina* and *T. dregeana* to partial dehydration and hydrated storage using a label-free LC-MS/MS method.

1.5 Outline of dissertation

The remainder of this dissertation is presented as a series of research articles, each containing an Abstract, Introduction, detailed Materials and Methods, Results, Discussion, and Concluding Remarks. These research articles form the four research chapters (Chapters two to five), which are followed by a Concluding chapter (Chapter 6).

In **Chapter 2** responses of *A. marina* and *T. dregeana* seeds to partial dehydration and storage are compared in terms of viability, ROS production and glutathione redox capacity. Germination data are related embryonic axis water content (g g^{-1}), rate of water loss, ROS production and glutathione redox capacity at different partial dehydration and storage intervals. Calorimetric assays and HPLC analysis was used to measure ROS production and total glutathione (GSH+GSSG) levels, respectively. The GSH:GSSG ratio was determined since it is known to be a reliable indicator of oxidative stress (Kranner *et al.*, 2006) .

In **Chapter 3** *A. marina* and *T. dregeana* are compared in terms of their water uptake characteristics, germination velocity and redox metabolism during germination. ROS production and glutathione redox capacity was measured at various points during germinative development. Calorimetric assays and HPLC analysis was used to measure ROS production and total glutathione (GSH+GSSG) levels, respectively. These germination associated data are then related to storage data for both species in order to explain their contrasting storage longevity.

Chapter 4 assesses the proteomic responses of *T. dregeana* seeds to partial dehydration and hydrated storage using iTRAQ. Proteins were identified and quantified and differentially expressed proteins were in turn arranged into metabolic pathways according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database using the Blast2GO tool. These data are then related to storage data for both species in order to explain their contrasting storage longevity.

Chapter 5 compares proteomic responses of *A. marina* and *T. dregeana* seed to partial dehydration and hydrated storage using a label-free LC-MS/MS method. The Blast2GO tool was also employed to arrange proteins identified in both species into metabolic pathways according to the KEGG Pathway Database. Differences in protein expression within treatments, between species were identified. These

data are then related to storage data for both species in order to explain their contrasting storage longevity.

The final chapter, **Chapter 6**, integrates the major findings of the various research chapters to provide the major conclusions of the study and make recommendations for future research on recalcitrant seeds and the phenomenon of desiccation sensitivity in general.

1.6 Study species

1.6.1 Avicennia marina (Forssk.) Vierh

Beachwood Mangroves Nature Reserve, Durban, South Africa was selected for the collection of *A. marina* seeds. The mangrove environment can be described as is a finely balanced intertidal ecosystem. The narrow zone where the land meets the sea is neither strictly terrestrial nor marine. The Beachwood mangroves Nature reserve lies within the littoral zone, which is continuous for thousands of kilometres, but is not uniform (Berjak *et al.*, 2011). Along these fringes of the estuarine banks within the reserve, are inlets where the shores are protected and gently sloping (Figure 1.1). The mangrove environment belongs to the littoral zone, but only to its warmest, most sheltered shores. The mangroves or mangrove trees grow within a tropical climate as they need consistently warm conditions for their development and survival (Berjak *et al.*, 2011). Although temperature is often regarded as the most important factor governing distribution, little is known with regards to specific temperature effects in mangroves in general. Berjak *et al*., (2011), found that there is no correlation between limits of mangrove occurrence and air temperature. It has been further suggested by these authors, that water temperature rather than air temperature may has a greater critical influence on mangroves.

There are two major areas that mangroves occur namely: the Americas-East-Atlantic and Indo-West-Pacific (Berjak *et al.*, 2011). The mangroves found in these contrasting biogeographic regions are characteristically different with each having a unique tree-species composition. Mangroves found along the east coast of Southern Africa are classified within the Indo-West-pacific biogeographic zone. Noticeable differences can be observed in the distribution of mangroves in this region for example, the mangroves found at Kosi Bay, KwaZulu-Natal (lat. 27 ºS) form thick woodlands over vast areas forming one community in which all species of the Southern African region can be found but it must be noted that this is as a result of development of the KwaZulu-Natal coast line which has drastically reduced the size of these mangroves. In comparison, mangroves found along the shores of the Eastern Cape Province (latitudes 31° to 33°S), have reached their limits of distribution and often there are patches of trees which

usually have a single species but there are exceptions such as the mangroves found at Mngazana on the wild coast (Berjak *et al.*, 2011).

Figure 1.1 A mangrove community growing along the estuarine banks at the Beachwood Mangroves Nature reserve (Image captured by Anushka Moothoo-Padayachie)

Figure 1.2 A mangrove community growing along a protected inlet, with *A. marina* trees at the front in the Beachwood Mangroves Nature reserve (Image captured by Anushka Moothoo-Padayachie)

A. marina is generally one of the most dominant trees in mangrove swamps. It is a medium sized tree (Fig. 1.3 b), that grows up to 12 m in height. Its leaves are small (65 × 25 mm), thick and leathery (Berjak *et al.*, 2011). They are simple and broadly lance-shaped. The upper surfaces of the leaves are smooth and olive green; their lower surfaces are matt, hairy, silver green and exude salty droplets in the early mornings (Berjak *et al.*, 2011). *A. marina* tress also contain specially adapted roots which grow upwards from the cable roots and reach a vertical height of 20- 690 mm. These pencil roots called 'pneumatophores' which allows the subterranean portion of the tree to be able to respire (Fig 1.3a). The flowers are inconspicuous visually, but are fragrant. The calyx is five-lobed. The corolla is tubular at the base, four or five lobed and is creamy yellow (Berjak *et al.*, 2011).

Figure 1.3 (a) New seedlings among pneumatophores of *A. marina* trees, (b) *A. marina* trees (Images captured by Anushka Moothoo-Padayachie)

The fruit and seeds are most noticeable from February-April, and are found in clusters of 1,2 or more capsules (Fig 1.4a). The size of each fruit is variable (average 25 mm, long). The fruits are ovoid laterally compressed with a pointed tip and contain a grey-green velvety coat (pericarp). Each fruit only contains a single seed (Fig. 1.4b). The fruit coat is intact until after propagule abscission, moisture (water brought in from tides) is required for the coat to be sloughed, after which germination is initiated and produces a seedling (Fig 1.4 b and c) (Farrant *et al.*, 1993a).

Figure 1.4 (a) *A. marina* seeds are found in clusters, (b) the pericarp sloughs off when the seed is in contact with water, (c) the seed germinated and the seedling very quickly 'rears up' on several roots to establish itself in the mud. (Images (a) and (b) captured by Anushka Moothoo-Padayachie; Image (c) courtesy of Patricia Berjak).

Seeds of *A. marina* contain a large axis (approx. 20 mm × 3mm) with cotyledons that enclose the axis except for a distal portion of the hypocotyl. The protruding hypocotyl tip contains a covering made-up of a thick mass of bristle-like hairs (Fig 1.5) that prevent the tip from being in direct contact with the surrounding environment once the pericarp has been sloughed (Farrant *et al.*, 1993c). The mass of hairs on the hypocotyl tip protect the five (sometimes more) root primordia that are enclosed by a very thin layer of hypocotyl tissue. Under moist conditions the pericarp is sloughed and when germination is initiated roots develop from the primordia.

Figure 1.5 *A. marina* seeds at different stages of germinative development with observable changes occurring at the root primordia. (Image courtesy of Patricia Berjak).

In South Africa, *A. marina* trees are the most abundant and widely distributed mangrove species in the mangrove belt region (Berjak *et al.*, 2011). Traditionally, *A. marina* leaves and seeds have been used in the treatment of ulcers and skin diseases (Bandaranayake, 1998, Kathiresan and Bingham, 2001). Chemical compounds such as steroids, triterpens, saponins, flavonoids, alkaloids, tannins and naphthoquinones have also been reported to be found in the bark, leaves, flowers and fruit of *A. marina* (Itoigawa *et al.*, 2001, Khafagi *et al.*, 2003, Zhu *et al.*, 2009). Numerous studies have also demonstrated the antiviral, antibacterial and antifungal effects of leaves of *A. marina* used to treat diseases such as urinary tract infections caused by bacterial pathogens and herpes simplex virus type 1 (Bandaranayake, 1998, Keivan *et al.*, 2009, Ravikumar *et al.*, 2010, Abeysinghe *et al.*, 2012). *A. marina* leaf extract has also been found to contain bioactive compounds with antimutagenic and antileukemic effects (Karami *et al.*, 2012).

1.6.2 *Trichilia dregeana* Sond.

Seeds of *T. dregeana* were collected directly from trees growing on the grounds of the University of KwaZulu-Natal, Westville campus, Durban, South Africa. *T. dregeana* is a member of the family *Meliaceae* and is a medium-sized to tall tree, that grows up to 30 m in height, with a round wide spreading crown (Pooley, 1993) (Fig. 1.6a). *T. dregeana* is an evergreen tree that has extensive distribution, stretching from Pondoland, KwaZulu-Natal and Mpumalanga in South Africa and northwards into tropical Africa (Pooley, 1997). It is found in regions of high rainfall and the flowers of this evergreen tree are visited by both bees and butterflies, as well as numerous birds that are known to feed on the seeds of the tree (Pooley, 1993). These trees also provide nestling sites for various species of birds (Pooley, 1993).

It has compound leaves and is imparipinnate with 3-5 leaflets and a terminal one. The leaflets are entire, opposite to alternate, glossy and dark green in colour (21 × 8.5 cm). The under surface of the leaves are hairless to slightly hairy, and notably paler than the upper surface. The flowers are inconspicuous, with a creamy-white colour and are sweetly scented. The five petals are velvety on both surfaces and are 14 × 24 mm in length (Pooley, 1993, Allaby, 2012). The fruits are velvety green three-lobed capsules that are 3 cm in diameter. In each capsule are 6 seeds, with a black seed coat and a bright waxy scarlet aril (Fig. 1.6b) (Pooley, 1993). It was observed that seeds of *T. dregeana* contained an axis (3 × 1.5 mm) with cotyledons that completely enclosed the axis (Fig. 1.7).

Figure 1.6 A fruiting *Trichilia dregeana* tree (a) and *T. dregeana* seeds within three lobed fruit (b) (Images captured by Anushka Moothoo-Padayachie)

Figure 1.7 *T. dregeana* seed with two cotyledons and embryonic axis (as indicated by arrow) (Image courtesy of Chandika Ramlall).

In the 19th century the wood from *T. dregeana* trees was used for furniture, household implements and even to repair ships in the Durban harbour in KwaZulu Natal (Pooley, 1993). *Trichilia dregeana* seeds are a potential source of oil (Grundy and Campbell, 1993). Various parts of the tree have also been used traditionally to treat common ailments related to inflammation such as bronchial inflammation, kidney pain, a sore back, fever and rheumatism (Watt and Breyer-Brandwijk, 1932, Hutchings *et al.*, 1996, Van Wyk *et al.*, 1997). Studies have shown that the aqueous leaf extracts of *T. dregeana* have antimicrobial properties (Hutchings *et al.*, 1996). Interestingly, Mulholland *et al*. (1980), isolated limonoids from *T. dregeana* seeds which are known to have antimicrobial and anti-inflammatory activities (Eldeen *et al.*, 2005, Eldeen *et al.*, 2007)

CHAPTER 2: A comparison of partial dehydration and hydrated storage induced changes in viability, reactive oxygen species production and glutathione redox capacity in two contrasting recalcitrant-seeded species

2.1 Abstract

Recalcitrant seeds are desiccation and/or chilling sensitive which curtails their storage lifespan. This study compared the responses of two recalcitrant-seeded species with contrasting storage longevity, *Avicennia marina* (Forssk.) Vierh. and *Trichilia dregeana* Sond., to partial dehydration and storage in terms of viability, reactive oxygen species (ROS) production and glutathione redox capacity. Seeds of *A. marina* exhibited a faster rate of water and viability loss (± 50% viability loss in 4d) during partial dehydration, compared with those of *T. dregeana* (± 50% viability loss in 14 d). In *A. marina* embryonic axes, ROS production peaked on 4 d of dehydration and was accompanied by an increase in the GSH:GSSG ratio. However, these seeds still lost ± 50% viability on day 4, implying that the glutathione system alone could not overcome dehydration-induced oxidative stress in this species. In *A. marina*, ROS and axis WC levels increased during storage and this was accompanied by a decline in the GSH:GSSG ratio and rapid loss of viability (4 d to reach 45%). In *T. dregeana* embryonic axes, ROS production (particularly hydrogen peroxide) initially increased and then decreased during both partial dehydration and hydrated storage. Unlike in *A. marina* embryonic axes, this reduced ROS production was accompanied by a decline in the GSH:GSSG ratio. The data suggest that while *T. dregeana* seeds may have incurred some oxidative stress during storage, a delay in and/ or suppression of the ROS-based trigger for germination may account for their significantly longer storage longevity relative to those of *A. marina*. These data suggest that the mechanisms of desiccation-induced seed viability loss may differ across recalcitrant-seeded species based on the rate and extent to which they lose water during partial drying and storage. Furthermore, while recalcitrant seed desiccation sensitivity and by implication storage longevity is modulated by redox metabolism, the specific ROS and antioxidants that contribute to this control may differ across species.

Keywords: *Avicennia marina, partial dehydration, reactive oxygen species, recalcitrant, storage, Trichilia dregeana.*

2.2 Introduction

Desiccation tolerance in seeds, a key feature of orthodox types (Roberts, 1973), is interpreted as an adaptive strategy to enable seed survival in natural seed banks and tolerance against severe environmental conditions (Ellis and Roberts, 1981) and is the prime basis of seed longevity and storability during *ex situ* storage (Berjak and Pammenter, 2008, Walters, 2015). Recalcitrant, unlike orthodox, seeds do not undergo maturation drying, are shed from the parent plant highly hydrated and remain desiccation sensitive throughout their development (Pammenter *et al.*, 1994) and are often damaged by loss of only a small proportion of water (Berjak and Pammenter, 2008). Additionally, many recalcitrant seeds are chilling sensitive (Berjak and Pammenter, 2008) and this together with their desiccation sensitivity makes them unamenable to conventional seed storage techniques (i.e. reduced temperature, seed moisture content and relative humidity [RH]) and severely curtails even their short- to medium-term storage lifespan under hydrated storage conditions (Pammenter *et al.*, 1994). Hydrated storage entails storing seeds at high RH, at or slightly below shedding water content [WC] at reduced (4-16°C) or ambient temperatures depending on their tolerance to chilling and susceptibility to fungal proliferation, which is species-specific. Even cryopreservation which is viewed as the only option for long-term conservation of the germplasm of recalcitrant-seeded species is not easily achieved due to their intolerance to desiccation and low temperatures (Berjak and Pammenter, 2013).

A plethora of literature over the past few decades have reported significant differences across recalcitrant-seeded species in terms of seed desiccation sensitivity and storage longevity (reviewed by Berjak and Pammenter, 2008). These studies indicate that there is great variability in (1) the degree of dehydration that the seeds of individual species will tolerate and (2) the rate at which they lose water(e.g. Farrant *et al*., (1989). Inter-species variability in seed desiccation sensitivity among recalcitrant-seeded species is widely reported; for example, *Quercus alba* seeds are more desiccation sensitive than *Q. nigra* (Connor *et al.*, 1996) and there are also even differences between different *Baccaurea* species (Normah *et al.*, 1997). Studies have shown that while some recalcitrant seeds are poised for germination in a matter of hours or days after shedding (Chaitanya and Naithani, 1994, Callistru *et al*., 2000), seeds of yet others have to undergo germinative development (Goveia *et al*., 2004). Some believe that these differences in developmental status have a significant effect on the degree of dehydration recalcitrant seeds will tolerate (Berjak and Pammenter, 2008). Studies have also shown that generally axes at higher WCs are more desiccation sensitive as shown for *Q. robur* (Finch-Savage, 1992), *Machilus thunbergii* (Lin and Chen, 1995) and *Theobroma cacao* (Li and Sun, 1999). Another factor contributing to variability in desiccation sensitivity among individual species is that their drying characteristics differ both intra- and inter-seasonally (Finch-Savage *et al.*, 1994).

This variability in desiccation sensitivity can in turn lead to difference in storability (Berjak and Pammenter, 2008). Recalcitrant seed storage longevity can range from a few days to few months. For example, seeds of *Shorea robusta* Gaertn F. (Chaitanya and Naithani, 1994) and *Avicennia marina* (Forssk.) Vierh. (Farrant *et al.*, 1997, Calistru *et al.*, 2000) can only be stored for a matter of days while seed of species like *T. dregeana* (Goveia *et al.*, 2004) and *Encephalartos natalensis* Dyer and Verdoorn (Woodenberg *et al.*, 2010) are shed considerably undeveloped and can be stored for several months during which time embryo development continues. Recalcitrant seeds that are not chilling sensitive (like many temperate species) may even have a longer storage longevity if storage conditions are optimised (Corbineau and Côme, 1989, Connor *et al.*, 1996).

The diversity in desiccation sensitivity among recalcitrant seeds has been explored but to a limited extent. Understanding the basis of seed desiccation sensitivity starts with knowledge of processes and mechanisms involved in the acquisition and maintenance of desiccation tolerance and investigating whether or not these occur in recalcitrant seeds, and if so, to what extent in different species (Berjak and Pammenter, 2013). Lack of ability for metabolic switch-off that occurs during maturation drying in orthodox seeds, is one of the basic reasons that recalcitrant seeds are desiccation sensitive (Berjak and Pammenter, 2013). When water is lost from recalcitrant tissues, and especially when dehydration proceeds slowly, metabolism is said to become unbalanced. This can result in considerable intracellular damage (termed metabolism-linked damage) and death of seeds/embryos at relatively high WCs (Pammenter *et al.*, 1998, Pammenter and Berjak, 1999, Walters *et al.*, 2001, Walther *et al.*, 2002, Berjak and Pammenter, 2008).In recalcitrant seeds, metabolism-linked damage is thought to be intimately associated with the uncontrolled generation of ROS under conditions in which the intracellular antioxidant defences are inadequate to quench them (Kranner and Grill, 1993). Desiccation induced oxidative stress has been widely reported to contribute to the loss of viability in recalcitrant seeds during drying (Roach *et al.*, 2008, Varghese *et al.*, 2011) and storage (Pukacka and Ratajczak, 2005). Desiccation interferes with metabolic processes resulting in the production of potentially harmful ROS such as singlet oxygen ($^1O_2$), superoxide ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2). Studies have shown that ROS may disrupt membrane integrity via peroxidation of membrane lipids (Pukacka and Ratajczak, 2006, Roach *et al.*, 2008), damage nucleic acids, and alter protein structure and activity through oxidative modifications such as carbonylation (Johansson *et al.*, 2004, Oracz *et al.*, 2007, Sweetlove and Møller, 2009).

While strict control of ROS is taken for granted in hydrated cells, possession and effective operation of a suite of both enzymic and non-enzymic antioxidants is of prime importance in tissues encountering various stresses including desiccation (Pammenter and Berjak, 1999, Kranner, 2002, Bailly, 2004, Kranner and Birtić, 2005, Varghese *et al.*, 2011, Chandrakar *et al.*, 2016). During the early stages of

desiccation, the glutathione-ascorbate cycle plays a crucial role in the detoxification of potentially toxic H_2O_2 by using antioxidant metabolites such as ascorbate, glutathione and NADPH, and enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) (Pukacka and Ratajczak, 2006; Varghese *et al*., 2011). Moreover, Kranner *et al*. (2006) have reported that the ratio of reduced glutathione (GSH) to its oxidised form (GSSG) can be used as a stress marker in seeds and there are reports that glutathione plays a key role in maintenance of axis viability in recalcitrant seeds during drying (Varghese *et al.*, 2011) and storage (Tommasi *et al.*, 2006).

Given our limited understanding of the mechanisms underlying inter-species differences in storage longevity and desiccation sensitivity in recalcitrant seeds the present study compared dehydration and storage induced changes in viability, ROS production and glutathione redox capacity in two recalcitrant-seeded species of contrasting storage longevity. The species investigated in this study included *A. marina* (highly recalcitrant) whose seeds can only survive for a matter of days in storage (Farrant *et al.*, 1993a, Moothoo-Padayachie *et al.*, 2016) and *T. dregeana* (less recalcitrant) whose seeds can be stored under hydrated storage conditions for several months before visible germination or decline of viability is observed (Goveia *et al.*, 2004, Moothoo-Padayachie *et al.*, 2016).

2.3 Materials and methods

2.3.1 Seed collection and processing

Mature fruits of *A. marina* were collected from the ground at the Beachwood Mangroves Nature Reserve, Durban, South Africa (29°48.470' S 31°02.384' E), during low tides. Care was taken in collecting only those seeds that showed no signs of pericarp browning, which typically occurs from 24 h after shedding (Calistru *et al.*, 2000, Moothoo-Padayachie *et al.*, 2016). Freshly harvested seeds of *A. marina* were soaked in distilled water for 30 min to permit sloughing of the pericarp (Calistru *et al.*, 2000) and blotted dry before use. Pericarp sloughing is part of the natural post-shedding physiology *in situ* and comes about when seeds are shed into water or are immersed by water after shedding. Mature and open capsules of *T. dregeana* were harvested directly from trees at the University of KwaZulu-Natal, Westville campus, Durban, South Africa (29°49.054' S 30°56.521' E). Seeds displaying any visible signs of damage and predation were removed prior to any further processing. Seeds were collected over two seasons (March-April for *A. marina* and May-July for *T. dregeana* in 2012 and 2013).

2.3.2 Desiccation

Pericarp removed seeds of *A. marina* were sown in soil collected from beneath the parent trees whereas freshly harvested seeds of *T. dregeana* were sown with aril intact (as this is how these seeds are shed *in situ*) in commercial potting soil (Grovida, South Africa). Both soils (mangrove and commercial potting soil) were dried for 24 h to remove any excess moisture, prior to sowing of seeds. Approximately 50 seeds per tray (30 × 20 cm; 15 cm deep) were randomly sown to a depth of 10 mm. The seeds were allowed to dry under ambient conditions at 25°C for a period of 14 d; various parameters were assessed over this period, as described below.

2.3.3 Hydrated storage

Naked seeds of *A. marina* were surface-sterilized in 1% sodium hypochlorite (NaOCl) for 20 min on a shaker. After brief rinsing, the seeds were left to dry for four hours on a bench top back to their shedding WC. Thereafter, these seeds were stored hydrated in a monolayer, on plastic mesh grids suspended about 100 mm over sterile moistened paper towels, within sealed, sterile opaque buckets at 25°C (Calistru *et al.*, 2000, Moothoo-Padayachie *et al.*, 2016). Fungal contamination was minimized by an initial application of 2.5 ml L^{-1} of a fungicide (PrevicurN®; active ingredient, propamocarb-HC [AgrEvo, Pietermaritzburg, South Africa]) and then spraying the fungicide at three day intervals for approximately 30 d (Calistru *et al.*, 2000).

For *T. dregeana*, the arils were removed from seeds before they were surface sterilized using a 1% NaOCl solution containing a few drops of Tween 20 for 20 min. Seeds were subsequently soaked in an antifungal cocktail comprising 0.5 ml L^{-1} Early Impact (active ingredient, triazole and benzimidizole; Zeneca Agrochemicals, South Africa) and 2.5 ml L^{-1} PrevicurN® (active ingredient, propamocarb; AgrEvo, South Africa) for 240 min (Calistru *et al.*, 2000, Berjak and Pammenter, 2004). The seeds were then dusted with Benlate (active ingredient: benomyl [benzimidazole], Dupont, USA), and stored hydrated (as described for *A. marina*) at 16°C (after Goveia *et al.*, 2004). Seeds of both species that germinated in hydrated storage were regularly removed from the buckets and discarded.

2.3.4 Viability assessment

Seeds of both species were retrieved from partial dehydration (daily for *A. marina* and at two-day intervals for *T. dregeana*) and hydrated storage (at five day intervals for *A. marina* and monthly for *T. dregeana*) treatments and assessed for viability. The seeds of *A. marina* and *T. dregeana* (n = 15 for each sampling day) were sown in mangrove and commercial potting soil respectively, within seedling trays (same dimensions used in partial drying experiments, with five seeds per tray). The soil was maintained at field capacity using sea water for *A. marina* and deionised water for *T. dregeana* for the

duration of the germination trial. These studies were conducted within a glasshouse (26/18°C, day/night; ambient light) on the grounds of the University of KwaZulu-Natal. Germination was defined as radicle emergence of at least 10 mm and was scored daily (30 d) until no further change in germination was recorded.

As mentioned above, the seeds that germinated in storage were discarded and considered non-viable because those seeds eventually died when additional water for germination to progress was not availed. Since few seeds of both species germinated in hydrated storage, care was taken in ensuring that stored seeds used for these assays showed no signs of in-storage germination.

2.3.5 Water Content determination

Freshly harvested, dehydrated and stored seeds were sampled for embryonic axis WC at intervals that coincided with the viability assays. Immediately after excision, embryonic axis WC (n=10) was measured gravimetrically using a 5-place balance (Mettler, Mt5, Germany). Axes were weighed before and after drying in an oven at 80°C for 48 h. Water content was expressed on a dry mass basis (dmb; g H_2O per g dry matter [g g^{-1}]) as described in Varghese *et al*. (2011).

2.3.6 Estimation of extracellular superoxide

Levels of extracellular $\cdot O_2^-$ production were determined spectrophotometrically at A_{490} following the NADH-mediated oxidation of epinephrine (Sigma, St. Louis, MO) to adrenochrome (Misra and Fridovich, 1972) using an extinction coefficient of 4.47 mM^{-1} cm^{-1} and expressed as nmol of epinephrine oxidized $s^{-1}g^{-1}$ DW. Embryonic axes excised from *A. marina* seeds following dehydration (after 0, 1, 2, 3 and 4 d) and storage (at 0, 5, 10, 15 and 20 d) were rinsed for approximately 20 s in distilled water and subsequently incubated at 100% RH for 5-10 min to allow any wound induced ROS to dissipate before assays were initiated (after Roach *et al.*, 2010). This also applied to axes of dehydrated (on 0, 2, 4, 7, 12 and 14 d) and stored (at 0, 1, 2, 3, 4, 5, 6, 9 and 12 months) *T. dregeana* axes. Each assay comprised six replicates of a single axis for *A. marina* and six replicates of five axes each for *T. dregeana*. The embryonic axes were gently shaken at 60 rpm in 2.0 ml of 1 mM epinephrine (pH 7.0) for 30 min in the dark at 25°C after which the absorbance of the assay mixture was read at 490 nm using a UV-Vis spectrophotometer (Shimadzu, UV-2600, Japan). The dry weight of the axes was estimated once they were dried in an oven at 80 °C for 48 h (after Roach *et al.*, 2008).

Epinephrine can be also oxidised non-specifically, and possibly by enzymes (e.g., tyrosinases; Baker and Orlandi, 1995). Therefore, the specificity of the assay was determined as ≥50% inhibition of $\cdot O_2^-$ production when 250 U. mL^{-1} of SOD was added to the assay mixture (Table 2.1). Furthermore, confirmatory tests included inhibition of $\cdot O_2^-$ production by both sources using chemical inhibitors: (a)

diphenylene iodonium (DPI) which is an inhibitor of NAD(P)H oxidase (Henderson and Chappell, 1996) and (b) sodium azide (NaN_3) which is an inhibitor of peroxidases (Liu *et al.*, 2006). For this, freshly excised embryonic axes of *A. marina* (three replicates of one axis each) and *T. dregeana* (three replicates of five axes each) were left to rest for 5-10 min and then placed in either 10 µM DPI (Whitaker *et al.*, 2010) or 1 mM NaN_3 (Kranner *et al.*, 2010) for 10 min, followed by incubation in epinephrine for 30 min, and rates of $\cdot O_2^-$ production were measured as discussed above.

2.3.7 Estimation of extracellular hydrogen peroxide

Extracellular H_2O_2 production by embryonic axes excised from fresh, dehydrated and stored seeds at various intervals was determined spectrophotometrically using the xylenol orange assay by Gay and Gebicki (2000). A working reagent was made by mixing 1 part of "Reagent A" (which comprised of 25 mM $FeSO_4$, 25 mM $(NH_4)_2SO_4$ and 2.5 M H_2SO_4) and 100 parts of "Reagent B" (containing 125 µM xylenol orange and 100 mM sorbitol) which was stirred for 15 min prior to performing the assay. Six replicates of single axis of *A. marina* and six replicates of five axes each for *T. dregeana* were gently shaken at 60 rpm in 2.0 mL of working reagent for 30 min in the dark at 25°C (after Minibayeva *et al.*, 2009), after which the absorbance of the assay mixture was read at 560 nm. H_2O_2 production was calculated using a standard curve with known concentrations of H_2O_2. The specificity of the assay was determined by ≥50% inhibition of H_2O_2 production when 250 U mL^{-1} of CAT was added to the assay mixture (Table 2.2).

2.3.8 Determination of intracellular GSH and GSH disulphide (GSSG)

For the determination of GSH and GSSG, three replicates of a single axis (±100 mg) at 0, 1, 2, 3, and 4 d after sowing were analysed for *A. marina* and for *T. dregeana* were analysed five axes (±40 mg) at 0, 2, 4,7, 14, 16, and 20 d after sowing were analysed. The embryonic axes were rapidly frozen in liquid nitrogen (LN) and ground to a fine powder in LN using a mortar and pestle. GSH and GSSG were extracted on ice in 2 mL 0.1 M HCl containing 1 mM EDTA and transferred into 2 mL amber Eppendorf® tubes. The extracts were then centrifuged at 14000 g for 15 min (after Varghese *et al.*, 2011). The supernatants were then diluted (1:1 ratio; v/v) using 20 mM potassium phosphate buffer (pH 2.7), and the pH of samples were adjusted to 3.4 using 0.004 mL of a saturated potassium hydroxide solution. A 0.2 mL aliquot of each sample was transferred into 0.3 mL clear inserts within 1.5 mL amber auto sampler vials (Macherey-Nagel GmbH and Co., Germany) for analyses using high pressure liquid chromatography (HPLC; FRC-10A, Shimadzu, Japan). All samples and buffers were filter-sterilised using a 0.2 µm syringe filter and bottle-top filter respectively (GVS filter technology, USA) prior to HPLC analyses. A modified method (adapted fromYilmaz *et al.*, 2009) was employed for the

separation of GSH and GSSG by separating both reduced (GSH) and oxidized (GSSG) forms of glutathione without further derivatization. Total glutathione (GSH + GSSG) was separated on a Synergi Hydro-RP C18 column (150×4.6 mm, 4 µm; Phenomenex, S/No. 620813-1, USA). The mobile phase consisted of a 20 mM potassium phosphate buffer (pH 2.7) - acetonitrile (99:1 ratio; v/v). Owing to the crude nature of the seed glutathione extract, the flow rate was adjusted to 0.5 mL min^{-1} for better peak separation. Detection was performed at 210 nm by a UV-vis detector (RID-10A) at 22 °C oven temperature. The analyses of glutathione molecules lasted for 10-15 min, after which the run was discontinued. Quantification was carried out by external standardization using LC Lab solutions software and a calibration curve of known standards of GSH and GSSG. Recovery of GSH and GSSG were evaluated with homogenates spiked with standards. Prior to HPLC analysis, 0.01 mL GSH and GSSG was added to the seed extract. The seed extracts were then blindly assayed and the concentration of GSH and GSSG was derived from the calibration curves. The spiking of GSH and GSSG was determined as a standard measured in the absence of the real sample. Accuracy was evaluated by comparing the estimated concentration with the known concentrations of both thiols (data not shown).

2.3.9 Statistical analysis

All data were analysed using IBM SPSS statistics version 22. Data were tested for normality using a Shapiro-Wilk test. Viability and water uptake percentages were √arsine transformed prior to any analyses. Viability, water uptake, $\cdot O_2^-$, H_2O_2 and glutathione data were subjected to analysis of variance (ANOVA), where data was parametric, to test for differences within species, across sampling intervals. Means were separated using a Tukey post-hoc test. Where data did not meet ANOVA assumptions, even after transformation, a Kruskal-Wallis test was applied. Relationships between parameters (viability, ROS production and water loss) were tested via Pearson (where data was parametric) and Spearman's rank (where data was nonparametric) correlation analyses. An independent-samples *t*-test was used to test for significant differences in ROS, GSH, GSSG and total glutathione levels between species at harvest and when 50% survival was attained. All differences were considered significant at the 0.05 level.

2.4 Results

2.4.1 Seed viability loss and WC in response to partial dehydration and storage

During partial dehydration, seeds of *A. marina* lost ± 50% viability within 4 d compared with *T. dregeana* seeds that lost ± 50% viability after 14 d under the same drying conditions (Figs. 2.1a and b). The rate of water loss was twice as fast in dehydrated *A. marina* (0.2 g d^{-1}) seeds than in dehydrated

T. dregeana (0.1 g d^{-1}) seeds (data not shown). Freshly excised embryonic axes of *A. marina* had an initial shedding WC of *c.* 1.7 g g^{-1}. However, following partial dehydration for 4 d when ± 50% viability was lost, embryonic axis WCs decreased significantly ($P < 0.05$) to *c.*1 g g^{-1} (corresponding to 40% water loss) and coincided with a significant ($P < 0.05$) increase in seed viability loss compared with the control. In contrast, *T. dregeana* seeds had an initial axis WC of *c.* 2.3 g g^{-1} but following partial dehydration for 14 d when ± 50% viability loss occurred axis WC dropped to *c.* 0.8 g g^{-1} (corresponding to *c*. 62% water loss) (Fig. 2.1a and b).

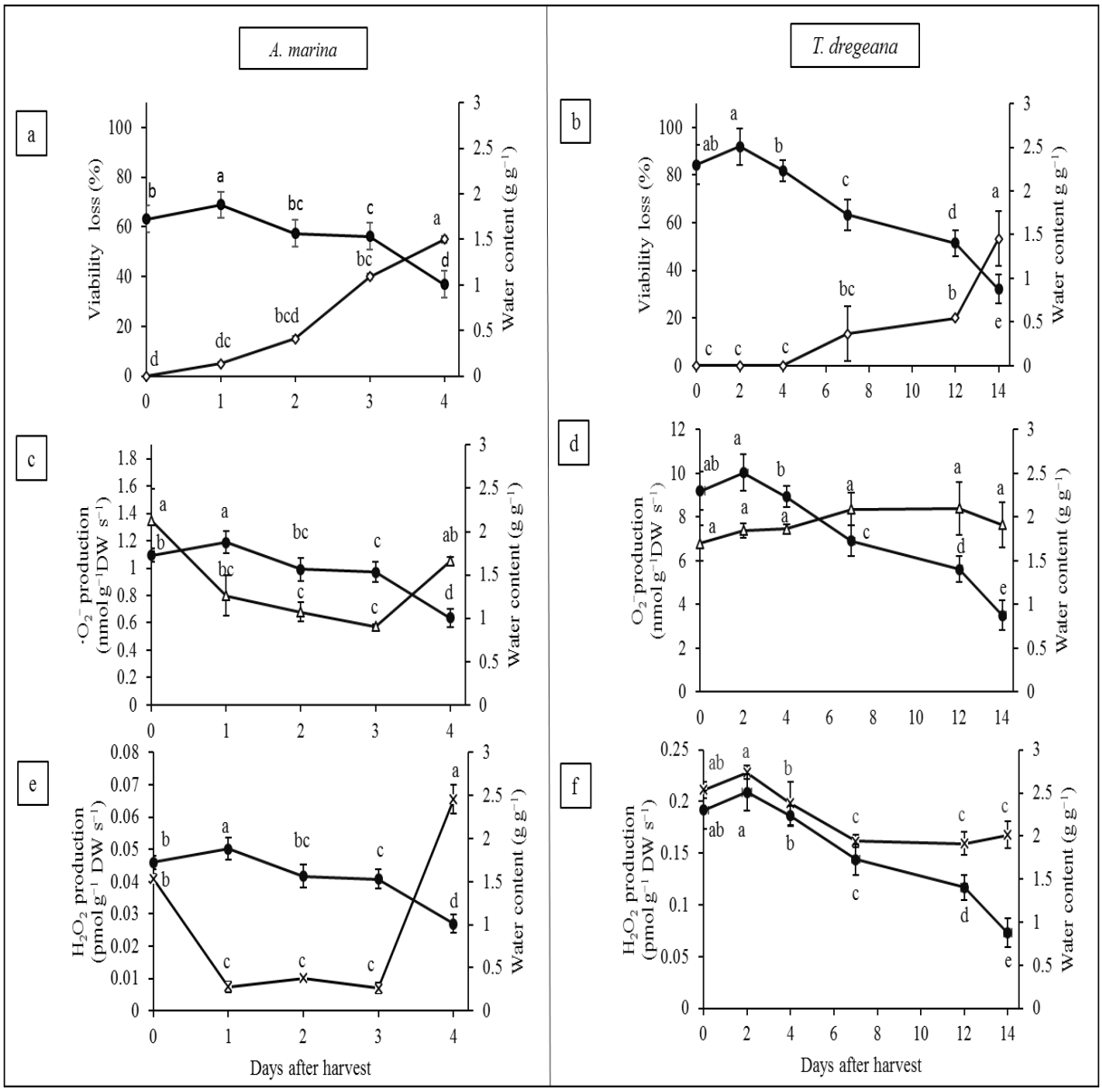

Figure 2.1 Percentage viability loss (◊; a and b), extracellular superoxide production (Δ; c and d) and extracellular hydrogen peroxide production (×; e and f) during partial dehydration of *A. marina* and *T. dregeana* seeds, respectively, following harvest in the absence of water. Embryonic axes WC (●) during various stages of partial dehydration is shown for both species in graphs 2.1a to 2.1f. Points labelled with different letters are significantly different when compared within species, across treatments ($P < 0.05$ in all cases; ANOVA)

During hydrated storage of *A. marina* seeds no significant difference ($P > 0.05$) in WC was found between 0, 5 and 10 d. However, a significant increase ($P < 0.05$) in WC was observed in embryonic axes when WC was estimated on 15 d and 20 d of hydrated storage, which coincided with 33% ± 6 and 53% ± 4 seed viability loss, respectively (Fig. 2.2a and b). Loss in *A. marina* seed viability post 20 d in hydrated storage was mainly due to seeds germinating in storage (data not shown). In contrast, a significant decrease ($P < 0.05$) in axis WC occurred in *T. dregeana* seeds in hydrated storage compared with freshly harvested seeds (control). A significant ($P < 0.05$) decrease in WC was measured in *T. dregeana* seeds over 5 months in storage and thereafter there were no significant differences ($P > 0.05$) in axis WC. At 12 months in hydrated storage, 54% ± 0.5 of *T. dregeana* seeds germinated in storage. Although, the non-germinated seeds remained 100% viable, embryonic axis WC of *T. dregeana* seeds dropped from *c.* 2.4 g g^{-1} at day 0 (control) to *c.* 2.0 g g^{-1} after 12 months in storage, implying that the seeds experienced a water stress.

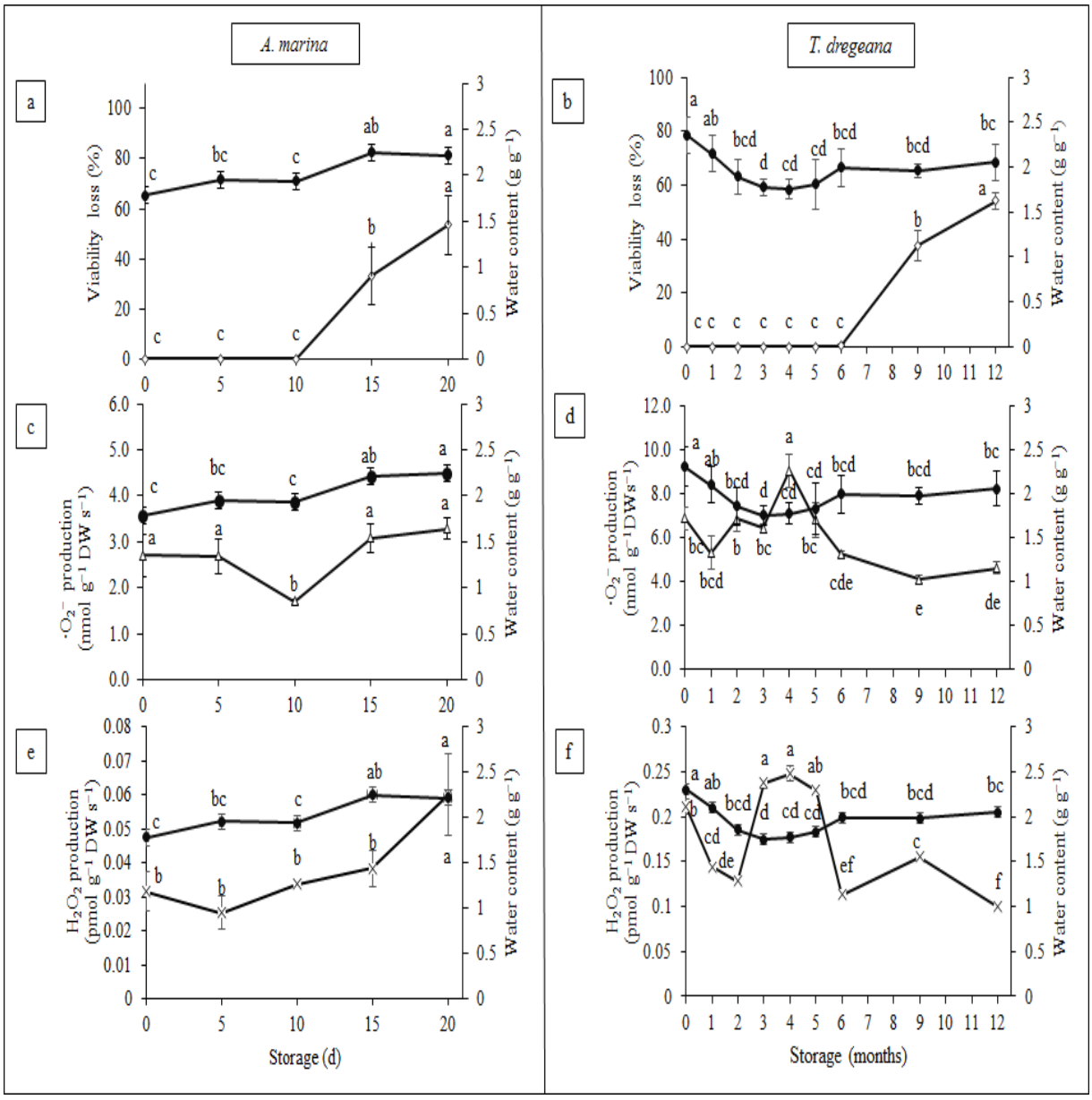

Figure 2.2 Percentage viability loss (◊; a and b), extracellular superoxide production (Δ; c and d) and extracellular hydrogen peroxide production (×; e and f) during hydrated storage of *A. marina* and *T. dregeana* seeds, respectively. Embryonic axes WC (●) during various stages of hydrated storage is shown for both species in graphs 2.2a to 2.2f. Points labelled with different letters are significantly different when compared within species, across treatments ($P < 0.05$ in all cases; ANOVA).

2.4.2 ROS production during partial dehydration

In embryonic axes of *A. marina*, there was an initial (between 0 d to 1 d) significant ($P < 0.05$) decrease in $\cdot O_2^-$ production, with similar levels of superoxide production in axes from 1 d till 3 d following partial dehydration (Fig. 2.1c). However, between 3 and 4 d following partial dehydration, there was a significant ($P < 0.05$) increase in $\cdot O_2^-$ production in axes of *A. marina* seeds which also coincided with a significant ($P < 0.05$) decrease in WC (Fig. 2.1c) and ± 50% viability loss (Fig 2.1a). A similar trend was also observed in terms of H_2O_2 production where levels in *A. marina* axes initially significantly ($P < 0.05$) decreased (between 0 and 1 d) but levels were comparable between 1 d and 3 d. Similarly, a significant increase in H_2O_2 production occurred in *A. marina* axes between 3 d and 4 d (Fig. 2.1e), when ± 50% viability was lost (Fig. 2.1a).

Although axis WC decreased during partial dehydration in *T. dregeana* and seed viability loss increased over time, no significant differences in superoxide production levels were found up until 14 d (Fig. 2.1d) when ± 50% viability loss occurred (Fig. 2.1b). In contrast, there was a significant decrease in H_2O_2 production in axes at 7 d of partial dehydration which was maintained constant until 14 d (Fig. 2.1e), when ± 50% viability loss occurred (Fig. 2.1b).

2.4.3 ROS production during hydrated storage

In the embryonic axes of stored *A.* marina seeds, there was no significant ($P > 0.05$) difference in $\cdot O_2^-$ production over 20 d, except at 10 d in storage when there was a significant ($P < 0.05$) decrease in $\cdot O_2^-$ production (Fig. 2.2c). Interestingly, following 10 d in storage, there was a significant ($P < 0.05$) increase in seed viability loss. During storage, the level of H_2O_2 production in the axes of stored *A. marina* seeds remained very similar over the first 15 d (Fig. 2.2e). However, after 15 d the level of H_2O_2 production significantly ($P < 0.05$) increased in axes. Levels of H_2O_2 production were particularly high at 20 d (Fig. 2e) which also coincided with ± 50% seed viability loss (Fig. 2.2a).

In the axes of stored *T. dregeana* seeds, $\cdot O_2^-$ production significantly ($P < 0.05$) increased and peaked at 4 months, thereafter $\cdot O_2^-$ production decreased, with levels being comparable between 6 and 12 months (Fig. 2.2c). A significant increase ($P < 0.05$) in seed viability loss occurred from six months onwards until ± 50% viability was lost after 12 months (since many seeds germinated in storage) (Fig. 2.2b). H_2O_2 production peaked at 3-5 months in the axes of stored *T. dregeana* seeds but thereafter significantly ($P < 0.05$) declined up until six months (Fig. 2.2f). Levels of H_2O_2 remained relatively constant between 6 and 12 months of storage but at 12 months H_2O_2 production was significantly ($P < 0.05$) lower than that at harvest (Fig 2.2d and f).

2.4.4 Confirmatory assays for ROS estimation in axes after partial dehydration and hydrated storage

The specificity of the assay used to measure the $\cdot O_2^-$ production described above was confirmed by the fact that exposure of excised axes of both *A. marina* and *T. dregeana* to SOD significantly ($P < 0.05$) decreased adrenochrome formation by >50% in both species (i.e., *c.* 52% and 65%, respectively; Table 2.1). Superoxide production in freshly excised axes of *T. dregeana* was also inhibited (>50%) by known enzyme inhibitors, viz., DPI and NaN3 (Table 2.1). Similarly, relatively lower but significant ($P < 0.05$) inhibition of $\cdot O_2^-$ production was also observed in freshly excised *A. marina* axes exposed to DPI (43% inhibition) and NaN3 (*c.* 36% inhibition) (Table 2.1). The specificity of the H_2O_2 assay used was confirmed by the fact that exposure of freshly excised axes to CAT significantly ($P < 0.05$) decreased H_2O_2 production by >50% in both species (Table 2.2).

Table 2.1: Effects of superoxide dismutase (SOD) and the enzyme inhibitors diphenylene iodonium (DPI) and sodium azide (NaN_3) on extracellular $\cdot O_2^-$ production (nmol g^{-1} DW s^{-1}) in *A. marina* and *T. dregeana* embryonic axes.

	$\cdot O_2^-$ production	
Inhibitor	*A. marina*	*T. dregeana*
Control (dH_2O)	2.3 ± 0.1 [a]	8.2 ± 0.1[a]
DPI (10 µM)	1.3 ± 0.1 (57%)[b]	2.9 ± 0.4 (35%)[b]
NaN_3 (1 mM)	1.5 ± 0.1 (64%)[b]	3.1 ± 0.4 (38%)[b]
SOD (250 U/ml)	1.1 ± 0.1 (48%)[b]	3.0 ± 0.1 (35%)[b]

Data represent mean ± SD (n=3 replicates of 1 axis for *A. marina* and n=3 replicates of 5 axes for *T. dregeana*). Values labelled with different letters are significantly different when compared within species, across treatments ($P < 0.05$ in all cases; ANOVA). Values within brackets reflect % increase/decrease relative to control.

Table 2.2: Effect of catalase (CAT) on extracellular H_2O_2 production in *A. marina* and *T. dregeana* embryonic axes. Data represent mean ± SD (n=3 replicates of 1 axis for *A. marina*, n=3 replicates of 5 axes for *T. dregeana*). Values labelled with different letters are significantly different when compared within species, across treatment ($P < 0.05$; ANOVA). Values within brackets reflect % increase/decrease relative to control.

	H_2O_2 production	
Inhibitor	*A. marina*	*T. dregeana*
Control (dH_2O)	3.2 ± 0.1[a]	14.9 ± 0.4[a]
CAT (250 U/ml)	0.7 ± 0.1 (21%)[b]	7.4 ± 0.1 (50%)[b]

2.4.5 Glutathione pool during partial dehydration

During partial dehydration, total glutathione levels (GSH+GSSG) increased over dehydration time in *A. marina* axes. Total glutathione levels were significantly ($P < 0.05$) higher dehydrated *A. marina* axes at 4 d of partial dehydration compared with the levels at harvest (Fig. 2.3a). The highest ratio of GSH:GSSG occurred in axes of seeds dried for 4 d (Fig. 2.3a) which coincided with ± 50% viability loss (Fig. 2.1a). During partial dehydration of *T. dregeana* seeds, total glutathione levels in the embryonic axes were comparatively lower initially (between 0 to 2 d) compared with all other days of estimation. However, total glutathione levels were significantly ($P < 0.05$) high at 4 d and 8 d compared with the control. Although total glutathione levels were significantly ($P < 0.05$) higher in axes at 14 d compared with the control, the ratio of GSH:GSSG was higher in axes at harvest (0 d) than 14 d, when ± 50% viability was lost.

2.4.6 Glutathione pool during hydrated storage

In the embryonic axes of stored *A. marina* seeds, total glutathione levels initially (between 0 d and 10 d) increased compared with the levels in axes from fresh seeds. However, following 10 d storage, total glutathione levels in the axes significantly ($P < 0.05$) decreased which coincided with an increase in seed viability loss (Fig. 2.2a). There was no significant ($P > 0.05$) difference in total glutathione levels in axes from seeds at harvest (control) compared with after seeds storage for 20 d. However, there was a higher ratio of GSH:GSSG in *A. marina* axes at harvest compared with at 20 d in storage. In the embryonic axes of stored *T. dregeana* seeds, the ratio of GSH:GSSG was higher at harvest than after 12 months in storage. However, in terms of total glutathione levels, a higher amount of total glutathione was found at 12 months (when ± 50% viability loss occurred) than at harvest. When ± 50% viability loss occurred in both species total glutathione levels were higher in *T. dregeana* seeds compared to *A. marina* seeds.

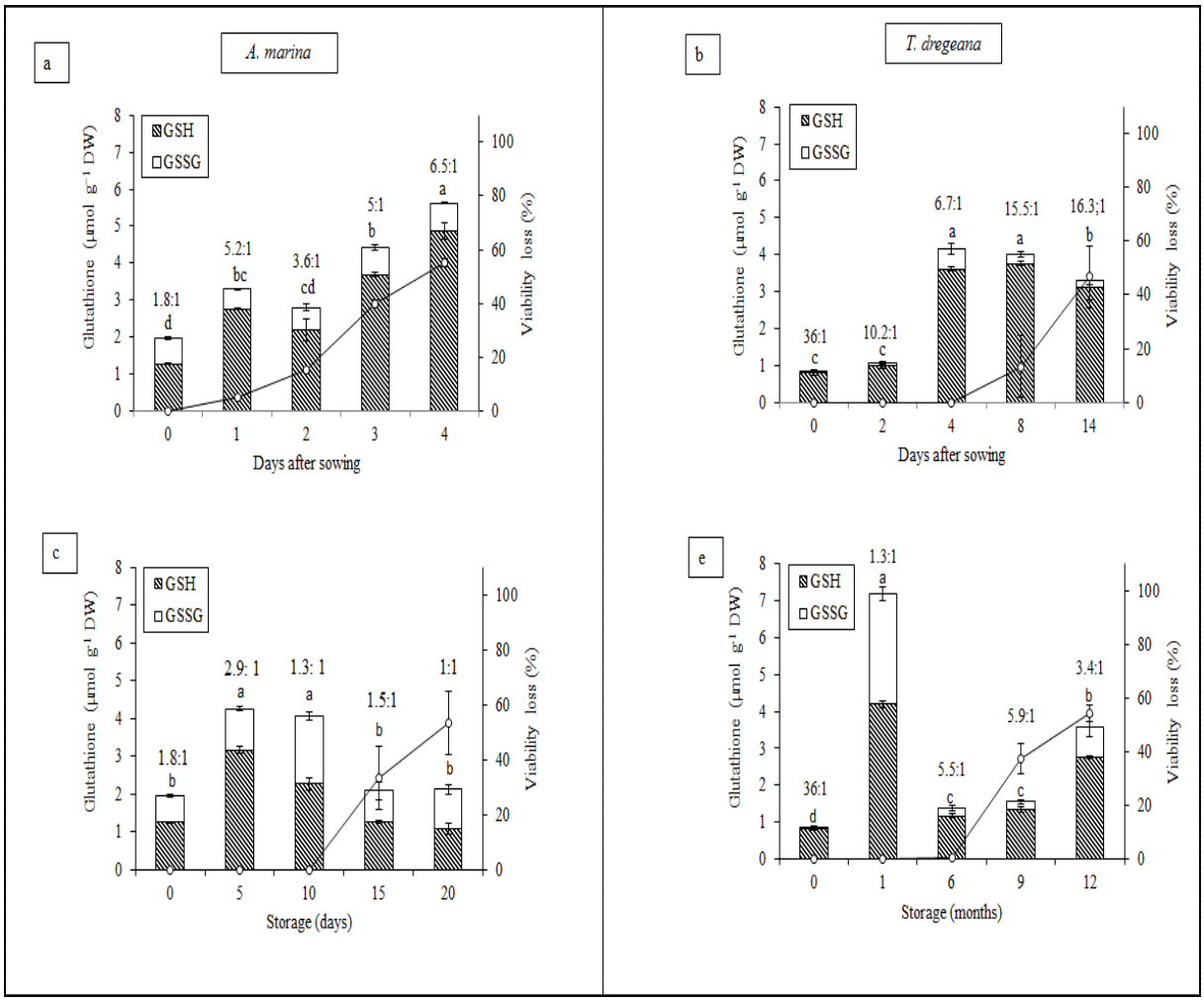

Figure 2.3 Reduced (GSH) and oxidized glutathione (GSSG), GSH:GSSG ratio and viability loss in embryonic axes from seeds that were partially dehydrated (a and b) and hydrated storage (c and d) of *A. marina* and *T. dregeana*, respectively. Values are the mean ± SD (*n* = 3 replicates of 1 axis for *A. marina* and 5 axes for *T. dregeana*); (*P* < 0.05 in all cases; ANOVA).

2.5 Discussion

To improve our current understanding of the biological basis for inter-species differences in desiccation sensitivity and storage longevity in recalcitrant seeds, the present study undertook a comparison of the responses of two recalcitrant-seeded species (*A. marina* and *T. dregeana*) to partial dehydration and hydrated storage. In accordance with the definition of recalcitrance (Roberts, 1973), both recalcitrant-seeded species were shed at high WCs (Figs. 2.1a and b). However, differences were found in the degree of dehydration both these individual species would tolerate. For example, *A. marina* seeds were much more desiccation sensitive than those of *T. dregeana* seeds: *A. marina* seeds lost ± 50% viability in only 4 d (corresponding to *c.* 40% water loss) compared with *T. dregeana* seeds which took 14 d to lose ± 50% viability (corresponding to *c.* 62% water loss).These differences in desiccation sensitivity could therefore be a consequence of relatively faster loss of water in *A. marina* seeds under the same drying conditions; the rate of water loss in *A. marina* axes (0.2 g d^{-1}) was twice as fast as that in *T. dregeana* axes (0.1 g d^{-1}). Farrant *et al*. (1989) also found that the rate of water loss can differ dramatically across recalcitrant seeds of different species (*Araucaria angustifolia, Scadoxus membranaceus and Landolphia kirkii*) dehydrated under identical conditions.

Desiccation-induced oxidative stress is a common cause of death in desiccation sensitive tissues in general and recalcitrant seeds in particular (Chaitanya and Naithani, 1994, Pukacka and Ratajczak, 2006, Roach *et al.*, 2008, Roach *et al.*, 2010, Sershen *et al.*, 2016). Oxidative stress is created when the antioxidant capacity of the tissues is not enough to quench the excessive ROS production as a result of various stresses including water loss, as in this study (Leprince *et al.*, 1999, Pukacka and Ratajczak, 2006, Roach *et al.*, 2010). In the present study, axis WC significantly decreased in *A. marina* during partial dehydration (between 3 d and 4 d) which also coincided with a significant ($P < 0.05$) increase in ROS production (Fig. 2.1c and e) and ± 50 viability loss (Fig. 2.1a). Numerous studies have shown that ROS accumulation and associated oxidative stress (especially during dehydration) is one of the major causes of loss of membrane structural integrity which leads to seed viability loss (Hendry *et al.*, 1992, Leprince *et al.*, 1999, Pukacka and Ratajczak, 2006). Increased ROS production during dehydration of *A. marina* seeds coincided with viability loss which is similar to results found in other recalcitrant-seeded species (Chaitanya and Naithani, 1994, Varghese and Naithani, 2002, Pukacka and Ratajczak, 2006, Roach *et al.*, 2008) such as *Acer saccharinum*, (Pukacka and Ratajczak, 2006) and *Camellia sinensis* (Chen *et al.*, 2011). Although there have been numerous studies on the role of ROS production during desiccation of recalcitrant seeds, the balance between the pro- and anti-oxidants during desiccation is still unclear. It is, however, well established that the presence and effectiveness of various antioxidant enzymes and compounds is of paramount importance to maintaining a redox state in viable cells. The ratio of GSH:GSSG especially, is a well-known indicator of oxidative stress in plants (Noctor

and Foyer, 1998) and orthodox (Tommasi *et al.*, 2001, Garnczarska, 2008) and recalcitrant (Varghese *et al.*, 2011) seeds. In axes of *A. saccharinum* seeds for example, the ratio of GSH:GSSG increased during initial dehydration. In the present study, at 4 d when ± 50% viability was lost in *A. marina* seeds, they displayed a high GSH:GSSG ratio, possibly in response to the high ROS levels (Fig. 2.1a), however, viability was still lost (at 7 d these seeds were 0% viable (data not shown). In *Acer saccharinum* seeds although total glutathione levels were initially high, with prolonged dehydration both the levels of GSH and GSSG declined with the final ratio of GSH:GSSG being higher in the control than after dehydration (Pukacka and Ratajczak, 2006). Thus, it is possible that total glutathione levels and ratio of GSH:GSSG may have declined in *A. marina* had these levels been measured beyond 4 d of drying.

In contrast, in *T. dregeana* axes no significant difference in $\cdot O_2^-$ production was found in seeds exposed to partial dehydration, even though the WC decreased over time (Fig. 2.1b) and ± 50% viability was lost at 14 d (Fig. 2.1d). Interestingly, H_2O_2 production decreased during partial dehydration with significantly ($P < 0.05$) lower levels in seeds exposed to 14 d partial dehydration when ± 50% viability was lost relative to the control (Fig. 2.1f). However, this decline in ROS was also accompanied by a decrease in the ratio of GSH:GSSG (Fig. 2.3b) on the day (14 d) that coincided with ± 50% viability loss (Fig. 2.1b). This suggests that these seeds may have incurred some oxidative stress even at reduced ROS levels, which may explain their viability loss. Alternatively, Varghese *et al*. (2011) also found that dehydration decreased axis ROS levels, the ratio of GSH:GSSG and germinability in this species, suggested that dehydration dampens the ROS-based trigger for germination in *T. dregeana* axes of *T. dregeana*. The response to dehydration in terms of ROS production and the glutathione system appears to differ between *T. dregeana* and *A. marina* seeds. Inter-species variability in ROS and antioxidant responses to dehydration appears to be a common observation (Farrant *et al.*, 1989, Ballesteros *et al.*, 2014, Sershen *et al.*, 2016).

As mentioned earlier, hydrated storage allows recalcitrant seeds to survive in the short- to medium- term by maintaining them close to their shedding WC. However, studies have shown that even in hydrated storage embryonic axes of recalcitrant seeds undergo germinative development and eventually lose viability due to a mild desiccation stress since additional water for germination is not supplied (Pammenter *et al.*, 1984, Farrant *et al.*, 1986b, Berjak and Pammenter, 2000). The results obtained for *A. marina* seeds here strongly contradict this suggestion in that WC in stored seeds remained relatively unchanged between 0 to 10 d but increased significantly after 10 d which coincided with significantly high levels of viability loss (Fig. 2.2a). This increase in axis WC could well be a consequence of the axis tissues actively absorbing water from the surrounding humid atmosphere with which they are in direct contact with due to the anatomy of these seeds. In contrast, in *T. dregeana* the axis is sandwiched

between large oily cotyledons and is not in direct contact with the humid atmosphere within the storage buckets, which may explain why axes of these seeds declined in WC as storage progressed (Fig. 2.2b). This increase in axis WC in *A. marina* axes was accompanied by an increase in H_2O_2 production from 15 d onwards (Fig. 2.2e). This increased ROS production (and WC) in *A. marina* seeds during hydrated storage may represent part of the biochemical trigger for germination described by Varghese *et al*. (2011) for this species. However, since this trigger was not accompanied by the provision of additional water for germination to progress in this case, the seeds lost viability (Fig. 2.2a). Total glutathione levels increased significantly initially (from 0 till 10 d) in these seeds, however, total glutathione levels significantly ($P < 0.05$) decreased on day 15 and 20 coinciding with high viability loss. The ratio of GSH:GSSG was also found to be lower in *A. marina* seeds stored for 20 d compared the control indicating that these seeds experienced oxidative stress due to an imbalance in pro- and antioxidants. Similar, findings i.e. reduced glutathione levels during storage have also been reported in recalcitrant *Ginkgo biloba* seeds (Tommasi *et al.*, 2006).

As alluded to above, *T. dregeana* seeds behaved different to those of *A. marina* during hydrated storage, in terms of axis water content. The data suggest that *T. dregeana* seeds experienced a mild dehydration stress (reduction in axis WC) as storage time progressed (Fig. 2.2b). Seeds of *T. emetica* have also been reported to encounter a mild desiccation stress during hydrated storage (Kioko *et al.*, 2006). At 4 months in hydrated storage there was an initial increase in ROS production in axes of *T. dregeana*, however, thereafter the levels of ROS decreased progressively (Figs. 2.2d and 2.2f) This decrease in ROS was accompanied by a decline in the ratio of GSH:GSSG (3.4:1 at 12 month)relative to the control (36:1). However, unlike in *A. marina* embryonic axes, in *T. dregeana* embryonic axes the decline in this ratio was accompanied by declining rather than increasing ROS levels. This may explain why *T. dregeana* seeds retained >50% of viability up until 12 months, relative to the 4 days exhibited by *A. marina* seeds. Since the decline in ROS beyond 4 months was not accompanied by a rapid decline in viability these results also suggest that the extended storability of these seeds relative to those of *A. marina* may be due to a delay in and/or suppression of the ROS-based trigger for germination. This may be a consequence of losing, as opposed, to taking up axis WCs (as in *A. marina*) during storage.

2.6 Concluding remarks and recommendations

The study confirmed that *A. marina* seeds are much more desiccation sensitive and have a shorter lifespan in hydrated storage compared with *T. dregeana* seeds. Partial dehydration of *A. marina* seeds was associated with a relatively faster rate of water loss compared with *T. dregeana* seeds and a spike in ROS production. Even though this increase in ROS was accompanied by an increase in the GSH:GSSG ratio *A. marina*, seeds lost ± 50% viability in 4 d, suggesting that the glutathione system alone is inadequate for overcoming dehydration-induced oxidative stress in this species. *T. dregeana*

seeds exhibited a comparatively lower rate of water loss during partial dehydration and decreased ROS production. However, dehydration reduced the GSH:GSSG ratio in *T. dregeana* seeds compared to the control which may have led to oxidative stress; hence the viability loss observed during dehydration. The results also suggest that during hydrated storage *A. marina* seeds may lose viability due to an earlier development of in the ROS-based trigger for germination which coincided with a reduced GSH:GSSG ratio. This peak in ROS may be brought about by the uptake of water during storage but this suggestion requires further investigation. In contrast, *T. dregeana* seeds appear to encounter a mild dehydration stress during storage and their extended storage lifespan appears to be a consequence of delaying and/or suppressing the ROS-based trigger for germination in storage. However, as in *A. marina* seeds storage was accompanied by a decline in the GSH:GSSG ratio which may explain why *T. dregeana* also eventually lost viability during storage. The mechanisms of desiccation-induced seed viability loss may therefore differ across recalcitrant-seeded species based on the rate and extent to which they lose water during partial drying and storage. Furthermore, while recalcitrant seed desiccation sensitivity and by implication storage longevity is modulated by redox metabolism, the specific ROS and antioxidants that contribute to this control may differ across species. To investigate some of the postulations made in this study, the next chapter presents data on water uptake, ROS production and glutathione redox capacity during germination in *A. marina* and *T. dregeana* seeds.

CHAPTER 3: Germination associated ROS production and glutathione redox capacity in two recalcitrant-seeded species differing in seed longevity

Anushka Moothoo-Padayachie, Boby Varghese, Norman W. Pammenter, Patrick Govender, and Sershen

3.1 Abstract

This study investigated the relationship between germination rate and storage lifespan in two recalcitrant-seeded species, *Avicennia marina* (Forssk.) Vierh. and *Trichilia dregeana* Sond., in relation to water uptake and oxidative metabolism. Seeds of *A. marina* had a higher germination rate and shorter hydrated storage lifespan than *T. dregeana*. Rapid germination of *A. marina* seeds was associated with high water uptake rates and an early increase in reactive oxygen species (ROS) production and decline in GSH:GSSG ratio. Slower germination in *T. dregeana* seeds was associated with lower water uptake rates, delayed onset of the ROS-based trigger for germination, and high GSH:GSSG ratio. Positive correlations ($p < 0.05$) between ROS production and percent water uptake, and inhibition of germination by ROS scavenging agents confirmed the requirement for heightened ROS levels for germination in both species. Germination rate in recalcitrant seeds appears to be governed by the rate of water uptake and ROS production; the latter being dependent on antioxidant activity. We propose that poor longevity in recalcitrant seeds, such as those of *A. marina*, is based on high rates of water uptake and low levels of ROS scavenging activity that promote the ROS-based trigger for germination during hydrated storage.

Keywords: *Avicennia marina, germination, reactive oxygen species, recalcitrant, storage, Trichilia dregeana.*

3.2 Introduction

Recalcitrant seeds are sensitive to desiccation and chilling, and therefore cannot be stored under conventional storage conditions of low water content (WC) and subfreezing temperatures used for the storage of desiccation tolerant, orthodox seeds (Pammenter and Berjak, 1999). Instead, recalcitrant seeds must be stored at or close to their shedding WC; even mild dehydration adversely affects viability (Eggers *et al.*, 2007). Short- to medium-term storage (days [d] to months) of recalcitrant seeds is achieved by hydrated storage which involves maintaining the seeds under conditions of saturated relative humidity (Berjak and Pammenter, 2004, FAO, 2013). However, such seeds eventually germinate or lose viability in hydrated storage (Farrant *et al.*, 1986b, 1989). Ultrastructural studies have

revealed that during hydrated storage, embryonic axes of recalcitrant seeds undergo germination-associated changes that are very similar to those occurring during orthodox seed germination; such changes include an increase in cell size, extensive vacuolisation, consumption of reserves, and development of mitochondria (Pammenter *et al.*, 1984, Farrant *et al.*, 1986b, Berjak and Pammenter, 2000). Furthermore, these changes imply that additional water is required to complete the process of germination, which if not supplied exposes the seeds to a water stress even under hydrated storage conditions (Farrant *et al.*, 1986b).

Whilst the basis of their loss of viability during storage appears to be common across species, recalcitrant seeds display vastly contrasting seed-storage longevity. Seeds of species such as *Avicennia marina* (Forssk.) Vierh., for example, are fully developed when shed and the period between shedding and visible germination is extremely short, placing major constraints on hydrated storage (16-21 d; Farrant *et al.*, 1997, Calistru *et al.*, 2000). On the other hand, seeds of *T. dregeana* are shed relatively immature, and there is a considerable lag between shedding and visible signs of germination; *T. dregeana* seeds can be successfully stored for several months in hydrated storage (Goveia *et al.*, 2004). Berjak *et al*. (1989) proposed that the hydrated storage lifespan of recalcitrant seeds is dependent on the rate at which the seeds germinate naturally. However, the physiological or biochemical basis of this suggestion is difficult to investigate/illustrate based on the fact that unlike orthodox seeds, recalcitrant seeds do not have a readily identifiable switch from developmental to germinative metabolism (Pammenter and Berjak, 2014). Reactive oxygen species (ROS) are known to play a dual role in seeds; although more widely recognised for their damaging role in cells, extracellularly produced ROS have been implicated in cell wall loosening and elongation (Müller *et al.*, 2009) in germinating orthodox (Liszkay *et al.*, 2004, Kranner *et al.*, 2010) and recalcitrant seeds (Roach *et al.*, 2010).

Compared with orthodox seeds (Wojtyla *et al.*, 2006, Bailly *et al.*, 2008, Gomes and Garcia, 2013), germination associated oxidative metabolism is not fully understood in recalcitrant types. However, Roach *et al.* (2010) have shown that excised embryonic axes of *Castanea sativa* seeds produced maximal $\cdot O_2^-$ just before elongation of axes, while Varghese *et al*. (2011) showed that slight dehydration of *T. dregeana* embryonic axes stimulated germination that was accompanied by high $\cdot O_2^-$ levels (which the authors termed "a biochemical trigger for germination"). Those authors postulated that the decrease in $\cdot O_2^-$ levels upon dehydration to relatively lower WC's (which they referred to as "a dampening of the biochemical trigger for germination") may be responsible for the decline in germination in partially dried *T. dregeana* axes. Additionally, many studies on storage of recalcitrant seeds have indicated that their survival and subsequent germination depends on the delicate balance between ROS production and antioxidant protection (Walters *et al.*, 2001, Tommasi *et al.*, 2006). If this balance is upset, free radical-

induced lipid peroxidation and oxidative stress can damage membranes, enzymes, and nucleic acids, resulting in loss of viability (Smith and Berjak, 1995, Bailly, 2004).

This redox balance in seeds is controlled by the action of enzymatic (e.g., glutathione reductase, ascorbate peroxidase, catalase [CAT], superoxide dismutase [SOD]), and non-enzymatic (e.g., α-tocopherol, flavonoids, phenolics, ascorbate, and reduced glutathione) antioxidants (Bailly, 2004, Kranner *et al.*, 2006). The glutathione reductase system in particular has been shown to be important in this regard, as it serves as a redox buffer in cells (Schafer and Buettner, 2001). The ratio of the components of glutathione/glutathione disulphide (GSH/GSSG) is frequently used as a marker of plant stress (Noctor and Foyer, 1998). Of even greater interest to the present study are reports that glutathione plays a key role in germination in orthodox seeds, with a rise in GSH levels and GSSG occurring just before radical protrusion (Tommasi *et al.*, 2001, Garnczarska and Wojtyla, 2008). Our study investigated the relationship between germination rate and storage lifespan in two recalcitrant-seeded species of contrasting storage longevity, in the context of water uptake rate and oxidative metabolism during germination. These studies were conducted on the seeds of *A. marina*, a mangrove tree, which produces very short-lived seeds (Berjak *et al.*, 1989, Farrant *et al.*, 1992b) and *T. dregeana*, a horticultural tree species, the seeds of which can be stored for much longer periods (Goveia *et al.*, 2004). The study first assessed storage longevity in *A. marina* and *T. dregeana* using hydrated storage and thereafter related germinability in each species to water uptake rate, $\cdot O_2^-$ and H_2O_2 production and ratio of glutathione to glutathione disulphide (GSH/GSSG).

3.3 Materials and Methods

3.3.1 Seed collection

The methods employed for this aspect of the study follow those described in section 2.3.1 of Chapter 2.

3.3.2 Hydrated storage of seeds

Seeds of *A. marina* seeds were soaked in distilled water for 30 min to permit sloughing of the pericarp (Calistru *et al.*, 2000). Naked seeds were then surface-sterilized in 1% sodium hypochlorite for 20 min. After brief rinsing, the seeds were left to dry for four h on a bench top back to their shedding WC. Thereafter, these seeds were stored hydrated in a monolayer, on plastic mesh grids suspended about 100 mm over sterile moistened paper towel, within sealed, sterile opaque buckets at 25 °C (Calistru *et al.*, 2000). Fungal contamination was minimized by an initial application of 2.5 ml L^{-1} of a fungicide

(PrevicurN; active ingredient, propamocarb-HC [AgrEvo, Pietermaritzburg, South Africa]) and then at three day intervals for approximately 30 d (Calistru *et al.*, 2000).

For *T. dregeana*, the arils were removed from seeds before they were surface sterilized using a 1% sodium hypochlorite solution, containing a few drops of Tween 80, for 20 min. Seeds were subsequently soaked in an antifungal cocktail comprising of 0.5 ml L^{-1} Early Impact (active ingredient, triazole and benzimidizole; Zeneca Agrochemicals, South Africa) and 2.5 ml L^{-1} PrevicurN (active ingredient, propamocarb; AgrEvo, South Africa) for 240 min (Calistru *et al.*, 2000, Berjak and Pammenter, 2004). The seeds were then dusted with Benlate (active ingredient: benomyl [benzimidazole]; Dupont), and stored hydrated (as described for *A. marina*) at 16 °C (after Goveia *et al.*, 2004). Seeds of both species that germinated in hydrated storage were regularly removed from the buckets and discarded.

3.3.3 Germination

Fresh and stored seeds of *A. marina* and *T. dregeana* were assessed for germinability (at 5 d intervals for the former and monthly interval for the latter) by sowing seeds (n = 15 for each season) retrieved from hydrated storage in seedling trays (five seeds per tray). Seeds of *T. dregeana* were sown (with aril intact) in commercial potting soil (Grovida), whereas *A. marina* seeds were sown in soil collected from underneath the parent trees. In each case, the soil was watered (with deionised water for *T. dregeana* and sea water for *A. marina*) to field capacity before the seeds were introduced and maintained as such for the duration of germination trial; *A. marina* seeds were watered daily with 500 mL of sea water whereas T. dregeana seeds were watered every other day with 500 mL of distilled water. These germination studies were conducted within a glasshouse (25-28 °C) on the grounds of the University of KwaZulu-Natal. Germination was defined as radicle emergence of at least 4 mm and 10 mm in seeds of *A. marina* (after Farrant et al., 1992a) and *T. dregeana* (after Varghese et al., 2011), respectively, and was scored daily until no further change in germination was recorded for 3 d. All assays were performed on the same batch of seeds. The experiment was performed separately over two seasons.

3.3.4 Water content determination

The methods employed for this aspect of the study follow those described in section 2.3.5 of Chapter 2. Water content was expressed on a dry mass basis (dmb; gram H_2O per gram dry matter [gg^{-1}]) as described in Varghese *et al*. (2011). However, since recalcitrant-seeded species vary in terms of embryonic axes shedding WCs (Ballesteros *et al.*, 2014), axes WCs for both species were normalised by calculating the percentage water uptake according to the following formula:

$$\% \text{ Water uptake} = \frac{\text{Final WC- Shedding WC}}{\text{Shedding WC}} \times 100$$

3.3.5 Estimation of extracellular superoxide

The methods employed for this aspect of the study follow those described in section 2.3.6 of Chapter 2.

3.3.6 Estimation of extracellular hydrogen peroxide

The methods employed for this aspect of the study follow those described in section 2.3.7 of Chapter 2.

3.3.7 Role of superoxide and hydrogen peroxide in seed germination

To evaluate the role of $\cdot O_2^-$ in recalcitrant seed germination, seeds of *A. marina* and *T. dregeana* were imbibed in 0 (control), 50, 100, or 150 µM (w/v) DPI, an inhibitor of NAD(P)H oxidase (Henderson and Chappell, 1996), for 8 h on a shaker at 60 rpm (after Jiang and Zhang, 2002, Xia *et al.*, 2009). Dimethylthiourea (DMTU; w/v), a scavenger of H_2O_2, was used to determine the effect of inhibition of H_2O_2 production on seed germination. Seeds of *A. marina* (with pericarp removed) and *T. dregeana* (with aril removed) were imbibed in 0 (control), 150, 1000, or 5000 µM DMTU for 8 h with continuous shaking at 60 rpm (after Jiang and Zhang, 2002, Xia *et al.*, 2009). Germination tests comprised of three replicates containing 10 seeds each and the entire experiment was repeated twice. Seeds were sown in sterile plastic buckets (0.5 L) containing moistened filter paper (with either 50, 100, or 150 M DPI; or 150, 1000 or 5000 µM DMTU, respectively) and maintained in a glasshouse at 25 °C under natural light and dark conditions. The filter papers were kept moistened with the corresponding concentration of DPI or DMTU, or distilled water for the control seeds, throughout the 30 d. To account for any lag in the onset of germination that may have been brought about by DPI and DMTU, the experiment was continued for an extended period of 30 d. Germination was scored positive when radicle emergence was at least 4 mm and 10 mm in *A. marina* and *T. dregeana* seeds, respectively.

To ensure that the inhibition of germination by DPI was not a consequence of cytotoxicity, following the 8 h treatment with DPI (50, 100, or 150 µM), the embryonic axes were excised and subjected to a 2,3,5 triphenyl tetrazolium chloride (TTC) test. Using the protocols of Harding and Benson (1995) and Verleysen *et al*. (2004), with modifications, embryonic axes (n=5 for both species) were incubated in 1.5 mL aqueous TTC solution (0.3 mL 2% TTC [w/v] in 0.05 M Tris-HCl buffer (pH 7.5) + 1.2 mL

0.05 M Tris-HCl buffer (pH 7.5), immediately after excision. The reduction of colourless TTC to insoluble pink/red triphenyl formazan was taken as a measure of respiratory activity, and hence, viability of the embryonic axis (Moore, 1962). Additionally, to determine whether the inhibitory effect of DPI on seed germination could be reversed in both species, seeds were treated with DPI (50, 100, or 150 µM) in the presence of 100 mM H_2O_2 as described by Ishibashi *et al*. (2010). Germination was scored positive when radicle emergence was at least 4 mm and 10 mm in *A. marina* and *T. dregeana* seeds, respectively.

3.3.8 Determination of intracellular GSH and GSH disulphide (GSSG)

The methods employed for this aspect of the study follow those described in section 2.3.8 of Chapter 2.

3.3.9 Statistical analysis

All data were analysed using IBM SPSS statistics version 22. Data were tested for normality using a Shapiro-Wilk test. Germination and water uptake percentages were √arsine transformed prior to any analyses. Germination, water uptake, superoxide, hydrogen peroxide, and glutathione data were subjected to analysis of variance (ANOVA), where data was parametric, to test for differences within species. Means were separated using a Tukey post-hoc test. Where data did not meet ANOVA assumptions, even after transformation, a Kruskal-Wallis test was applied. Relationships between parameters (germination, ROS production, water uptake, DPI and DMTU concentration) were tested via Pearson (where data was parametric) and Spearman's rank (where data was nonparametric) correlation analyses. An independent-samples *t*-test was used to test for significant differences in ROS, GSH, GSSG and total glutathione levels between species at harvest and when maximum germination was attained. All differences were considered significant at the 0.05 level.

3.4 Results

3.4.1 Storage longevity

After 40 d of storage, *A. marina* seeds displayed high levels of fungal infection despite regular antifungal treatments, and germinability was reduced from 100% (on day 0) to 35% after 40 d in hydrated storage (Fig. 3.1a). Seeds of *T. dregeana* had a relatively longer storage lifespan, displaying 100% germinability after one year in storage (Fig. 3.1b). This data confirmed that the two species have highly contrasting storage longevity.

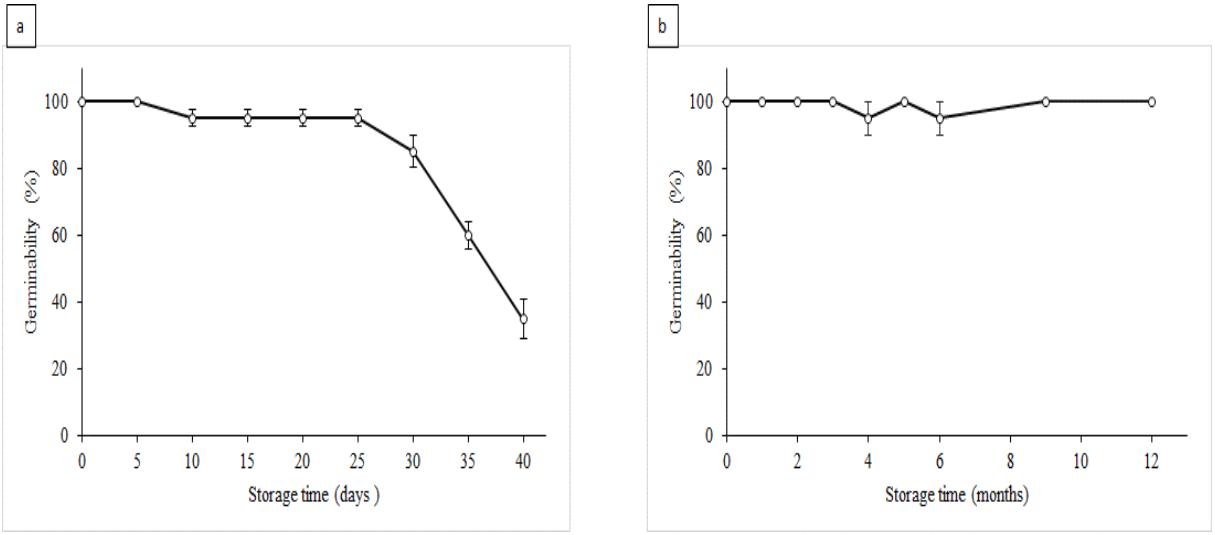

Figure 3.1 *A. marina* (a) and *T. dregeana* (b) seed germinability following hydrated storage at 25 °C and 16 °C respectively. Values represent mean ± SD (*n* = 30).

3.4.2 Water uptake, germination and ROS production

The initial (0-3 days after sowing [DAS]) rate of water uptake, measured in terms of change in embryonic axis WC after sowing in soil watered to field capacity, was markedly higher in *A. marina* (0.4 g d^{-1}) than in *T. dregeana* (0.08 g d^{-1}) (Fig. 3.2a and 2b). More specifically, WC increased by >70% 3 d in *A. marina* but by only ±7% in *T. dregeana* embryonic axes over the same time period. Axis WC increased by >70% 20 DAS in *T. dregeana*, which also coincided with the attainment of 100% germination (Fig.3.2a and b). While 100% of *A. marina* seeds started germinating 3 DAS, only 27% of *T. dregeana* seeds showed signs of germination 7 DAS. In both species, percent germination, and rate of $\cdot O_2^-$, and H_2O_2 production (Figs. 3.2a-2f) increased as percent water uptake increased. A strong positive correlation was found between percent water uptake and rate of $\cdot O_2^-$ production in *A. marina* ($r = 0.977$, $p = 0.023$) and *T. dregeana* ($r = 1$, $P < 0.01$). Similarly, a strong positive correlation existed between percent water uptake and rate of H_2O_2 production in *A. marina* ($r = 0.991$, $P = 0.009$) and *T. dregeana* ($r = 0.954$, $p = 0.001$).

In both species, maximum percent germination coincided with a peak in percent water uptake and ROS production. In *A. marina*, percent germination was positively correlated with percent water uptake ($r = 0.775$), rate of $\cdot O_2^-$ ($r = 0.775$) and H_2O_2 ($r = 0.775$) production, but as germination in these seeds was highly synchronous (100% of the seeds germinated on 3 DAS), these relationships were not significant ($P > 0.05$ in all cases). In *T. dregeana* seeds, percent germination was strongly positively

correlated with percent water uptake ($r = 0.962$, $p = 0.001$), and rate of $\cdot O_2^-$ ($r = 0.964$, $P < 0.001$) and H_2O_2 ($r = 0.938$, $P = 0.002$) production; germination in this species was asynchronous. However, while percent water uptake and ROS production peaked at maximum percent germination in both species, this process occurred 6.67 times faster in *A. marina* seeds. When ROS levels at harvest and when 100% germination was attained were compared statistically between species; $\cdot O_2^-$ levels at harvest were significantly ($P < 0.001$) higher (3.4-times) in the embryonic axes of *T. dregeana* than in *A. marina*. Levels of H_2O_2 at harvest were also significantly ($P < 0.001$) higher (5-times) in *T. dregeana* than in *A. marina*. When germination reached its maximum in both species, $\cdot O_2^-$ levels were significantly ($P < 0.001$) higher (6.6-times) in the embryonic axes of *T. dregeana* than *A. marina*. Furthermore, H_2O_2 levels at this point were also significantly ($P < 0.001$) higher (3.5 times) in the embryonic axes of *T. dregeana* compared to *A. marina*.

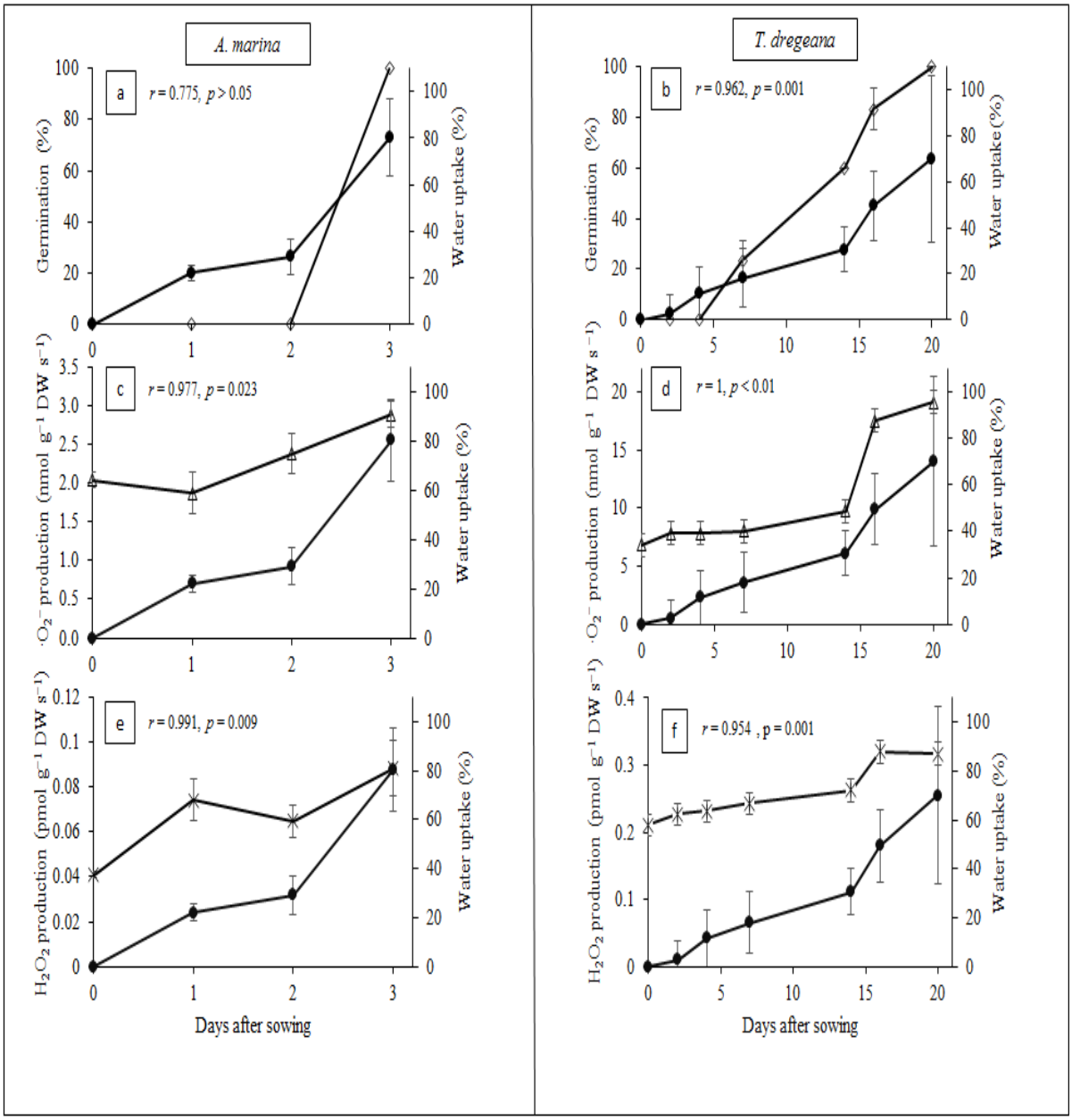

Figure 3.2 Percentage germination (◊; a and b), extracellular superoxide production (△; c and d) and extracellular hydrogen peroxide production (×; e and f) during germination of *A. marina* and *T. dregeana* seeds respectively, sown in soil maintained at field capacity. Embryonic axes percentage water uptake (•) during various stages of germination is shown for both species in graphs 2a to 2f. The results of the correlation between variables (*r* and *p* values) is given in each case.

3.4.3 Role of ROS production during germination

The specificity of the assay used to measure the $\cdot O_2^-$ production described above was confirmed by the fact that exposure of excised axes of both *A. marina* and *T. dregeana* to SOD significantly ($P < 0.05$) decreased adrenochrome formation by >50% in both species (i.e. 52% and 65%, respectively; in section 2.4.4, Table 2.1). Superoxide production in freshly excised axes of *T. dregeana* was also inhibited (>50%) by known enzyme inhibitors, viz. DPI and NaN_3 (section 2.4.4, Table 2.1). Similarly, relatively lower but significant ($P < 0.05$) inhibition of $\cdot O_2^-$ production was also observed in freshly excised *A. marina* axes exposed to DPI (43% inhibition) and NaN_3 (36% inhibition) (section 2.4.4, Table 2.1). The specificity of the H_2O_2 assay used was confirmed by the fact that exposure of freshly excised axes to CAT significantly ($P < 0.05$) decreased H_2O_2 production by >50% in both species (section 2.4.4, Table 2.2).

Given the positive results of the confirmatory assays for both ROS species, we investigated the effects of DPI and DMTU treatment on germination in freshly harvested *A. marina* and *T. dregeana* seeds. Pre-treatment of *A. marina* seeds with 50, 100, or 150 µM DPI, a potent inhibitor of NADPH oxidase, resulted in a significant ($P < 0.05$) decline in total germination (100% in control seeds *vs* 50% at 50 µM and 0% in 150 µM DPI-treated seeds after 30 DAS; Fig. 3.3a). The concentration of DPI was strongly negatively correlated ($r = -0.960$, $p = 0.04$) with germination in *A. marina* seeds. Although lower concentrations of DPI had no significant effect on onset of germination or time to reach 50% germination (T_{50}) in these seeds (Fig. 3.3a and 3.3c), 150 µM DPI inhibited germination completely. Pre-treatment of *T. dregeana* seeds with 50 or 100 µM DPI also had no effect on the onset, T_{50} or total germination relative to untreated (control) seeds (100% germination). However, treatment with 150 µM DPI inhibited germination completely. This inhibition was unlikely a consequence of cytotoxicity since all (100%) DPI treated seeds (50, 100, or 150 µM, for both species) stained positively when subjected to a TTC test. Furthermore, the inhibitory effects of DPI (50, 100, or 150 µM, for both species) were fully reversed (i.e., 100% germination) when DPI treated seeds were allowed to germinate in the presence of H_2O_2.

Pre-treatment of *A. marina* seeds with increasing concentrations of DMTU, a H_2O_2 scavenger (Jiang and Zhang, 2002, Xia *et al.*, 2009), led to a decrease in germination, but lower concentrations of DMTU (150 and 1000 µM DMTU) had no effect on the onset and T_{50} germination compared with untreated seeds (Fig. 3b and 3d). At the highest concentration (5000 µM DMTU), germination was completely inhibited (Fig. 3.3b). There was a very strong negative correlation ($r = -0.963$, $P = 0.037$) between % germination and DMTU concentration for *A. marina*. However, unlike *A. marina*, the seeds of *T. dregeana* were unaffected by DMTU treatment, in terms the onset, T_{50} and total germination, irrespective of DMTU concentration used in the study (Fig. 3.3b, 3.3d and 3.3f).

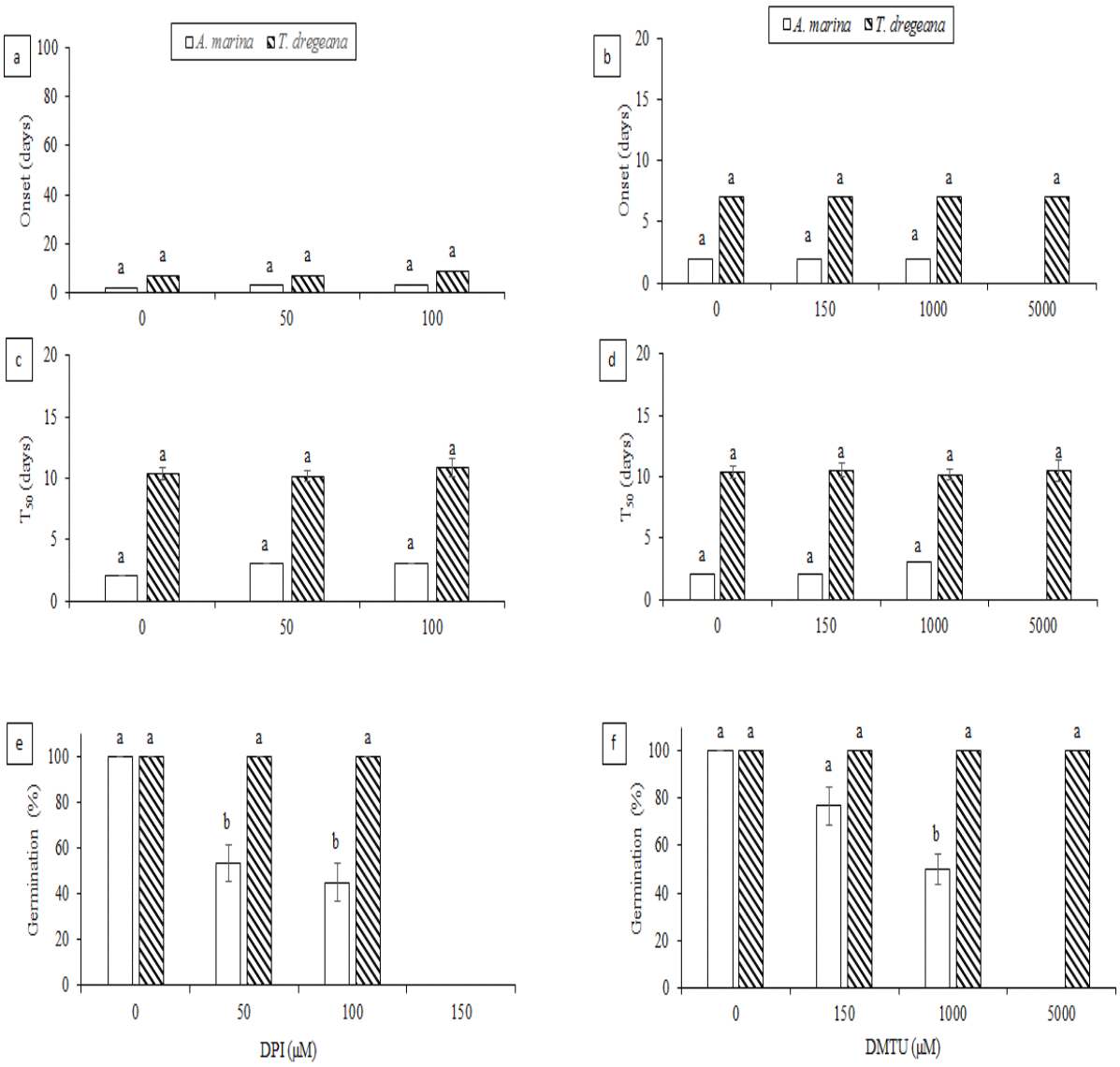

Figure 3.3 Onset (a and b), time taken for 50% of total germination (T_{50}) (c and d) and final percentage germination (e and f) in seeds of *A. marina* and *T. dregeana* respectively, treated with various concentrations of diphenylene iodonium (DPI) and dimethylthiourea (DMTU). Values represent mean ± SD (n = 60). Bars labelled with different letters are significantly different when compared within species, across treatments (p <0.05 in all cases). When germination was correlated with DPI concentration, r = −0.960 and p = 0.04 in *A. marina* and r = −0.963 and p = 0.037 in *T. dregeana*.

3.4.4 Glutathione pool and germination

In the embryonic axes of *A. marina*, GSH levels and the GSH: GSSG ratio were initially high at harvest and shortly after sowing (1 DAS), but thereafter decreased, with the lowest levels of GSH and GSH: GSSG ratio being recorded 3 DAS, when maximum germination was attained (Fig. 3.4a). In comparison, GSH levels in the embryonic axes of *T. dregeana* far exceeded those of GSSG at harvest and throughout the period leading up to, and including the attainment of 100% germination (Fig. 3.4b). Total glutathione (GSH+GSSG) in *A. marina* axes was significantly ($P < 0.001$) higher at harvest than when maximum germination occurred. However, in the axes of *T. dregeana*, total glutathione levels at harvest were significantly ($p < 0.001$) lower than when maximum germination was reached. When the levels of GSH and GSSG were compared separately between species at harvest and when germination occurred, the embryonic axes of *A. marina* exhibited significantly ($P < 0.001$) higher GSH levels than *T. dregeana* at harvest; however, when germination peaked GSH levels in *T. dregeana* were significantly ($P = 0.001$) higher than in *A. marina*. Axes of *A. marina* also possessed significantly ($P < 0.001$) higher levels of GSSG than *T. dregeana* at harvest. When germination peaked, GSSG levels in *A. marina* were still significantly ($P < 0.001$) higher (4-times) than in *T. dregeana*.

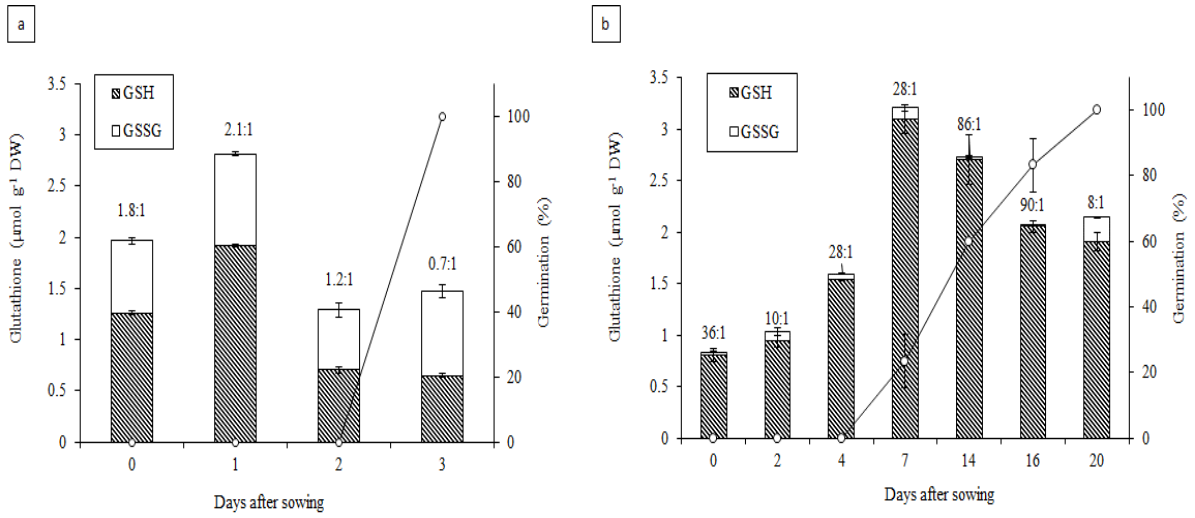

Figure 3.4 Glutathione (GSH) and oxidized glutathione (GSSG), GSH/GSSG ratio and germination in embryonic axes of *A. marina* (a) and *T. dregeana* (b) seeds during different stages of germination. Germination data are represented by circles. Values represent mean ± SD ($n = 3$ replicates of 1 axis for *A. marina* and 5 axes for *T. dregeana*). $p < 0.05$ when initial and final total glutathione (GSH+GSSG) were compared between species on day 0 and on the day coinciding with maximum germination.

3.5 Discussion

The study began by determining the hydrated storage lifespan of two recalcitrant-seeded species *A. marina* and *T. dregeana*. Seeds of *A. marina* exhibited a substantial decline in germinability after 25 d in storage and succumbed to fungal infection despite regular treatment with a variety of antifungal agents (Fig. 3.1a). Although the use of fungicides may reduce contamination and increase storage lifespan (Calistru *et al.*, 2000), seed associated micro-organisms are systemic, thus surface sterilization is not completely effective. Some *A. marina* seeds even began germinating in storage after 5 d, which is a phenomenon reported in recalcitrant seeds of other species (Sershen *et al.*, 2008). However, seeds of *T. dregeana* remained 100% germinable even after one year in hydrated storage. This is in agreement with previous findings that both species have contrasting storage longevities (Berjak *et al.*, 1989, Farrant *et al.*, 1993c).

According to Berjak *et al*. (1989), the hydrated storage lifespan of recalcitrant seeds is dependent on the rate at which these seeds germinate naturally. Our results support this hypothesis; when freshly harvested seeds of both species were put out to germinate, the rate of water uptake and germination was far more rapid in seeds of *A. marina* than *T. dregeana* (Fig.3. 2a and 3.2b). By comparison, 100% of *A. marina* seeds germinated 3 DAS, while only 27% of *T. dregeana* seeds showed signs of germination 7 DAS. Previous studies have shown that *A. marina* seeds accumulate soluble sugars instead of more complex carbohydrates such as heteropolysaccharides prior to germination, which allows for immediate transport and utilization thus facilitating the rapid onset of germination that follows shedding (Farrant *et al.*, 1992b). The results obtained in this study also suggest that rapid germination in species like *A. marina* may be facilitated by the rapid uptake of water preceding germination: percent water uptake in the embryonic axes of *A. marina* was >70% 3 DAS but only ±7% over the same period of time in *T. dregeana* (Fig. 3.2a and 2b). This may simply be a consequence of differences in seed anatomy; the pericarp surrounding *A. marina* seeds breaks open shortly after exposure to water (Farrant *et al.*, 1993a), whilst water uptake is hampered in seeds of *T. dregeana* seeds by a waxy aril which decomposes over time. These difference in water uptake and germination rate most likely have an ecological basis: *A. marina* is a tree species that grows within mangroves in which rapid germination is necessary to exploit the availability of water during high tide events and ensure the avoidance of desiccation stress during low tide events (Farrant *et al.*, 1992b). In contrast, *T. dregeana* is a tree species within sub-tropical forests in which slow germination can allow for opportunistic seedling establishment, i.e., when water and/or light becomes available (Ramlall *et al.*, 2015). Ultrastructural evidence in germinating *A. marina* seeds suggest the early onset (once germination is initiated) of photosynthetic activity in the chlorophyllous cotyledons and embryos of these seeds (Farrant et al. 1992), which could explain their rapid water uptake and germination rate. Similarly, photosynthetic activity of *Posidonia oceanica* seeds has been found to improve the rate of germination and seedling growth (Farrant *et al.*, 1992b, Celdrán

and Marín, 2013). Unlike the cotyledons of *A. marina*, those of *T. dregeana* seeds only show signs of photosynthetic activity after the primary root has been established (>10 days after germination has occurred; Sershen, author's unpublished observations).

In both species, maximum germination coincided with a peak in percent water uptake and ROS production (Fig. 3.2a – 2f); there was also a strong positive correlation between percent water uptake and rate of ROS production in both species. Thus, regardless of differences in the rate of germination between the two species, both $\cdot O_2^-$ and H_2O_2 appear to play a crucial role in germination. Levels of ROS have also been shown to peak at maximum germination in orthodox seeds, e.g., *Lupinus luteus* L. (Garnczarska and Wojtyla, 2008) and *Pinus pinea* L. (Tommasi *et al.*, 2001). Additionally, our results suggest that hydration level (which influences cytoplasmic viscosity) is intimately linked to germination associated ROS production in both rapid and slow germinators. In orthodox seeds too, ROS are mainly able to fulfil their functions as cellular messengers when seeds are hydrated during imbibition and germination (Bailly *et al.*, 2008, Kranner *et al.*, 2010). The faster germination rate in *A. marina* appears to be simply associated with an earlier peak in ROS production compared with *T. dregeana*. Inter-species differences in the timing of germination associated ROS production may have implications on their seed longevity. Varghese *et al*. (2011) have suggested that the peak in extracellular ROS production represents a biochemical trigger for germination. In *A. marina*, this trigger may develop earlier than in *T. dregeana* during hydrated storage, as recalcitrant seeds progress towards germinative metabolism during such storage (Farrant *et al*. 1989). However, in hydrated storage this trigger is not followed by the supply of additional water for germination to progress to completion, and hence results in viability loss.

The results mentioned above strongly suggest that ROS play a role during germination in recalcitrant seeds as in orthodox seeds (Liszkay *et al.*, 2004, Kranner *et al.*, 2010). For $\cdot O_2^-$, this was confirmed by the fact that germination in *A. marina* and *T. dregeana* was inhibited in a dose-response manner when seeds were exposed to DPI (Fig. 3.3e), which is a potent NADPH oxidase inhibitor (after Jiang and Zhang, 2002, Xia *et al.*, 2009). NADPH oxidase is thought to be the major enzyme involved in $\cdot O_2^-$ production. It is, however, acknowledged that DPI is not a specific inhibitor for NADPH oxidase and can inhibit a number of flavoproteins in plant cells, crossing cell membranes, entering cells/subcellular compartments, and blocking respiration irreversibly (Ishibashi *et al.*, 2010). The positive results of the TTC test carried out on DPI treated seeds suggest that respiration was not blocked and that the inhibition of germination by DPI was not a consequence of cytotoxicity.

Furthermore, percent germination in *A. marina* seeds was also compromised in a dose-response manner with exposure to DMTU (Fig. 3f); DMTU is a known H_2O_2 scavenger (Jiang and Zhang, 2002, Xia *et*

al., 2009). Therefore, these results show that H_2O_2 also plays a role in the germination of *A. marina* seeds. The seeds of *T. dregeana*, unlike those of *A. marina*, remained unaffected by treatment with DMTU, irrespective of the concentration used (Fig. 3f). However, the role of H_2O_2 in germination of *T. dregeana* seeds cannot be ruled out since the concentration of DMTU used may have been insufficient to scavenge the relatively high levels of H_2O_2 produced in *T. dregeana* seeds (5-times higher than in *A. marina*). It should be noted though, that the inhibitory effects of DPI on seed germination were completely reversed by exogenously supplied H_2O_2, which is a downstream product of $\cdot O_2^-$ metabolism. Previous studies that have shown that the inhibitory effects of DPI (Ishibashi *et al.*, 2010) and DMTU (De Agazio and Zacchini, 2001) on germination can be reversed by H_2O_2. The results obtained here therefore lend support to the suggestion that ROS are required for germination in *A. marina* and *T. dregeana*.

Whilst the stimulatory role of ROS in germination has been suggested in other recalcitrant seeds (Roach *et al.*, 2010), the balance between pro- and anti-oxidants, and how this is achieved during germination in such seeds still remains unclear. The GSH:GSSG ratio has been well recognized as an indicator of oxidative stress in plant tissues (Noctor and Foyer, 1998), including embryonic axes of recalcitrant seeds (Varghese *et al.*, 2011). In *A. marina* axes, GSH levels and the GSH: GSSG ratio were initially high at harvest, whilst the lowest levels of GSH were recorded when maximum germination was attained (Fig. 3.4a). Similarly, total glutathione (GSH+GSSG) levels were highest at harvest (0 DAS) in this species and significantly lower at the point of maximum germination (3 DAS). These data suggest that when *A. marina* seeds reach the point at which germination occurs (3 DAS), ROS production peaks, while glutathione levels wane and may not be sufficient to prevent the germination associated oxidative burst. In contrast, in the slower germinating *T. dregeana* GSH levels far exceeded those of GSSG at harvest and throughout the period leading up to and including maximum germination (Fig. 3.4b). In orthodox seeds GSH levels have also reported to be maintained at a higher ratio to GSSG levels throughout the process of germination (Tommasi *et al.*, 2001, Garnczarska and Wojtyla, 2008). These results suggest that antioxidant protection may be sustained during hydrated storage in *T. dregeana* seeds, delaying germination associated metabolism i.e., by supressing the ROS signal that triggers germination, and hence, extending their storage lifespan relative to *A. marina* seeds.

3.6 Concluding remarks and recommendations

This study confirmed that *A. marina* had a higher germination rate and shorter storage lifespan than *T. dregeana*. This rapid germination rate in *A. marina* is associated with faster rates of water uptake, earlier spikes in ROS production, and reduced levels of antioxidant activity as germination is approached. This may also explain why recalcitrant-seeded species such as *A. marina* lose viability much earlier during hydrated storage than slower germinating species like *T. dregeana*: the earlier ROS-

based trigger for germination may develop earlier in *A. marina* in hydrated storage and when additional water is not supplied, viability is lost. Rapid water uptake and germination in *A. marina* seeds may also have an ecological basis: this species occurs in mangroves where rapid germination and establishment is necessary to avoid desiccation stress during low tide events.

The study also showed that ROS, as in orthodox seeds, plays a crucial role in recalcitrant seed germination, the timing of which depends on the delicate balance between pro- and anti-oxidant processes. Thus, this study supports findings of Berjak *et al*. (1989), who suggested that hydrated storage lifespan of recalcitrant seeds may be a reflection of the rate at which the seeds germinate naturally. Our current investigations involve measuring parameters related to oxidative metabolism during hydrated storage in *A. marina* and *T. dregeana* in order to validate the propositions made here.

CHAPTER 4: Uncovering the basis of viability loss in desiccation sensitive *Trichilia dregeana* seeds using differential quantitative protein expression profiling by iTRAQ

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4.1 Abstract

Recalcitrant seeds, unlike orthodox types, are desiccation sensitive and hence, cannot be stored using conventional seed storage methods. In this study, relative changes of protein expression in *T. dregeana* seeds during desiccation and hydrated storage (a short- to medium-term storage method) were analysed to understand the basis of their desiccation- and storage-induced viability loss. Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) were used to compare (selected) protein expression levels across fresh, partially dehydrated and stored seeds. A total of 114 proteins were significantly differentially expressed in embryonic axes of fresh seeds and those seeds exposed to dehydration and hydrated storage (which exposed seeds to a mild dehydration stress). Proteins involved in protein synthesis (glycine-tRNA ligase) were up-regulated in stored and dehydrated seeds, possibly in response to dehydration-induced repair processes and/or germinative development. A range of proteins related to antioxidant protection (L-ascorbate peroxidase and glutathione peroxidase) were variably up- and down-regulated in stored and dehydrated seeds respectively. Additionally, a class I heat shock protein was down-regulated in dehydrated and stored seeds; no late embryogenesis abundant proteins were identified in both stored and dehydrated seeds; and storage and dehydration up-regulated proteins involved in the provision of energy for cell survival. The results suggest that dehydration- and storage-induced viability loss in recalcitrant seeds may be based on proteomic changes that lead to cellular redox imbalance and increased cell energy demands. This, together with the absence/down-regulation of proteins associated with desiccation tolerance in plant tissues may form part of the proteomic footprint for desiccation sensitivity in seeds.

Keywords: *desiccation; hydrated storage; proteomics; recalcitrant seeds; redox metabolism*

4.2 Introduction

Recalcitrant seeds, unlike orthodox types, are desiccation and often chilling sensitive (Roberts, 1973, Pammenter and Berjak, 1999). Thus, recalcitrant seeds are not well suited to long-term germplasm conservation using conventional seed storage methods. They can, however, be stored in the short- to medium-term using hydrated storage, which involves maintaining seeds under conditions of reduced temperature and saturated relative humidity (Eggers *et al.*, 2007). Studies have shown though, that even under these conditions, embryonic axes of recalcitrant seeds undergo germinative development and if additional water is not supplied to complete this process the seeds lose viability as a consequence of a mild desiccation stress (Pammenter *et al.*, 1984, Farrant *et al.*, 1986b, Berjak and Pammenter, 2000).

Desiccation sensitivity in recalcitrant seeds has been attributed to the absence or poor expression of a range of mechanisms associated with desiccation tolerance (Pammenter and Berjak, 1999), which is a polygenic trait (Dussert *et al.*, 2004). Examples of the mechanisms activated during drying in orthodox seeds include the following: the active down-regulation/ "switching off" of metabolism (Leprince *et al.*, 2000), the accumulation of sucrose and other oligosaccharides (Horbowicz and Obendorf, 1994), the production of late embryogenesis abundant (LEA) proteins (Farrant *et al.*, 1993c), the presence and operation of repair mechanisms during rehydration (Oliver *et al.*, 1998), and the up-regulation of "housekeeping" antioxidants that control reactive oxygen species (ROS) generation during water loss (Pukacka and Ratajczak, 2007).

Some of the mechanisms associated with desiccation tolerance mentioned above have been shown to be absent, poorly expressed or compromised by dehydration in the recalcitrant seeds of a range of species. For example, a range of enzymic and non-enzymic antioxidants involved in the quenching of ROS in seeds (Bailly, 2004, Kranner *et al.*, 2006), appear to be either inadequate or compromised during desiccation (Hendry *et al.*, 1992, Varghese and Naithani, 2002) and hydrated storage (Tommasi *et al.*, 2006) in recalcitrant seeds. Additionally, in recalcitrant *Acer saccharinum* (Pukacka and Ratajczak, 2006) and *T. dregeana* (Whitaker *et al.*, 2010) seeds, desiccation-induced ROS production, particularly hydrogen peroxide (H_2O_2), has been found to be largely responsible for the loss of structural and functional properties of cell membranes and hence, viability loss. However, there are also reports of antioxidants being enhanced during desiccation stress in recalcitrant seeds. For example, components of the glutathione-ascorbate cycle, a metabolic pathway that detoxifies H_2O_2 using antioxidant metabolites such as ascorbate, glutathione and NADPH, and enzymes like ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR), were enhanced at the early stages of desiccation in *A. saccharinum* seeds (Pukacka and Ratajczak, 2006).

Whilst aspects related to desiccation-induced oxidative stress (Varghese and Naithani, 2002), metabolic disruption (Roach *et al.*, 2008) and ultrastructural damage (Berjak and Pammenter, 2000) have been relatively well researched in recalcitrant seeds, the proteomic basis of their desiccation sensitivity remains unclear and under-explored. This is largely because recalcitrant seeds contain many interfering compounds that present numerous challenges to proteomic studies (Garnczarska and Wojtyla, 2008, Parkhey *et al.*, 2015). Despite these challenges, some proteomic studies have shown that desiccation induces a rapid accumulation of antioxidant enzymes including APX, superoxide dismutase (SOD) and proteins in recalcitrant seeds (Chen *et al.*, 2011). However, Bai *et al.* (2011) found that APX, GR, MDHAR and DHAR enzyme activities are induced during the early stages of desiccation and then decline upon further dehydration, resulting in the inefficient removal of ROS.

Additionally, considerable interest has been directed towards the presence/absence of specific proteins known to play a role in desiccation tolerance (Tunnacliffe and Wise, 2007, Battaglia *et al.*, 2008), for example LEA proteins in *Castanospermum australe* (Delahaie *et al.*, 2013) and *A. marina* (Farrant *et al.*, 1996). In a comparative study of the heat-stable proteome of the recalcitrant seeds of *C. australe* and an orthodox legume *Medicago truncatula*, it was shown that for 12 LEA genes, polypeptides were either absent or strongly reduced in *C. austral*e compared with *M. truncatula* (Delahaie *et al.*, 2013). Though those authors showed non-seed specific dehydrins to accumulate at high levels in the cotyledons of recalcitrant *C. austral*e compared with orthodox *M. truncatula* seeds, no dehydrins have been found in the recalcitrant seeds of *A. marina* (Farrant *et al.*, 1996) and *T. dregeana* (Han *et al.*, 1997). It has been speculated that the desiccation sensitivity of seeds is at least partially due to the insufficient accumulation and/or absence of certain dehydrins (Vertucci and Farrant, 1995, Panza *et al.*, 2007) . However, it should be noted that proteomic studies conducted on recalcitrant seeds to date are largely restricted to studies that have employed two dimensional electrophoresis (2-DE) with subsequent protein identification by mass spectrometry (MS/MS) (Bai *et al.*, 2011, Chen *et al.*, 2011, Delahaie *et al.*, 2013, Parkhey *et al.*, 2015). Although 2-DE protein separation can be used to produce insightful protein maps, there are several limitations to this approach related to technical reproducibility, correct spot matching and the low number of proteins identified (Balbuena *et al.*, 2011). A further drawback of at least one of these key studies cited above is that it only examined the heat-stable proteome extracted from the cotyledons, rather than the more metabolically active, developmentally important embryonic axis (Delahaie *et al.*, 2013).

In light of the above, and in line with current proteomic research, the present study aimed to explore the total proteome extracted from the embryonic axes of the desiccation sensitive species *T. dregeana* using a high-throughput technique called Isobaric Tags for Relative and Absolute Quantification (iTRAQ), coupled to mass spectrometry. iTRAQ is a powerful gel-free proteomic method, considered to be one

of the most robust techniques for differential quantitative proteomic analyses (Latterich *et al.*, 2008, Wilm, 2009). The study attempts a global characterization of protein functions, i.e. the functional characterization of proteins in terms of their involvement in cellular pathways in recalcitrant seeds. This was done by identifying, quantifying, annotating and comparing proteins expressed in *T. dregeana* seeds exposed to partial dehydration and hydrated storage, both of which result in viability loss in recalcitrant seeds, with those expressed in freshly harvested (i.e. unstressed) seeds. The data presented allows for a more fundamental understanding of the proteomic basis of desiccation sensitivity in recalcitrant seeds.

4.3 Materials and methods

4.3.1 Seed collection

Seeds of *T. dregeana* were obtained from mature and open capsules harvested directly from trees growing on the Westville campus (29°49.054' S 30°56.521' E) of the University of KwaZulu-Natal, Durban, South Africa. Seeds displaying any visible signs of damage and predation were removed prior to any further processing for reasons discussed in Moothoo-Padayachie *et al*. (2016). Seeds were collected over two seasons (April-June in 2012 and 2013).

4.3.2 Water content determination

The methods employed for this aspect of the study follow those described in section 2.3.5 of Chapter 2. Water content was expressed on a dry mass basis (dmb; g H_2O per g dry matter [g g^{-1}]) as described in Varghese *et al*. (2011).

4.3.3 Hydrated storage treatment

The arils of *T. dregeana* seeds were removed prior to surface sterilization with 1% sodium hypochlorite solution containing a few drops of Tween 20 for 20 min. The seeds were subsequently soaked in an antifungal cocktail comprising of 0.5 ml L^{-1} Early Impact (active ingredient, triazole and benzimidizole; Zeneca Agrochemicals, South Africa) and 2.5 ml L^{-1} PrevicurN® (active ingredient, propamocarb; AgrEvo, South Africa) for 4 h (Calistru *et al.*, 2000, Berjak and Pammenter, 2004). The seeds were then dusted with Benlate (active ingredient: benomyl [benzimidazole], Dupont, USA), and stored hydrated at 16°C (after Goveia *et al.*, 2004). Seeds that germinated in hydrated storage were regularly removed from the buckets and discarded. This was because germinated seeds in the buckets would eventually die and become a source of fungal inoculum for non-germinated ones (Goveia *et al.*, 2004). As alluded to in the Introduction, recalcitrant seeds progress towards germination in hydrated storage which progressively leads to a mild dehydration stress, which terminates in death if additional water is not supplied (Pammenter and Berjak, 1999). In the present study, after 12 months of storage >50% of

the seeds (n=3) had to be removed from the buckets as they showed signs of germination. The remaining non-germinated seeds were assessed for viability (germinability) and used for protein analyses described below.

4.3.4 Desiccation treatment

Freshly harvested seeds were sown in commercial potting soil (Grovida, Durban, South Africa); prior to sowing, the soil was dried for 24 h to remove any excess moisture. Approximately 50 seeds per tray were randomly sown to a depth of 10 mm with the aril intact. The seeds were allowed to dry under glasshouse conditions at 25°C for a period of 20 d. Seed germination was assessed as described below. Fourteen days after sowing, seeds subjected to 62% loss in axis water content which led to ±50% viability loss, were used for the protein analyses described below.

4.3.5 Germination assessment

Seeds were retrieved from the storage (at monthly intervals) and desiccation (at two day intervals) treatments and assessed for germinability. The seeds (n = 15) were sown in commercial potting soil within seedling trays (five seeds per tray) and the soil was maintained at field capacity using deionised water for the duration of the trial. These studies were conducted within a glasshouse (26/18°C, day/night; ambient light) on the grounds of the University of KwaZulu-Natal. Germination was defined as radicle emergence of at least 10 mm and was scored daily until no further change in germination was recorded for 30 d.

4.3.6 Protein extraction

For protein extraction, four replicates of ±100 excised embryonic axes each (± 400 mg) were used for treatments (partially dehydrated and hydrated stored) and the control (freshly harvested). These replicates were generated using two independent experiments, each involving two biological replicates. Total soluble proteins were extracted from the embryonic axes according to Boudet *et al*. (2006) and all chemicals used were reagent grade (Sigma-Aldrich, Germany, unless otherwise stated). Embryonic axes were snap-frozen in liquid nitrogen and then ground in the presence of PVPP (1:1). The powder was then suspended in 1 ml of cold extraction buffer (50 mM HEPES, 1 mM EDTA, complete protease inhibitor cocktail [Roche, Germany]). After centrifugation at 20 000 *g* (4°C) for 15 min the supernatant was removed and 20% trichloroacetic acid (v/v) was added. The mixture was stored at -20°C overnight and thereafter centrifuged at 20 000 *g* (4°C) for 15 min. The supernatant was carefully removed and discarded. The protein pellet was then sequentially washed with 100 µl of ice cold 100% acetone, 80% methanol and 100% methanol. The protein pellet was then centrifuged at 20 000 *g* (4°C), allowed to dry

for 25 min under a fume hood and resuspended in 4% sodium dodecyl sulphate overnight. The protein concentration was determined using a BCA protein assay kit (Pierce, USA) (Smith *et al.*, 1985).

4.3.7 In-solution Trypsin Digestion and iTRAQ Labeling

Total protein (400 µg) was reduced by adding 0.1 volume of dithiothreitol and incubated at 60°C for 1 h. The reduced total protein was then concentrated down to approximately 30 µl. The 30 µl retentate was alkylated with UT buffer (8 M urea, 500 mM triethylammonium bicarbonate [TEAB] and 15 mM methyl methanethiosulfonate [MMTS]) and incubated at 20°C for 15 min. Thereafter, SDS was removed by repeated washes with UT buffer (excluding MMTS). Sample volumes were normalised using 50 mM TEAB buffer. In filter trypsin digestion was performed by adding a trypsin buffer (3 µg of sequencing grade trypsin [Promega, USA] in 500 mM TEAB) to the retentate to produce a final protein:trypsin ratio of 100:1. Any remaining peptides were washed through by adding TEAB. Labelling of the samples with iTRAQ 8-plex reagents (Applied Biosystems, USA) was performed according to the manufacturer's recommendations. After 2 h of labelling at room temperature, 2 µl from the control and treatment samples (was pooled for a test run on the liquid chromatography-mass spectrometer (LCMS) to confirm that the labelling was successful. Millipore water was added to each sample and incubated at room temperature to hydrolyse the labelling reaction. All samples were pooled and ~ 30 µg of total protein was desalted on a C18 spin column (Pierce, USA).

4.3.8 LC-MS/MS analysis

LC−MS/MS analysis was conducted with a Q-Exactive quadrupole-Orbitrap mass spectrometer coupled with a Dionex Ultimate 3000 nano-HPLC system (Thermo Fisher Scientific, USA). The mobile phases consisted of solvent A (0.1% formic acid in water) and solvent B (80% acetonitrile, 10% water, and 0.1% formic acid). Desalted labelled peptides were dissolved in sample loading buffer (95% water, 5% Acetonitrile, 0.05% trifluoroacetic acid) and an estimated 1 µg was then loaded onto a C18 trap column (100 µm × 20 mm × 5 µm). Chromatographic separation was performed with an Acclaim Pep Acclaim (Thermo Fisher Scientific, USA) C18 column (75 µm × 250 mm × 3 µm). The linear gradient for peptide separation was generated at 250 nL/min as follows: time change=148 min, gradient change=6-30% for Solvent B; time change=34 min, gradient change=30-60% for Solvent B; time change=0.1 min, gradient change=60- 90% for Solvent B. The gradient was held at 90% Solvent B for 15 min before dropping down to 50% Solvent B for 15 min and finally re-equilibrated to 6% Solvent B for 15 min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 250°C. The applied electrospray voltage was 1.95 kV.

4.3.9 Database searching

All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1) and X! Tandem (The GPM, thegpm.org; version CYCLONE [2010.12.01.1]). Mascot was set up to search the Sapindales database (579914 entries) assuming the digestion enzyme trypsin. X! Tandem was set up to search a subset of the SwissProt_Sapindales database also assuming trypsin as the digestion enzyme. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.100 Da and a parent ion tolerance of 10 ppm. Methylthio of cysteine was specified in Mascot and X! Tandem as a fixed modification. Glutamine->pyro-glutamine of the n-terminus, ammonia-loss of the n-terminus, glutamine->pyro-glutamine of the n-terminus, oxidation of methionine and iTRAQ8plex of lysine and the n-terminus were specified in X! Tandem as variable modifications. Oxidation of methionine and iTRAQ8plex of lysine and the n-terminus were specified in Mascot as variable modifications.

4.3.10 Criteria for protein identification and quantification

The criteria for protein identification were as follows: Scaffold (version Scaffold_4.4.8, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications; peptide identifications were accepted if they could be established at >95.0% probability by the Scaffold Local False Discovery Rate (FDR) algorithm; protein identifications were only accepted if they could be established at >99% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii *et al.*, 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Only proteins that were identified in both of the two independent experiments were considered. Sequences from the keratins, trypsin and species other than plants were not considered. For protein quantification, the filters were set as follows: (1) mean was chosen for the protein ratio type; (2) minimum peptides was set to two, and only unique peptides were used for quantitation and (3) normalization by mean and outliers were removed automatically. The peptide threshold was set as above for identity. Differentially expressed proteins (DEPs) were determined using an analysis of variance (ANOVA, $n = 4$) at the 0.05 level of significance; an identified protein was considered significantly increased or decreased in abundance if the fold change met the threshold criterion of an iTRAQ ratio of 1.5.

4.3.11 Bioinformatics analysis

Functional analysis of proteins identified was conducted using the Blast2GO tool (Conesa and Götz, 2008) . FASTA format sequences of the identified and quantified protein set was input to Blast2GO. Blast2GO was initially run to incorporate a sequence description by performing a BLASTp search against the Swissprot database (e-value cut-off of 1×10^{-50}, 20 for the retrieved number of BLAST hits,

33 for the highest scoring pair (HSP) length cut-off). Subsequently Blast2GO was employed to map the gene ontology (GO), Enzyme Commission (EC) numbers and Interpro terms, and to annotate the sequences (e-value hit filter of 1×10^{-6}, a Hsp-hit coverage cut-off of 0, an annotation cut-off of 55, and a GO weight of 5). The automatic annotation performed by Blast2GO was manually revised to guarantee accurate assignment. The Blast2GO tool was also used to conduct metabolic pathway analyses of identified proteins according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (http://www.genome.jp/kegg/pathway.html).

4.4 Results

4.4.1 Germination responses to partial dehydration and storage

The shedding WC of embryonic axes excised from *T. dregeana* seeds was 2.5 g g^{-1}. Following partial dehydration, axis WC dropped to 0.9 g g^{-1} and germinability in *T. dregeana* seeds was lost rapidly from 100% (on day 0) to ±50% within 14 d (data not shown). After 12 months of hydrated storage 54.2 ± 3.2% of seeds germinated in storage. Viability of the remaining non-germinated seeds was 100% but these non-germinated seeds showed a significant ($P < 0.05$) reduction in water content (2.0 g g^{-1}) during storage, implying that they were water-stressed during storage.

4.4.2 Proteins identified by iTRAQ and their functional classification

The total proteins extracted from the embryonic axes of freshly harvested (control), hydrated stored and dehydrated *T. dregeana* seeds was explored and compared using the iTRAQ technique (Fig. 4.1). Analysis of protein extracts from all samples resulted in the identification of 311 proteins (5065 spectra) in total that met the criteria for identification as discussed above. Of these, 114 proteins showed significant ($P < 0.05$) differential expression. Functional classification of the differentially expressed proteins (DEP) demonstrated that they were associated with a wide range of biological processes in plants. Based on the GO analysis, of all the processes observed three major biological processes were prominent namely: 'cellular process', 'metabolic process' and 'response to stimulus', representing 80%, 79% and 63% of protein sequences annotated for these GO-terms, respectively (Fig. 4.2a). The cellular component was divided into 10 categories of which 87% of the sequences represented the cell and cell part, 82% the organelle, and only 39% the extracellular region (Fig. 4.2 b). In terms of molecular function, the proteins were divided into four major categories: binding, catalytic activity, structural molecule activity and antioxidant activity representing 71%, 49%, 23% and 10% of all sequences, respectively (Fig. 4.2c). This study, however, focused only on the catalytic activity and antioxidant activity which included oxidoreductase activity and peroxidase activity as depicted in the molecular functional analysis of the DEP (Fig. 4.3).

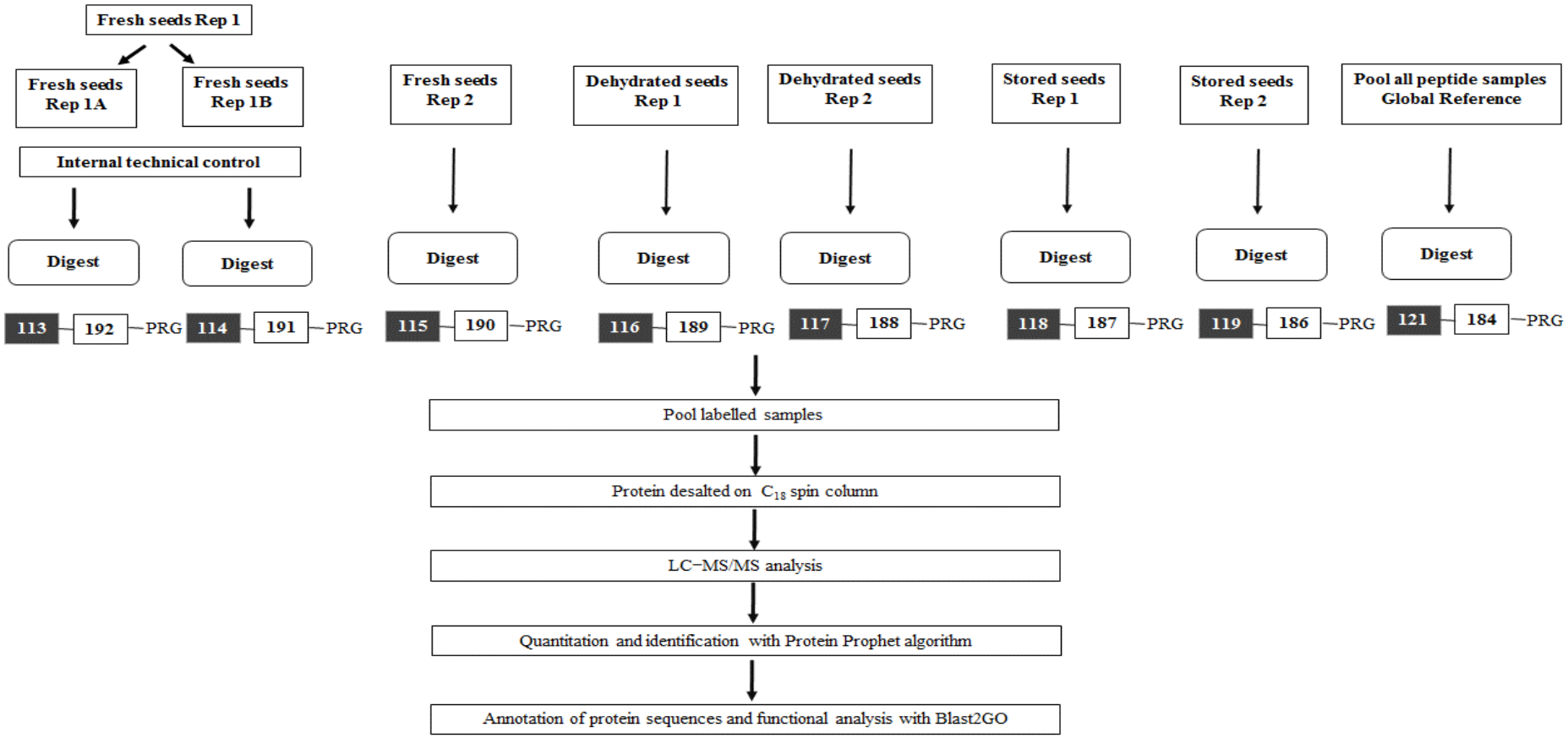

Figure 4.1 Workflow of the iTRAQ experiments for the fresh, partially dehydrated and stored seeds. For each experiment, the extracted proteins were trypsin-digested and peptides labelled with iTRAQ tags. After labelling peptides were pooled and desalted on a C_{18} spin column. The sample was then analysed by LC−MS/MS analysis conducted with a Q-Exactive quadrupole-Orbitrap mass spectrometer coupled with a Dionex Ultimate 3000 nano-HPLC system. Searches and quantitation was done using Scaffold_4.4.8, Proteome Software. Finally, the identified proteins in the set were annotated and functionally analyzed using the Blast2GO tool based on gene ontology (GO) terms.

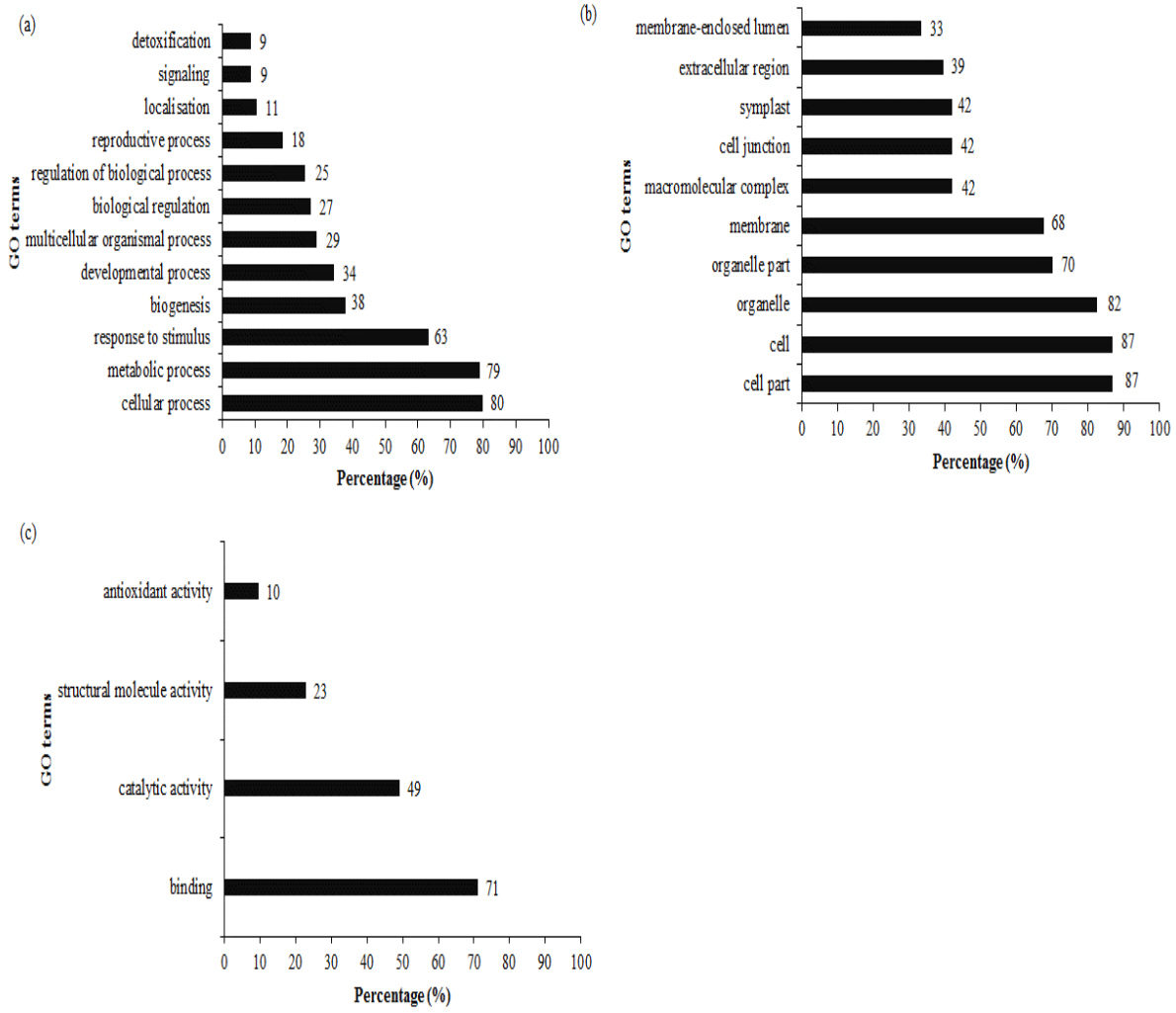

Figure 4.2 Gene ontology annotations for differentially expressed proteins in *T. dregeana* axes: (a) biological process (b) cellular distribution and (c) molecular functions.

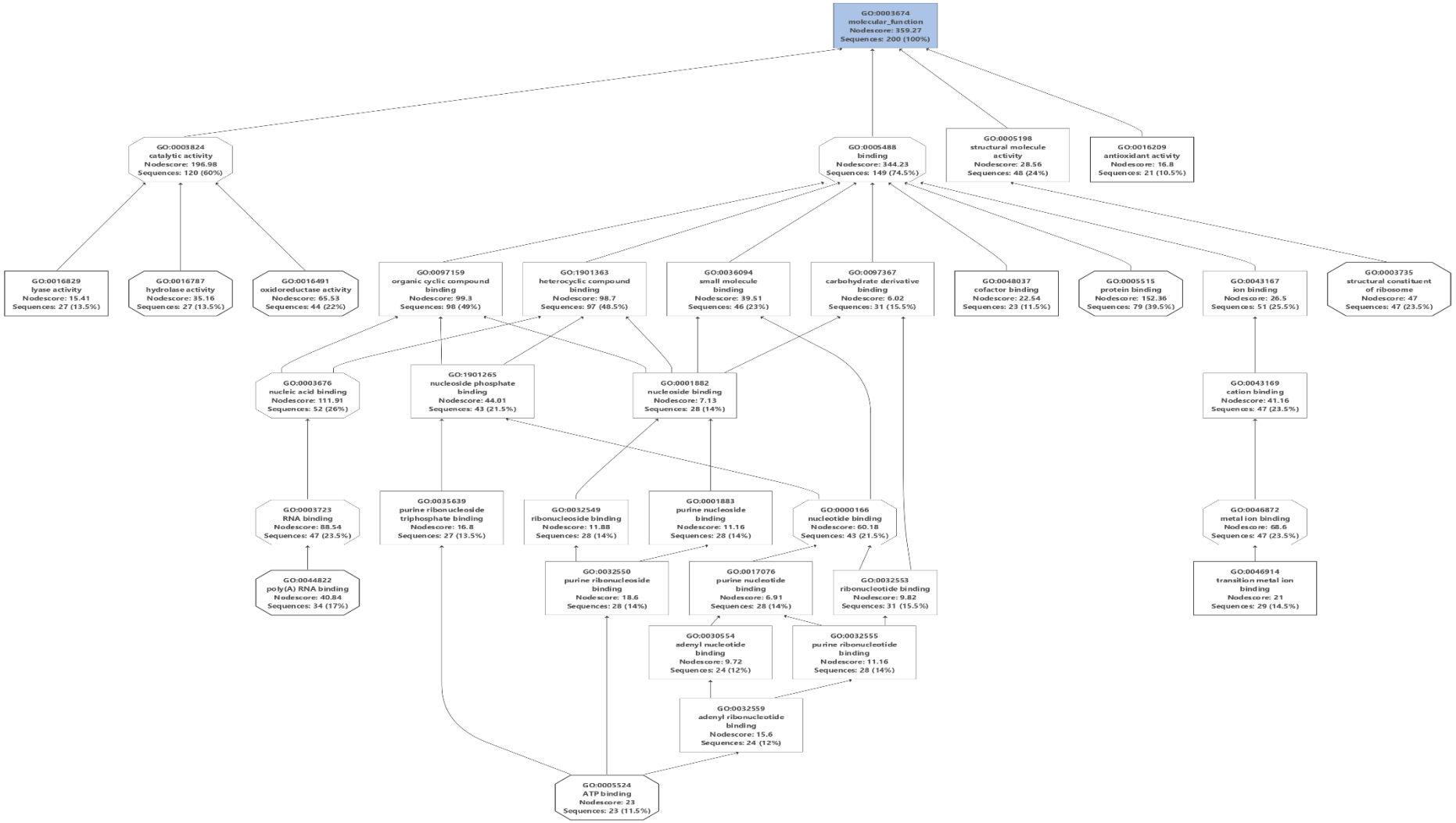

Figure 4.3 Molecular functional groups of 114 differentially expressed proteins in embryonic axes of *T. dregeana* seeds.

4.4.3 Proteome changes in response to dehydration and hydrated storage

Proteins that met a quantification ratio cut-off of 1.5 fold (ratio <0.6 for the down-regulated and >1.5 for the up-regulated proteins), with a significant *P*-value ($P < 0.05$) were considered differentially expressed. Compared with the axes of freshly harvested seeds, 35 proteins were significantly ($P < 0.05$) up-regulated whilst 38 were down-regulated in axes of dehydrated seeds (Fig. 4.4a). With hydrated storage 45 proteins were significantly ($P < 0.05$) up-regulated and 48 were down-regulated, compared with axes from freshly harvested seeds. The average and highest fold change of that set of proteins are shown in Fig. 4.4b. When the amplitude of change is considered, the average fold change in the embryonic axes of dehydrated and stored seeds was not significantly different from each other but the highest fold change in terms of up-regulation was for the protein glycine tRNA ligase 1 **(**O23627**)** in the axes of dehydrated seeds, and for down-regulation of an uncharacterized protein (V4RZM7), which plays a functional role in translation, in axes of stored seeds.

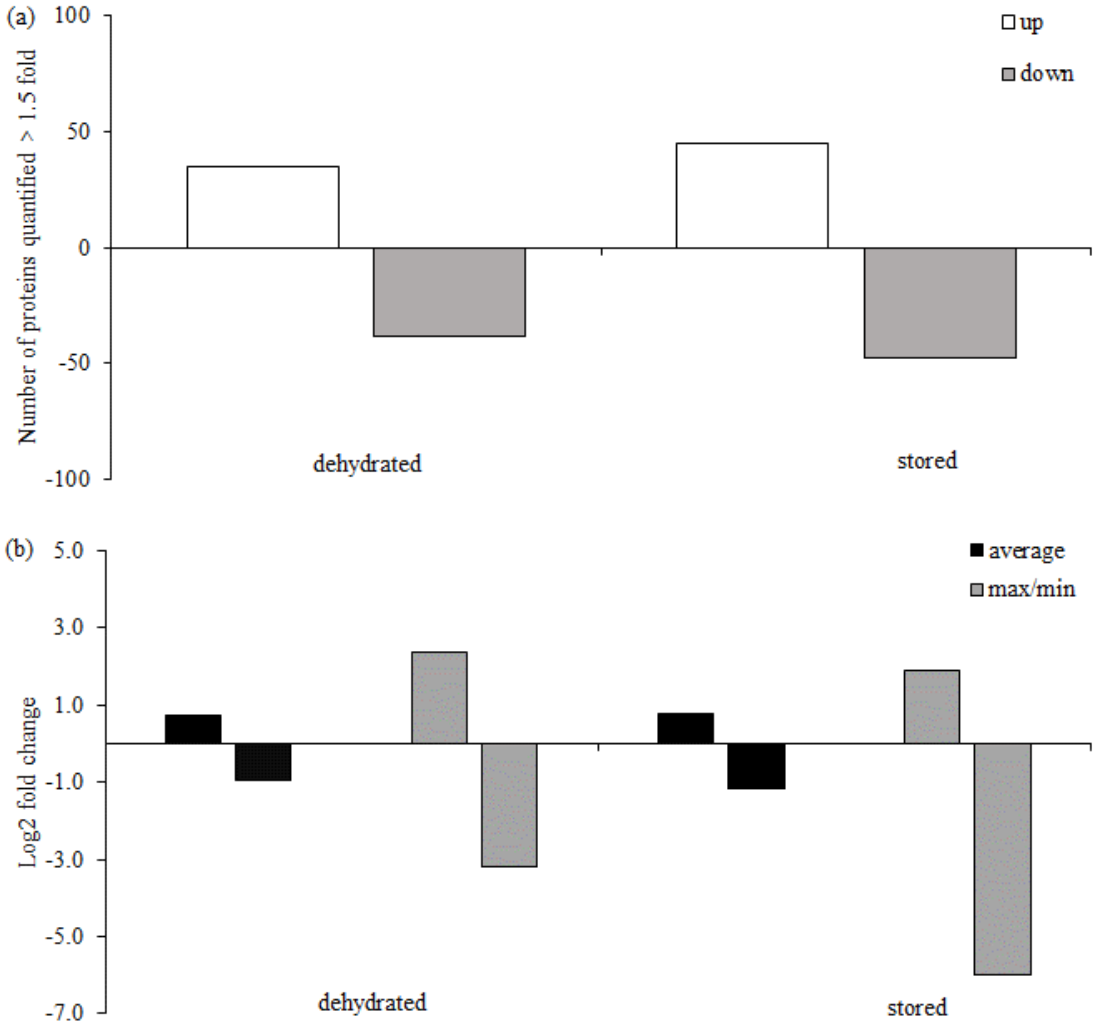

Figure 4.4 Overall changes in the protein level in embryonic axes of partially dehydrated and stored seeds: (a) the number of sequences quantified between the two treatments - for all the proteins that were up- (white bars) and down-regulated (grey bars), and (b) the fold-change of the proteins represented as mean (black) and max/min (grey bars) changes. An arbitrary fold change cut-off of ± 1.5 was used to select the protein subsets.

4.4.4 Molecular functional analysis of dehydrated and stored seeds

The DEPs identified in the embryonic axes of dehydrated and stored seeds were compared and arranged according to their functional categories. Major molecular functions up-regulated in the axes of dehydrated and stored seeds included binding, catalytic activity, structural molecule activity and antioxidant activity (Fig. 4.3). Further comparison of the individual functional categories revealed that proteins up-regulated in response to dehydration and storage were associated with protein binding (GO: 0005515), organic cyclic compound binding (GO: 0097159), ion binding (GO: 0043167), heterocyclic compound binding (GO: 1901363), small molecule binding (GO: 0036094), carbohydrate derivative binding (GO: 0097367), cofactor binding (GO: 0048037), lyase activity (GO: 0016829), oxidoreductase activity (GO: 0016491), hydrolase activity (GO: 0016787) and transferase activity (GO: 0016740) (Table 4.1). However, functional proteins grouped under the structural constituent of ribosome (GO: 0003735) and peroxidase activity (GO: 0004601), were only found to be up-regulated in embryonic axes of dehydrated seeds. Similarly, proteins up-regulated under the functional categories, like amide binding (GO: 0033218) and isomerase activity (GO: 0016853), were found exclusively in axes of stored seeds. The functional categories associated with down-regulation in both the axes of dehydrated and stored seeds include protein binding (GO: 0005515), ion binding (GO: 0043167), organic cyclic compound binding (GO: 0097159), heterocyclic compound binding (GO: 1901363), structural constituent of ribosome (GO: 0003735), peroxidase activity (GO: 0004601) and oxidoreductase activity (GO: 0016491). However, proteins under the functional category SOD activity (GO: 0004784) were only significantly ($P < 0.05$) down-regulated in axes of dehydrated seeds (Table 4.2).

Table 4.1: Molecular function of related up-regulated proteins in embryonic axes of stored and dehydrated *T. dregeana* seeds

Molecular function (GO ID)	Term	Protein number		
		Stored	Dehydrated	Shared [a]
GO: 0033218	amide binding	4	–	0
GO: 0003735	structural constituent of ribosome	–	4	0
GO: 0004601	peroxidase activity	–	3	0
GO: 0005515	protein binding	19	12	9
GO: 0016491	oxidoreductase activity	11	8	6
GO: 0016740	transferase activity	5	5	3
GO: 0016787	hydrolase activity	6	6	3
GO: 0016829	lyase activity	11	9	8
GO: 0036094	small molecule binding	19	14	10
GO: 0043167	ion binding	29	23	14

Table 4.1: Continued Molecular function of related up-regulated proteins in embryonic axes of stored and dehydrated *T. dregeana* seeds

		Protein number		
Molecular function (GO ID)	Term	Stored	Dehydrated	Shared [a]
GO: 0097159	organic cyclic compound binding	24	22	13
GO: 0097367	carbohydrate derivative binding	16	9	6
GO: 1901363	heterocyclic compound binding	23	21	13
GO: 0016853	isomerase activity	6	−	0

[a] Shared indicates proteins with a shared molecular function in the dehydrated and stored seeds

Table 4.2: Molecular function of related down-regulated proteins in embryonic axes of stored and dehydrated *T. dregeana* seeds

Molecular function (GO ID)	Term	Protein number		
		Stored	Dehydrated	Shared [a]
GO: 0003735	structural constituent of ribosome	24	9	9
GO: 0004601	peroxidase activity	3	5	2
GO: 0004784	superoxide dismutase activity	–	2	0
GO: 0005515	protein binding	13	9	5
GO: 0016491	oxidoreductase activity	3	8	2
GO: 0043167	ion binding	6	8	1
GO: 0097159	organic cyclic compound binding	19	9	6
GO: 1901363	heterocyclic compound binding	19	9	6

[a] Shared indicates proteins with a shared molecular function in the dehydrated and stored seeds

4.4.5 Stress response proteins

From the functional categories oxidoreductase, peroxidase and superoxide activity (Table 4.2), a group of important scavenging enzymes were found to be differentially expressed in the axes of dehydrated and stored seeds. In both treatments, the highest percentage of DEPs in response to stress (GO: 0006950) were related to oxidative stress (GO: 0006979) and cellular stress [GO: 0033554] (Fig. 4.5 a-d). These proteins included the up-regulation of L-ascorbate peroxidase (Q10N21), 2-Cys peroxiredoxin (P80602), and isocitrate dehydrogenase (Q8LPJ5) in both treatments (Table 4.3). Interestingly, SOD (V4VU13, A8ICW9) and peroxidase (B7UCP4, V4V6S4) were down-regulated significantly ($P < 0.05$) in axes from dehydrated seeds and catalase (CAT) (V4TFV8) was significantly ($P < 0.05$) down-regulated in axes of stored seeds, while glutathione peroxidase was down-regulated in both axes of dehydrated (B6DVI8, V4VJI5, V4VT52) and stored seeds (B6DVI8, V4VJI5) (Table 4.3). The remaining proteins which were categorised into oxidative stress (GO: 0006979) and cellular stress (GO: 0033554) were up-regulated and related to protein synthesis (B2M1Y5), protein folding (A1ECK2, V4TWG4), other metabolic processes (P26520, V4VKP2, V4TM67), or were uncharacterized (Table 4.3). Most uncharacterized proteins identified in this study showed sequence similarity to proteins from the non-orthodox species *Citrus clementina* (Table 4.3).

Proteins grouped under the sub-category in response to heat (GO:0009408), that is FAM10 family protein At4g22670 (Q93YR3), was found to be down-regulated in axes of dehydrated seeds and a 16.9 kDa class I heat shock protein (Q943E7) was found to be down-regulated in both axes of dehydrated and stored seeds (Table 4.3). Proteins under the sub-category response to water deprivation that were down-regulated in axes of dehydrated seeds were similarly down-regulated in axes of stored seeds. Of these proteins, glutathione peroxidase (V4VT52, B6DVI8, V4VJI5) and peroxidase (B7UCP4) exhibited the most marked changes. However, unlike in the axes of dehydrated seeds in which 12% of the proteins were up-regulated in response to water deprivation, no proteins related to water deprivation were up-regulated in axes of stored seeds (Fig. 4.5a and b). In terms of the percentage of proteins down-regulated in response to water deprivation, 20% of proteins were found to be down-regulated in axes of dehydrated seeds and 13% in axes of stored seeds. In response to stress (GO:0006950), more than 50% of up-regulated proteins included those that are reportedly up-regulated in response to cold stress (GO:0009409) (Fig. 4.5b); this included proteins such as L-ascorbate peroxidase (Q10N21), 2-Cys peroxiredoxin (P80602), ribulose-1,5-bisphosphate carboxylase (RuBisCo) large subunit-binding protein (P08927), ATP synthase subunit alpha (P24459), as well as several metabolic proteins (Table 4.3). Proteins down-regulated in response to cold stress (GO:0009409) in embryonic axes of stored seeds were found to be mainly involved in protein synthesis and included Elongation factor 2 (O23755), 60S acidic ribosomal protein (O04204, O50003) and an uncharacterized protein (V4TJQ9) (Table 4.3).

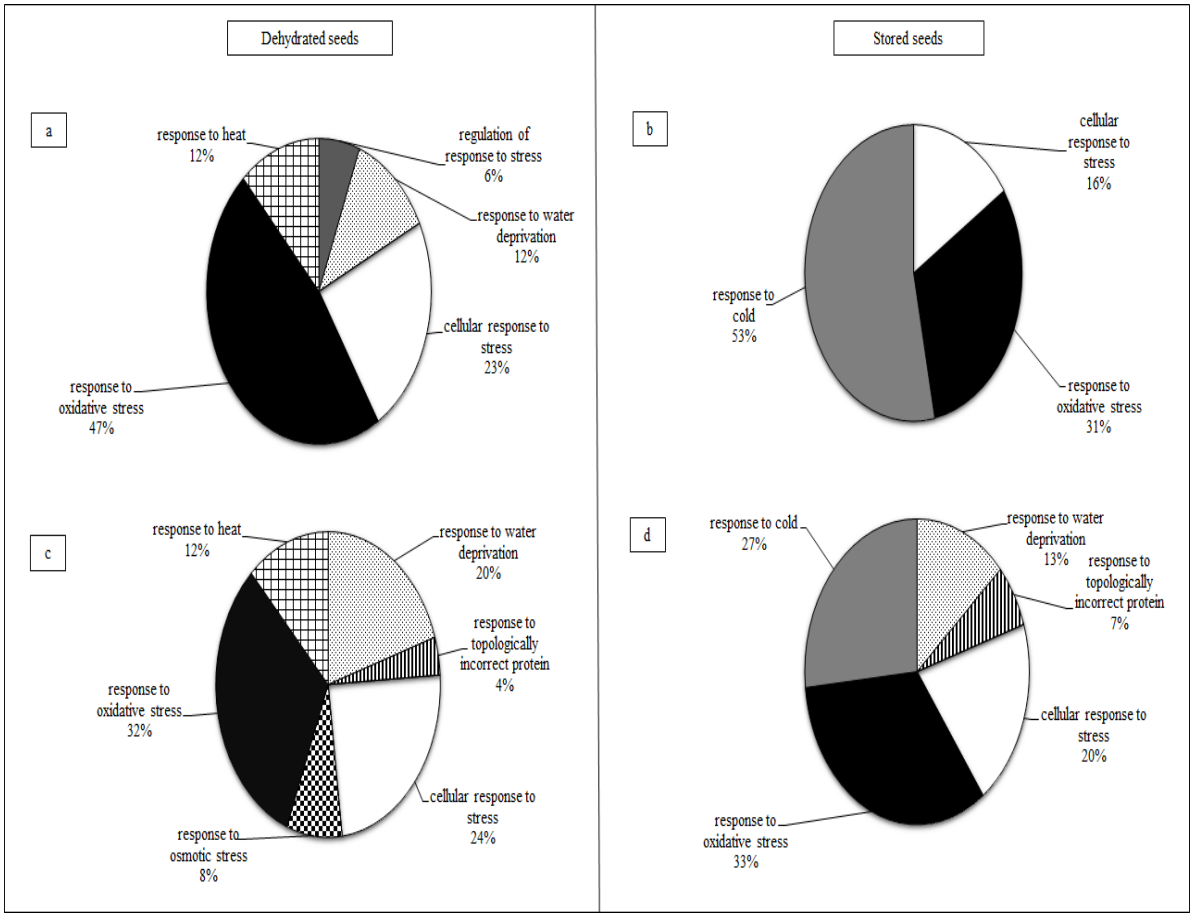

Figure 4.5 Molecular functional groups of proteins associated with response to stress were up-regulated (a and b) and down-regulated (c and d) in the embryonic axes of dehydrated (a and c) and stored (b and d) *T. dregeana* seeds.

Table 4.3: Identification of differentially expressed proteins (DEPs) in *T. dregeana* embryonic axes

Protein description	MW[a]	Acc. No.[b]	SC% [c]	P value[d]	Species	Regulation in dehydrated seeds[e]	Regulation in stored seeds[f]
Regulation of response to stress (GO:0080134)							
Uncharacterized protein	38 kDa	V4VRW5	15	0.005	*Citrus clementina*	↑	
Response to water deprivation (GO:0009414)							
Uncharacterized protein	81 kDa	V4TM62	13	0.0023	*Citrus clementina*	↑	
Uncharacterized protein	26 kDa	V4TPW1	4	<0.0001	*Citrus clementina*	↑	
Glutathione peroxidase	23 kDa	V4VT52	44	0.02	*Citrus clementina*	↓	
Glutathione peroxidase	19 kDa	B6DVI8	31	<0.0001	*Litchi chinensis*	↓	↓
Peroxidase 4	39 kDa	B7UCP4	7	<0.0001	*Litchi chinensis*	↓	
Uncharacterized protein	38 kDa	V4V6S4	5	<0.0001	*Citrus clementina*	↓	
Glutathione peroxidase	19 kDa	V4VJI5	13	<0.0001	*Citrus clementina*	↓	↓
Uncharacterized protein	65 kDa	V4TJQ9	6	<0.0001	*Citrus clementina*	↓	
Response to oxidative stress (GO:0006979)							
Glyceraldehyde-3-phosphate dehydrogenase	37 kDa	P26520	23	<0.0001	*Petunia hybrid*	↑	↑
Uncharacterized protein	65 kDa	V4RNH3	17	<0.0001	*Citrus clementina*	↑	↑
L-ascorbate peroxidase 1	27 kDa	Q10N21	9	<0.0001	*Oryza sativa* subsp. *Japonica*	↑	↑
Uncharacterized protein	77 kDa	V4SVQ9	22	<0.0001	*Citrus clementina*	↑	↑
Uncharacterized protein	26 kDa	V4TPW1	4	<0.0001	*Citrus clementina*	↑	
Aconitate hydratase 2	98 kDa	D3GQL2	10	0.0004	*Citrus clementina*	↑	↑
Uncharacterized protein	38 kDa	V4VRW5	15	0.005	*Citrus clementina*	↑	↑
Glyceraldehyde-3-phosphate dehydrogenase	37 kDa	V4VKP2	44	0.0002	*Citrus clementina*	↑	

Table 4.3: continued Identification of differential expressed proteins (DEPs) in *T. dregeana* embryonic axes

Protein description	MW[a]	Acc. No.[b]	SC% [c]	P value[d]	Species	Regulation in dehydrated seeds[e]	Regulation in stored seeds[f]
Glutathione peroxidase	23 kDa	V4VT52	12	0.02	*Citrus clementina*	↓	
Superoxide dismutase [Cu-Zn]	15 kDa	A8ICW9	34	0.0001	*Dimocarpus longan*	↓	
Peroxidase	39 kDa	B7UCP4	7	<0.0001	*Litchi chinensis*	↓	
Glutathione peroxidase	19 kDa	B6DVI8	31	<0.0001	*Litchi chinensis*	↓	↓
Uncharacterized protein	38 kDa	V4V6S4	5	<0.0001	*Citrus clementina*	↓	
Glutathione peroxidase	19 kDa	V4VJI5	13	<0.0001	*Citrus clementina*	↓	↓
Superoxide dismutase [Cu-Zn]	16 kDa	V4VU13	15	0.0001	*Citrus clementina*	↓	
Uncharacterized protein	65 kDa	V4TJQ9	6	<0.0001	*Citrus clementina*	↓	↓
Peptidyl-prolyl cis-trans isomerase	18 kDa	A1ECK2	5	0.018	*Citrus hybrid cultivar*		↑
Peptidyl-prolyl cis-trans isomerase	29 kDa	V4TWG4	10	0.0033	*Citrus clementina*		↑
Fructose-bisphosphate aldolase	43 kDa	V4TM67	6	<0.0001	*Citrus clementina*		↑
Uncharacterized protein	71 kDa	V4S4E8	36	0.0002	*Citrus clementina*		↑
Uncharacterized protein	18 kDa	V4SLT9	18	<0.0001	*Citrus clementina*		↓
Catalase	57 kDa	V4TFV8	9	<0.0001	*Citrus clementina*		↓
Cellular response to stress (GO:0033554)							
Uncharacterized protein	65 kDa	V4RNH3	17	0.0001	*Citrus clementina*	↑	↑
Uncharacterized protein	77 kDa	V4SVQ9	22	0.0001	*Citrus clementina*	↑	↑
Uncharacterized protein	26 kDa	V4TPW1	4	0.0001	*Citrus clementina*	↑	
Uncharacterized protein	38 kDa	V4VRW5	15	0.005	*Citrus clementina*	↑	↑
Superoxide dismutase [Cu-Zn]	15 kDa	A8ICW9	34	0.0001	*Dimocarpus longan*	↓	
Glutathione peroxidase	23 kDa	V4VT52	12	0.02	*Citrus clementina*	↓	
Glutathione peroxidase	19 kDa	B6DVI8	31	<0.0001	*Litchi chinensis*	↓	↓
Glutathione peroxidase	19 kDa	V4VJI5	13	<0.0001	*Citrus clementina*	↓	↓

Table 4.3: continued Identification of differential expressed proteins (DEPs) in *T. dregeana* embryonic axes

Protein description	MW[a]	Acc. No.[b]	SC% [c]	P value[d]	Species	Regulation in dehydrated seeds[e]	Regulation in stored seeds[f]
Superoxide dismutase [Cu-Zn]	16 kDa	V4VU13	15	0.0001	*Citrus clementina*	↓	
Uncharacterized protein	65 kDa	V4TJQ9	6	<0.0001	*Citrus clementina*	↓	↓
Cell division cycle protein	90 kDa	B2M1Y5	15	0.0214	*Dimocarpus longan*		↑
Uncharacterized protein	71 kDa	V4S4E8	36	0.0002	*Citrus clementina*		↑
Response to heat (GO:0009408)							
Uncharacterized protein	81 kDa	V4TM62	13	0.0023	*Citrus clementina*	↑	
Uncharacterized protein	37 kDa	V4VKP2	44	0.0002	*Citrus clementina*	↑	
FAM10 family protein At4g22670	47 kDa	Q93YR3	6	<0.0001	*Arabidopsis thaliana*	↓	
16.9 kDa class I heat shock protein	17 kDa	Q943E7	9	0.03	*Oryza sativa* subsp. *Japonica*	↓	
Uncharacterized protein	65 kDa	V4TJQ9	6	<0.0001	*Citrus clementina*	↓	
Response to topologically incorrect protein (GO:0035966)							
Uncharacterized protein	65 kDa	V4TJQ9	6	<0.0001	*Citrus clementina*	↓	↓
Response to osmotic stress (GO:0006970)							
Peroxidase	39 kDa	B7UCP4	7	<0.0001	*Litchi chinensis*	↓	
Uncharacterized protein	38 kDa	V4V6S4	5	<0.0001	*Citrus clementina*	↓	
Response to cold (GO:0009409)							
Uncharacterized protein	65 kDa	V4RNH3	17	<0.0001	*Citrus clementina*		↑
Uncharacterized protein	77 kDa	V4SVQ9	22	<0.0001	*Citrus clementina*		↑

Table 4.3: continued Identification of differential expressed proteins (DEPs) in *T. dregeana* embryonic axes

Protein description	MW[a]	Acc. No.[b]	SC% [c]	P value[d]	Species	Regulation in dehydrated seeds[e]	Regulation in stored seeds[f]
2-phospho-D-glycerate hydrolase	48 kDa	D7NHW9	39	<0.0001	*Poncirus trifoliata*		↑
Uncharacterized protein	65 kDa	V4VZ80	28	0.0016	*Citrus clementina*		↑
Uncharacterized protein	31 kDa	V4VGJ1	7	<0.0001	*Citrus clementina*		↑
Enolase	48 kDa	Q43321	37	0.036	*Alnus glutinosa*		↑
Malate dehydrogenase, mitochondrial	36 kDa	P46487	16	<0.0001	*Eucalyptus gunnii*		↑
Uncharacterized protein	61 kDa	V4SV61	10	<0.0001	*Citrus clementina*		↑
RuBisCo large subunit-binding protein subunit beta, chloroplastic	63 kDa	P08927	32	<0.0001	*Pisum sativum*		↑
L-ascorbate peroxidase 1, cytosolic	27 kDa	Q10N21	9	<0.0001	*Oryza sativa* subsp. *Japonica*		↑
2-Cys peroxiredoxin BAS1, chloroplastic (fragment)	23 kDa	P80602	8	0.0042	*Triticum aestivum*		↑
Enolase	48 kDa	P26300	20	0.0014	*Solanum lycopersicum*		↑
Uncharacterized protein	62 kDa	V4SL39	20	<0.0001	*Citrus clementina*		↑
Uncharacterized protein	71 kDa	V4S4E8	36	0.0002	*Citrus clementina*		↑
ATP synthase subunit alpha, mitochondrial	55 kDa	P24459	5	<0.0001	*Phaseolus vulgaris*		↑
Phosphoglycerate kinase	42 kDa	V4S9G5	20	0.002	*Citrus clementina*	↑	
Lactoylglutathione lyase	40 kDa	V4WDC9	7	0.0024	*Citrus clementina*	↑	
60S acidic ribosomal protein	34 kDa	O04204	10	<0.0001	*Arabidopsis thaliana*	↓	
60S acidic ribosomal protein	18 kDa	O50003	22	<0.0001	*Prunus armeniaca*	↓	
Elongation factor 2	94 kDa	O23755	7	0.0016	*Beta vulgaris*	↓	
Uncharacterized protein	65 kDa	V4TJQ9	6	<0.0001	*Citrus clementina*	↓	

[a] Molecular weight of protein
[b] Accession number in Uniprot database
[c] Sequence coverage
[d] Protein with a significant change in abundance (fold change) by a factor > 1.5-fold compared to the freshly harvested seeds (control) by ANOVA ($p<0.05$)
[e] Proteins up -/down-regulated in dehydrated seeds
[f] Proteins up-/down-regulated in stored seeds

4.4.6 Primary metabolic processes

The DEP's in both the embryonic axes of dehydrated and stored seeds were categorized into several metabolic processes (Fig. 4.6). Proteins relating to energy metabolism were up-regulated under the primary metabolic processes and included the carbohydrate metabolic process and the tricarboxylic acid cycle in both dehydrated and stored seeds (Fig. 4.6 a and b). In axes of stored seeds, proteins associated with lipid metabolic processes were also up-regulated. A large percentage of proteins that were down-regulated in the axes of dehydrated (75%) and stored seeds (82%) were grouped under protein metabolic process (Fig. 4.6 c and d).

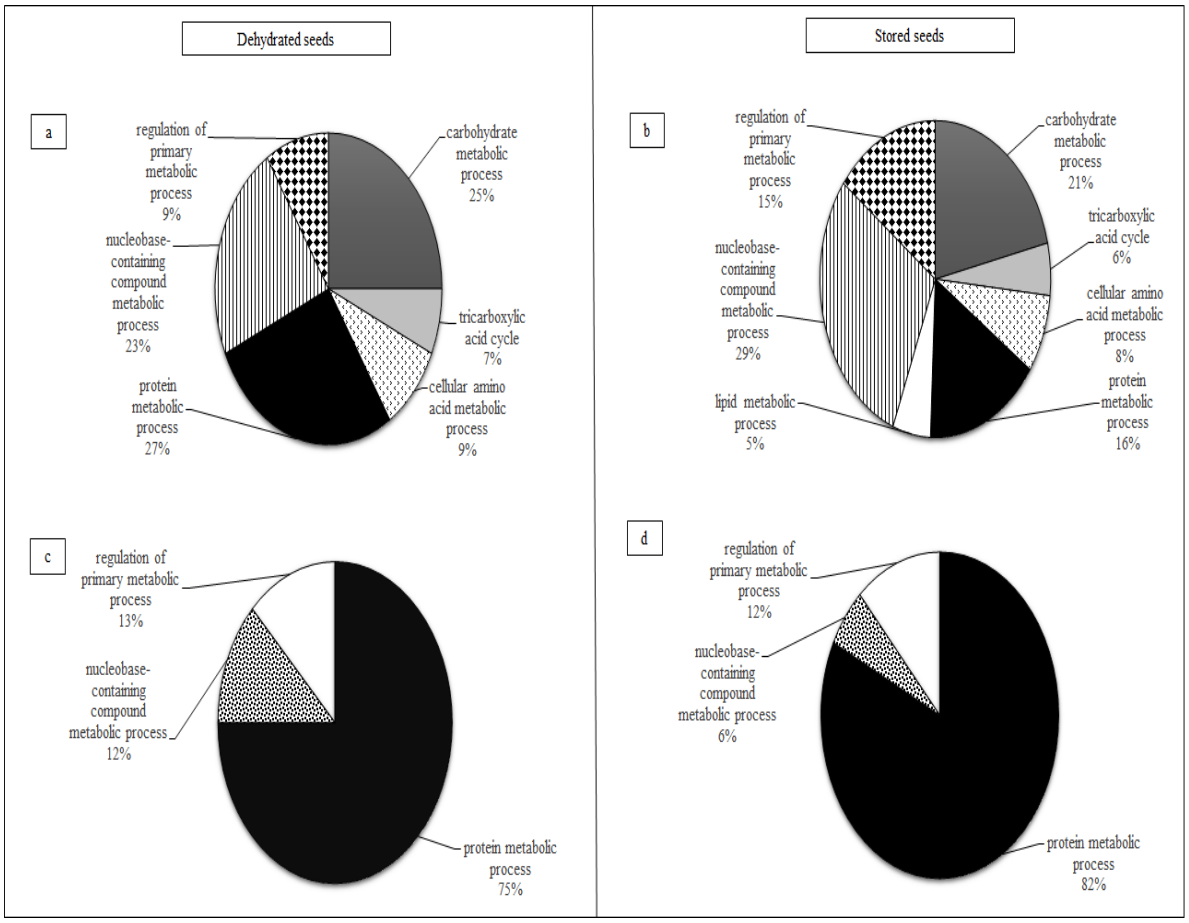

Figure 4.5 Molecular functional groups of proteins associated with primary metabolic processes were up-regulated (a and b) and down-regulated (c and d) in the embryonic axes of dehydrated (a and c) and stored (b and d) *T. dregeana* seeds.

4.4.7 Functional classification based on KEGG Pathway analysis

As an alternative to categorizing unique sequences by biochemical functions, sequences were assigned to metabolic pathways via KEGG (Kanehisa *et al.*, 2015) using enzyme commission (Ec) numbers as the basis for assignment. Major pathways were selected based on the highest number of proteins and enzymes found. These pathways include glutathione metabolism, glycolysis/gluconeogenesis, citrate cycle (TCA cycle) and pyruvate metabolism (Table 4.4). The enzymes involved in glutathione metabolism in both axes of dehydrated and stored seeds showed comparable regulation (Fig. 4.7). However, transferase (Ec: 2.5.1.18) was only found to be up-regulated in the dehydrated seeds. The majority of enzymes involved in glycolysis, TCA cycle, and pyruvate metabolism were up-regulated in axes of both partially dehydrated and stored seeds (Table 4.4).

Table 4.4: KEGG metabolic pathways for *T. dregeana* embryonic axes

Pathway	Dehydrated seeds		Stored seeds	
	Sequences	Up/down-Regulated	Sequences	Up-/down-regulated
Glutathione metabolism				
Dehydrogenase Ec: 1.1.1.42	Q8LPJ5	↑	Q8LPJ5	↑
Peroxidase Ec: 1.11.1.9	V4VJI5, V4VT52, B6DVI8	↓	V4VJI5, V4VT52, B6DVI8	↓
L-ascorbate peroxidase Ec: 1.11.1.11	Q10N21	↑	Q10N21	↑
Glutathione peroxidase Ec: 1.11.1.12	V4VJI5, V4VT52, B6DVI8	↓	V4VJI5, V4VT52, B6DVI8	↓
Transferase Ec: 2.5.1.18	V4TPW1	↑	-	-
Thioredoxin peroxidase Ec: 1.11.1.15	P80602	↑	P80602	↑
Glycolysis/ Gluconeogenesis				
Hydratase Ec: 4.2.1.11	D7NHW9, P26300,	↑	D7NHW9, P26300, Q43321	↑
Kinase Ec: 2.7.2.3	V4S9G5	↑	V4S9G5	↑

Dehydrogenase (NADP +) Ec:1.2.1.9	P26520, V4VKP2	↑	P26520	↑
Dehydrogenase (phosphorylating) Ec: 1.2.1.12	P26520, V4VKP2	↑	P26520	↑
Aldolase Ec: 4.1.2.13	-	-	V4TM67	↑
Isomerase Ec: 5.3.1.1	-	-	V4T6F5	↑
Decarboxylase Ec: 4.1.1.1	-	-	V4SUL7	↓
Hydratase Ec: 4.2.1.11	-	-	D7NHW9	↑
Citrate cycle (TCA cycle)				
Dehydrogenase Ec: 1.1.1.37	V4U2L0	↑	P46487, V4U2L0	↑

Table 4.4: Continued KEGG metabolic pathways for *T. dregeana* embryonic axes

Pathway	Dehydrated seeds Sequences	Stored seeds Up/down-Regulated	Sequences	Up-/down-regulated
Carbon fixation in photosynthetic organisms				
Dehydrogenase Ec: 1.1.1.37	V4U2L0	↑	P46487, V4U2L0	↑
Dehydrogenase (decarboxylating) Ec: 1.1.1.39	P51615	↑	P51615	↑
Kinase Ec: 2.7.2.3	V4S9G5	↑	V4S9G5	↑
Dehydrogenase (oxaloacetate-decarboxylating) (NADP+) Ec: 1.1.1.40	P51615	↑	P51615	↑
Dehydrogenase (phosphorylating) Ec: 1.2.1.12	P26520	↑	P26520	↑
Isomerase Ec: 5.3.1.1	-	-	V4T6F5	↑
Aldolase Ec: 4.1.2.13	-	-	V4TM67	↑
Pyruvate metabolism				
Dehydrogenase Ec: 1.1.1.37	V4U2L0	↑	P46487,	↑
Lyase Ec: 4.4.1.5	V4WDC9	↑	V4WDC9	↑
Dehydrogenase (decarboxylating) Ec: 1.1.1.39	P51615	↑	P51615	↑
Dehydrogeanse (oxaloacetate-decarboxylating) Ec: 1.1.1.38	P51615	↑	P51615	↑
Decarboxylase Ec: 4.1.1.3	P51615	↑	P51615	↑
Dehydrogenase (oxaloacetate-decarboxylating) (NADP+) Ec:1.1.1.40	P51615	↑	P51615	↑
Dehydrogenase (NAD+) Ec:1.1.1.82	-	-	P21528	↑

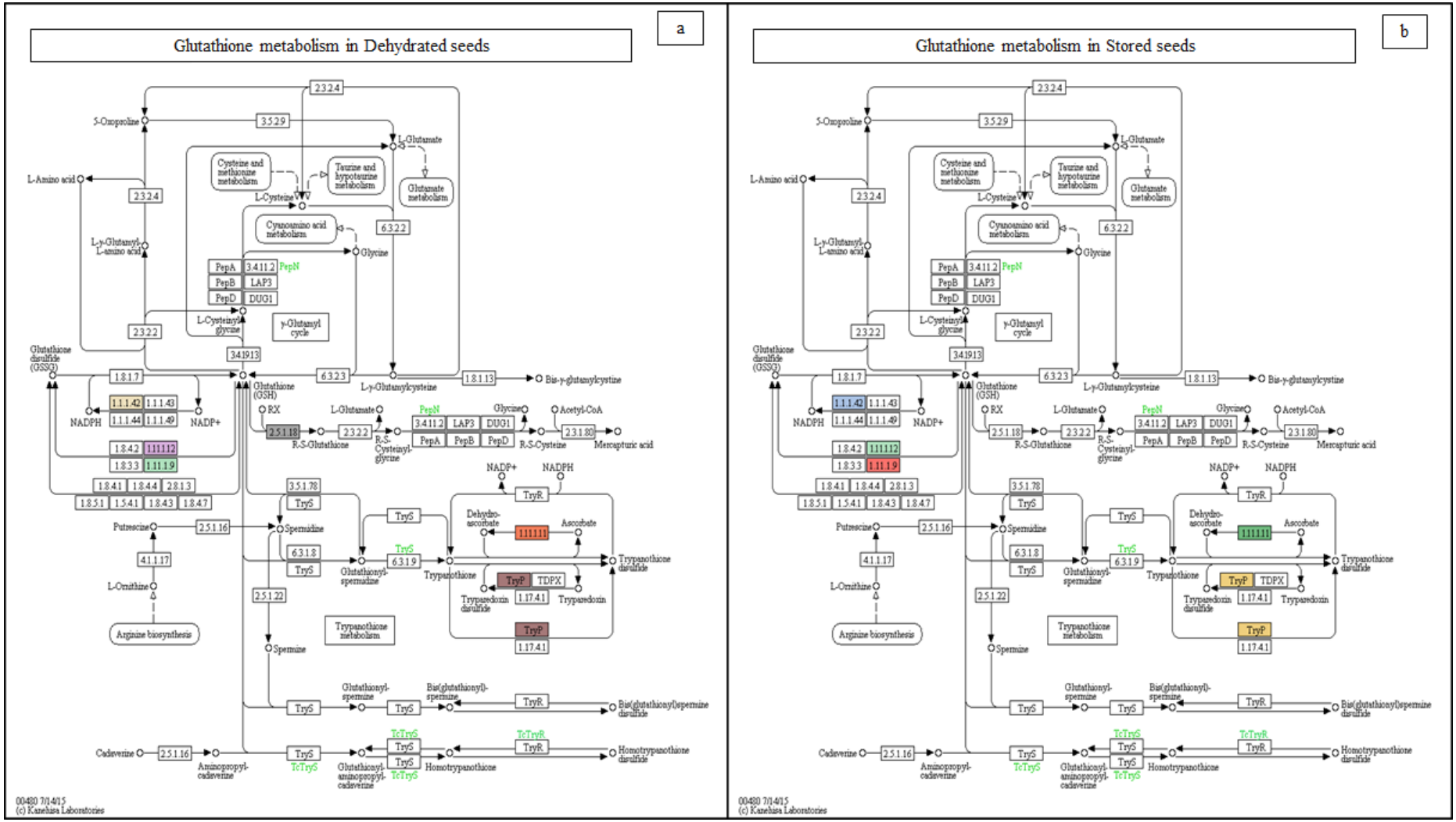

Figure 4.6 KEGG pathway map of glutathione metabolism in embryonic axes of (a) partially dehydrated seeds and (b) stored seeds

4.5. Discussion

Via a comparison of a range of stress biomarkers Sershen *et al*. (2016) illustrated the importance of considering both the type and degree of damage incurred when assessing desiccation stress in recalcitrant seeds. Though recalcitrant seed responses to desiccation and hydrated storage are relatively well characterized in terms of redox metabolism, ultrastructure, physiology and general metabolism, changes at the proteomic level are relatively poorly understood. The present study used a proteomic approach to explore the possible mechanisms underlying viability loss in *T. dregeana* seeds during partial dehydration and hydrated storage. In the present study, *T. dregeana* seeds lost 50% viability after partial dehydration to 0.9 g g^{-1} WC and >50% of the seeds germinated in storage after 12 months. Though the non-germinated seeds were viable after 12 months storage, in recalcitrant seeds hydrated storage is accompanied by the development of a mild dehydration stress as a consequence of germinative development which culminates in viability loss if additional water is unavailable (Pammenter *et al.*, 1994). The germination and significant reduction in axis water content observed during storage of *T. dregeana* seeds in this study therefore suggests that the non-germinated seeds were water-stressed.

In terms of the total average number of fold changes of proteins in the embryonic axes of partially dehydrated and stored *T. dregeana* seeds, there did not seem to be a significant difference (Fig. 4.4a). However, glycine tRNA ligase 1 (O23627) in the axes of dehydrated seeds had the highest fold change in terms of up-regulation while in the axes of stored seeds an uncharacterized protein (V4RZM7) which plays a functional role in translation displayed the highest fold change in terms of down-regulation (Fig. 4.4b). Mitochondrial glycine tRNA ligase 1 plays a crucial role in protein synthesis in plants (Maréchal-Drouard *et al*., 1993, Duchêne *et al.*, 2001). Studies have shown mild/initial dehydration to enhance the rate of protein synthesis in recalcitrant seeds (Farrant *et al.*, 1992a, Sershen *et al.*, 2016). The up-regulation of protein synthesis in the axes of *T. dregeana* seeds during partial dehydration may be associated with mechanisms involved in the repair of the variety of physical and biochemical lesions induced by dehydration, which can lead to metabolic impairment and/or cell death if unrepaired (Sershen *et al.*, 2016). Alternatively, slight dehydration has been shown to stimulate germination in *T. dregeana* (Varghese *et al*., 2011), implying that this enhancement in glycine tRNA ligase 1 may be related to germinative development as it is one of the key steps in orthodox (Cheung *et al.*, 1979) and recalcitrant (Gumilevskaya *et al.*, 2003) seed germination is *de novo* protein synthesis, mediated by preformed mRNA.

Interestingly, ribulose-1,5-bisphosphate carboxylase (RuBisCo) which regulates photosynthetic carbon assimilation in plants was found to be up-regulated in stored seeds (Table 4.3). Recalcitrant seeds of some species like *T. dregeana* can be likened to developing seedlings, since their chlorophyllous

cotyledons and axes show signs of being photosynthetically active leading up to and following germination (Ramlall *et al.*, 2015). The up-regulation of RuBisCo in the axes of stored *T. dregeana* seeds could therefore be linked to the progression of germinative development in storage. Moothoo-Padayachie *et al.* (2016) also suggested that the longevity of recalcitrant seeds like those of *T. dregeana* in hydrated storage is based on the rate and timing of the ROS-based trigger for germination. Furthermore, a large body of evidence suggests that the inability of recalcitrant seeds to retain germinability after desiccation (Pukacka and Ratajczak, 2006, Roach *et al.*, 2010, Sershen *et al.*, 2016) and during hydrated storage (Hendry *et al.*, 1992, Chaitanya and Naithani, 1994) is related to elevated ROS levels and inadequate ROS quenching capacity, particularly in relation to ascorbate and glutathione levels (Tommasi *et al.*, 2001, Pukacka and Ratajczak, 2007). This may explain why in the present study, the majority of DEPs that arose in response to stress were related to oxidative and cellular stress (Fig. 4.5 a-d) and these are discussed below.

Many proteins regulating redox status were up-regulated after partial dehydration and storage. These included L-ascorbate peroxidase, 2-cys peroxiredoxin and thioredoxin peroxidase (Table 4.4) and proteins related to glutathione metabolism (Fig. 4.7). Ascorbate peroxidase is present in all cell compartments and has a high affinity for hydrogen peroxide (H_2O_2), making it an important enzyme in ROS detoxification (Noctor and Foyer, 1998), including in recalcitrant seeds (Pukacka and Ratajczak, 2006, Chen *et al.*, 2011). Pukacka and Ratajczak (2006) found ascorbate peroxidase activity to increase in *A. saccharinum* embryonic axes during desiccation and attributed this to the need to counteract ROS production. Similarly, in a study on recalcitrant *Camellia sinensis* seeds Chen *et al*. (2011) showed L-ascorbate peroxidase to be up-regulated in response to desiccation. However, those authors also showed that with prolonged dehydration ascorbate peroxidase activity decreased.

The proteins 2-cys peroxiredoxin and thioredoxin peroxidase are also involved in protecting lipids, enzymes, and DNA against ROS damage (Rouhier and Jacquot, 2005). Their up-regulation in axes of *T. dregeana* seeds here, suggests that these proteins play a protective role during drying and storage in recalcitrant seeds. In a study by José *et al*. (2009), *Magnolia ovata* seeds were dried to different water contents to assess their viability and their gene expression at the mRNA level in relation to seed development and desiccation tolerance. Those authors found that the expression of the 2-cys-peroxiredoxin gene showed no significant difference in expression between fresh and partially dried *M.. ovata* seeds. However, during the first days of germination this gene was found to be up-regulated in both the fresh and partially dried *M. ovata* seeds. Thus, the results in the current study suggest that the up-regulation of 2-cysperoxiredoxin in partially dehydrated seeds and stored *T. dregeana* seeds could be linked to the progression of germinative development, particularly in storage. Furthermore, 2-cysperoxiredoxin may be required to protect the seed during early germination because of a rise in ROS

levels which serves as a biochemical trigger for germination in *T. dregeana* seeds (Moothoo-Padayachie *et al.*, 2016).

Interestingly, KEGG pathway analysis showed glutathione S-transferase, a major antioxidative enzyme capable of catalysing GSH-dependent reduction of H_2O_2 (Noctor and Foyer, 1998), to be up-regulated but only in axes of partially dehydrated seeds (Fig. 4.7). This result corroborates other suggestions that only selected antioxidant enzymes are enhanced in response to desiccation- versus storage-induced stress in recalcitrant seeds (Pukacka and Ratajczak, 2006, Cheng and Song, 2008, Chen *et al.*, 2011). This selective response of the antioxidant system may be modulated by the intensity and duration of the desiccation stress; hydrated storage has been likened to a mild dehydration stress with low levels of water loss and stress over an extended period of time [weeks to months], whilst natural drying involves much more rapid water loss and intense stress over a shorter period of time (Varghese *et al.*, 2011).

In the present study, partially dehydrated *T. dregeana* seeds showed a 50% decline in germinability after just 14 d, while stored seeds showed signs of a mild dehydration stress: after 12 months >50% of stored seeds germinated in storage and though the non-germinated seeds showed a significant decline in axis water content, they were viable. This may also explain (1) why certain antioxidant enzymes (*viz*. glutathione peroxidase) were down-regulated in axes of partially dehydrated and stored *T. dregeana* seeds and (2) why certain antioxidant enzymes (viz. SOD and peroxidase) were only down-regulated in axes of dehydrated seeds, whilst others (viz. catalase) were only down-regulated in axes of stored seeds. Superoxide dismutase and catalase have been reported as important ROS scavengers during the desiccation (Song *et al.*, 2004, Chen *et al.*, 2011) and storage (Pukacka and Ratajczak, 2005) of recalcitrant seeds. These findings are in agreement with those of Song *et al*. (2004), who also found SOD and CAT activities to decrease significantly in embryonic axes of *T. dregeana* seeds during dehydration, and others (Chaitanya *et al.*, 2000, Varghese and Naithani, 2002, Varghese *et al.*, 2011) who have shown a correlation between dehydration- and storage-induced loss in seed viability and a decline in antioxidant capacity in recalcitrant seeds of a number of species. Furthermore, in a proteomic study on recalcitrant seeds of *C. sinensis* Chen *et al*., (2011) also showed prolonged desiccation to result in the down-regulation of antioxidant enzymes. In the present study, two proteins identified as glutathione peroxidase were down-regulated only in axes of dehydrated seeds while a third was found to be downregulated in axes of stored seeds. Peroxidase was also found to be down-regulated in axes of dehydrated seeds. Glutathione peroxidase and peroxidase play an important role in the detoxification of H_2O_2 (Noctor and Foyer, 1998) . These results may explain the loss in *T. dregeana* seed viability upon partial dehydration and storage in this study. This is in agreement with findings of Chen *et al*. (2011) as mentioned above who found that with prolonged desiccation putative antioxidant enzymes involved in counteracting ROS were down-regulated and this was accompanied by viability loss.

A 16.9 kDa class I heat shock protein identified in *Oryza sativa* (rice) was also found to be down-regulated in both axes from partially dehydrated and stored *T. dregeana* seeds in this study. Wehmeyer and Vierling (2000) showed a correlation between the reduction in small heat shock proteins (sHSPs) and desiccation intolerance in *Arabidopsis* seeds, suggesting that sHSPs play a role in desiccation tolerance. In this regard, two general roles of heat shock proteins (HSPs) have been suggested for helping cells cope with stress-induced damage to proteins: (1) HSPs can promote the degradation of abnormal proteins; (2) HSPs can reactivate stress-damaged proteins and function as "molecular chaperones" to prevent the aggregation or promote the proper refolding of denatured proteins (Parsell and Lindquist, 1993).

Another set of stress-related proteins that has long been implicated in the acquisition of desiccation tolerance are the LEA proteins (Galau *et al.*, 1986). Delahaie *et al*. (2013) found that polypeptides of 12 LEA genes identified in the heat stable genome of an orthodox-seeded species, *M. trancatula*, were either absent or poorly expressed in the cotyledonary tissues of the closely related recalcitrant-seeded species *C. australe* upon dehydration. However, their study was conducted on cotyledonary tissue and hence, could not establish the implications of this dehydration-induced absence/poor expression of LEA proteins in the more developmentally important axis tissues of these recalcitrant seeds. As in the axes of other recalcitrant-seeded species (e.g. *A. marina* [Farrant *et al*., 1996]), the present study identified no LEA proteins in *T. dregeana* embryonic axes. The absence of LEA proteins in *T. dregeana* axes and the down-regulation of sHSPs like16.9 kDa class I heat shock proteins during drying and storage may have therefore also contributed to their desiccation sensitivity and reduced storage lifespan.

Energy demand for cell survival increased in both partially dehydrated and stored seeds of *T. dregeana*, as seen by the up-regulation of key enzymes involved in glycolysis: phosphopyruvate hydratase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase (NADP+) and glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) (Table 4.4). These enzymes are essential for the glycolytic pathway (Plaxton, 1996) and a possible explanation for their up-regulation is the heavy ATP demand associated with desiccation stress as shown in desiccation tolerant plant tissues (Farrant *et al.*, 2015). The up-regulation of phosphoglycerate kinase is particularly significant as it catalyzes the reaction which is the first ATP-generating step of glycolysis, i.e. substrate level phosphorylation (which involves a phosphoryl group transfer from the 'high-energy' mixed anhydride 1,3-bisphosphoglycerate to ADP, generating ATP). Interestingly, certain glycolytic enzymes (aldolase, isomerase and decarboxylase) were also found to be up-regulated in axes of stored seeds but not in axes of dehydrated ones (Table 4.4), which suggests that stored and dehydrated seeds may differ in terms of their energy demands. This could be related to the fact that while stored seeds were experiencing germinative development in

parallel with a prolonged mild dehydration stress, partially dried seeds were subjected to a more intense dehydration stress over a relatively shorter duration.

Lastly, the demand for ATP when *T. dregeana* seeds were exposed to a desiccation stress is further demonstrated by the up-regulation of enzymes involved in the TCA cycle (Table 4.4). These enzymes, viz. malate dehydrogenase, isocitrate dehydrogenase (NADP+) and aconitate hydratase, were up-regulated in both partially dehydrated and stored seeds. Furthermore, in both stored and dehydrated seeds enzymes up-regulated in pyruvate metabolism were found to be associated mainly with the carboxylation of pyruvate to produce oxaloacetate which is one of the citric acid cycle intermediates. The citric acid cycle enzymes up-regulated are involved in the conversion of S-malate to oxaloacetate, citrate to isocitrate and isocitrate to oxalosuccinate, suggesting that pyruvate is being fed into the TCA cycle and driving ATP production, possibly needed in repair mechanisms in dehydrated seeds, and in repair and germination in stored seeds.

4.6 Concluding remarks and recommendations

The nature and degree of response of recalcitrant seeds to desiccation stress is based on both the intensity and duration of the stress. This was evidenced in the present study by the differential seed viability and proteomic responses of *T. dregeana* seeds to partial dehydration (more intense desiccation stress, over short duration) and hydrated storage (mild stress over a prolonged duration). Despite these differences, both stresses (partial dehydration and storage) perturbed parts of the proteome responsible for protein synthesis, maintenance of cellular redox balance, stress tolerance and provision of energy for cell survival significantly. Additionally, LEAs which have been shown to be essential for desiccation tolerance in seeds and plant tissues in general were not expressed in *T. dregeana* axes. Future research should focus on how the over-expression of certain antioxidant enzymes shown to be up-regulated during drying and storage in this study (e.g. SOD and peroxidase) will influence desiccation tolerance in plants and their propagules. The results presented here are in agreement with other proteomic studies on recalcitrant seeds and though more comparative studies on moderately and highly recalcitrant-seeded species are needed, these data represent at least part of the proteomic footprint for desiccation sensitivity in seeds.

CHAPTER 5: Proteomic responses of recalcitrant *Avicennia marina* Forssk. Vierh and *Trichilia dregeana* Sond. seeds to partial dehydration and hydrated storage

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5.1 Abstract

Recalcitrant seeds are desiccation and generally chilling sensitive which severely curtails their hydrated storage lifespan. The aim of the present study was to compare seed physiological and proteomic responses of a highly (*A. marina* (Forssk.) Vierh.) and moderately (*T. dregeana*) recalcitrant-seeded species to partial dehydration and hydrated storage using a label-free LC-MS/MS method. A total of 30 proteins were identified for the embryonic axes of *A. marina* and 105 for those of *T. dregeana*; these proteins were thereafter classified according to their molecular function and arranged into metabolic pathways. Differences in protein expression within treatments, between species were evidenced by proteins such as superoxide dismutase (SOD), adenosylhomocysteinase and calmodulin which was found in higher abundance in dehydrated *T. dregeana* than *A. marina* axes. A glyceraldehyde-3-phosphate dehydrogenase and a 70 kDa heat shock protein was also found in higher abundance in axes of stored seeds of *T. dregeana* compared with axes of stored *A. marina* seeds. In embryonic axes of dehydrated *A. marina* seeds triose phosphate isomerase and a proteasome subunit alpha type were found in higher abundance than in the axes of dehydrated *T. dregeana* seeds. Interestingly, in axes of stored seeds of *A. marina* proteins related to photosynthesis (photosystem II reaction center protein) and a nucleotide diphosphate kinase were found in higher abundance than in axes of stored *T. dregeana* seeds. These results support previous suggestions that the higher metabolic activity and faster germinative development in *A. marina* relative to *T. dregeana* seeds, are responsible for the reduced seed storage lifespan of the former. The relatively higher abundance of proteins such as superoxide dismutase (SOD) adenosylhomocysteinase and calmodulin protein in *T. dregeana* seeds may also provide reasons for why *T. dregeana* seeds are less desiccation sensitive than those of *A. marina*.

Key words: *desiccation sensitive; dehydration; storage; proteins; LC-MS/MS*

5.2 Introduction

Recalcitrant, unlike orthodox, seeds remain sensitive to dehydration throughout their development (Chin and Roberts, 1980, Berjak and Pammenter, 2008), placing major constraints on both their short- to medium- term and long-term storage (Farrant *et al.*, 1997, Calistru *et al.*, 2000). Therefore, to develop more effective germplasm storage methods for recalcitrant-seeded species, it is essential to understand the mechanisms underlying the desiccation sensitivity of their seeds. Many studies on storage of recalcitrant seeds have indicated that their survival during hydrated storage, a method which involves maintaining the seeds under conditions of saturated relative humidity (Berjak and Pammenter, 2004, FAO, 2013), depends on the delicate balance between reactive oxygen species (ROS) production and antioxidant protection (Walters *et al.*, 2001, Tommasi *et al.*, 2006). Studies have shown that in recalcitrant seeds desiccation interferes with metabolic processes leading to an accumulation of potentially toxic ROS such as superoxide ($\cdot O_2^-$), singlet oxygen, hydrogen peroxide (H_2O_2) and hydroxyl radicals (Pukacka and Ratajczak, 2006, Roach *et al.*, 2008, Roach *et al.*, 2010). In plants and their propagules, ROS have been reported to disrupt membrane integrity via lipid peroxidation (Pukacka and Ratajczak, 2006, Roach *et al.*, 2008); damage nucleic acids; and alter protein structure and activity through oxidative modifications such as carbonylation (Johansson *et al.*, 2004, Oracz *et al.*, 2007, Sweetlove and Møller, 2009).

To avoid oxidative damage, seeds contain antioxidant systems which regulate redox metabolism by quenching ROS; these antioxidants include both enzymatic (e.g. glutathione reductase, ascorbate peroxidase, catalase [CAT], superoxide dismutase [SOD] and non-enzymatic (e.g. α-tocopherol, flavonoids, phenolics, ascorbate and reduced glutathione) types (Bailly, 2004, Kranner *et al.*, 2006). However, desiccation, depending on its intensity and duration, can lead to the accumulation of ROS, if not efficiently removed by increased levels of antioxidants (Chen *et al.*, 2011). For example, in *Ginkgo biloba* seeds enzymatic antioxidants were found to be inadequate for counteracting oxidative stress during storage (Tommasi *et al.*, 2006), which itself has been shown to impose a low intensity but protracted water stress (Pammenter *et al.*, 1994). Similarly, dehydration of *T. dregeana* Sond. embryonic axes, has been shown to compromise superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities and result in elevated levels of ROS (Song *et al.*, 2004). Additionally, those authors showed that catalase (CAT), glutathione reductase (GR), and dehydroascorbate reductase (DHAR) activities increases during the initial phase of dehydration but rapidly decreased with further drying. Moothoo-Padayachie *et al.* (2016) (results featured in Chapter 3) also showed that rapid germination of *A. marina* seeds was associated with an increase in ROS production and decline in GSH: GGSG ratio. However, slower germination in *T. dregeana* seeds was associated with a delayed onset of the ROS-based trigger for germination and a high GSH:GSSG ratio. The authors proposed that poor storage

longevity in recalcitrant seeds of species such as *A. marina* may be based on low levels of ROS quenching which allow for the ROS-based trigger for germination to progress during hydrated storage.

Though recalcitrant seed responses to desiccation and hydrated storage are relatively well characterized in terms of redox metabolism, ultrastructure, physiology and general metabolism, the exact mechanisms underlying their desiccation sensitivity are still unclear. Proteomic responses are particularly poorly understood, given the fact that comparisons among species whose seeds differ in their degree of desiccation sensitivity are rare (Balbuena *et al.*, 2011). This is mainly due to the many challenges recalcitrant seeds pose to current proteomic methods; these challenges are largely a consequence of the interfering compounds they contain (Balbuena *et al.*, 2011). Nevertheless, a study by Chen *et al*. (2011) on recalcitrant *Camellia sinensis* embryonic axes found that desiccation induced a rapid accumulation of antioxidant enzymes including APX, SOD and other proteins. In a study on *Antiaris toxicaria* seeds Bai *et al*., (2011) found that APX, GR, MDHAR and DHAR enzyme activities were induced during the early stages of desiccation but then declined with further dehydration, resulting in the inefficient removal of ROS. In both proteomic studies mentioned above, 2DE-gels and LC/MS/MS shotgun proteomics was employed. This method is, however, very limited in its sensitivity, has low dynamic range, and is limited in its reproducibility (Panchaud *et al.*, 2008); although, reproducibility can be enhanced with the use of differential imaging gel electrophoresis (DIGE) (Ünlü *et al.*, 1997, Tonge *et al.*, 2001).

An alternative to this approach involves the use of non-gel based LC-MS/MS (Wang *et al.*, 2008, Schulze and Usadel, 2010). One particular gel-free proteomic method, isobaric tag for relative and absolute quantitation (iTRAQ), has been shown to be among the most robust techniques for differential quantitative proteomic analyses (Latterich *et al.*, 2008, Wilm, 2009). However, labelling of proteins in the presence of interfering compounds can be challenging, especially in recalcitrant-seeded species (Balbuena *et al.*, 2011). Label-free proteomics allows for the quantification of peptides using spectral characteristics, such as retention time, m/z ratio and peak intensity, by comparing the direct mass spectrometric signal intensity for any given peptide or by counting the number of acquired tandem mass spectra matching (Old *et al.*, 2005, Stevenson *et al.*, 2009). It is a relatively new approach that has been successfully used in different systems such as humans (Old *et al.*, 2005), yeast (Foss *et al.*, 2007) and fly (Xun *et al.*, 2009) but reports of its application in plants are rare (Katz *et al.*, 2010).

In light of the need to understand the molecular mechanisms underlying desiccation sensitivity in recalcitrant seeds, and recent advances in proteomic research, the present study compared the seed physiological and proteomic responses of a highly (*A. marina* Forssk. Vierh.) and moderately (*T. dregeana*) recalcitrant-seeded species to partial dehydration and hydrated storage using a label-free LC-MS/MS method.

5.3 Materials and methods

5.3.1 Seed collection

The methods employed for this aspect of the study follow those described in section 2.3.1 of Chapter 2.

5.3.2 Desiccation treatment

The methods employed for this aspect of the study follow those described in section 2.3.2 of Chapter 2.

5.3.3 Hydrated storage of seeds

The methods employed for this aspect of the study follow those described in section of Chapter 2.3.3 of Chapter 2.

5.3.4 Viability assessment

Seeds were retrieved from storage (at monthly intervals; n = 15) and desiccation (at two day intervals; n = 15) treatments and assessed for viability as described in section 2.3.4 of Chapter 2.

5.3.5 Protein extraction

Three replicates of two excised embryonic axes (±300 mg) for *A. marina* and ±100 axes (±400 mg) for *T. dregeana* axes belonging to partial dehydration and hydrated storage intervals associated with ±50% viability loss and the control (freshly harvested seeds) were extracted for total soluble proteins according to Boudet *et al*. (2006). All chemicals used were reagent grade (Sigma-Aldrich, Germany) unless otherwise stated. Embryonic axes were snap-frozen in liquid nitrogen and frozen material was ground in the presence of PVPP (1:1). The powder was then suspended in 1 ml of cold extraction buffer (50 mM HEPES, 1 mM EDTA, protease inhibitor [Roche, Germany]). After centrifugation at 20 000 *g* (4°C) for 15 min the supernatant was removed and 20% trichloroacetic acid (v/v) was added. The mixture was stored at -20°C overnight and thereafter centrifuged at 20 000 *g* (4°C) for 15 min. The supernatant was carefully removed and discarded. The protein pellet was then sequentially washed with 100 µl of ice cold 100% acetone and twice with 80% methanol. The protein pellet was centrifuged at 20 000 *g* (4°C), allowed to dry for 15 min under a fume hood and re-suspended in 4% sodium dodecyl sulphate overnight. The protein concentration was determined using a BCA protein assay kit (Pierce, USA) (Smith *et al.*, 1985).

5.3.6 In-solution digest

All chemicals used were analytical grade (Sigma-Aldrich, Germany) unless otherwise stated. Samples (200 µg of protein each) were reduced by adding 50 mM triscarboxyethyl phosphine (TCEP; Fluka) in 100 mM TEAB (final concentration 5mM TCEP) for 30 min at 37°C. Following reduction, cysteine residues were modified to methylthio using 200 mM methyl methanethiosulphonate (MMTS) in 100 mM triethylammonium bicarbonate (TEAB) solution (final concentration of 20 mM) for 30 min. After modification, the samples were diluted to 98 µL with 100 mM TEAB. Proteins were digested by adding 5 µL trypsin (Pierce) solution (1 mg/1 mL) and incubated for 18 h at 37°C. The samples were then dried down and re-suspended in 50 µL of 2% acetonitrile: water; containing 0.1% formic acid (v/v).

5.3.7 Desalting

Residual digest reagents were removed using an in-house manufactured C_{18} stage tip (Empore Octadecyl C_{18} extraction discs; Supelco). The samples were loaded onto the stage tip after activating the C_{18} membrane with 30 µL methanol (Sigma) and equilibration with 30 µL 2% acetonitrile: water; 0.05% trifluoroacetic acid (TFA) (v/v). The bound sample was washed with 30 µl 2% acetonitrile: water; 0.1% formic acid (FA) (v/v) before elution with 30 µL 50% acetonitrile: water; 0.1% FA. The eluate was evaporated to dryness. The dried peptides were dissolved in 25 µL 2% acetonitrile: water; 0.1% FA for LC-MS/MS analysis as described below.

5.3.8 Liquid chromatography

Liquid chromatography was performed on a Thermo Scientific Ultimate 3000 RSLC equipped with a 2 cm × 100 µm C_{18} trap column and a 35 cm × 75 µm in-house manufactured C_{18} (Luna C_{18}; 5 µm; Phenomenex) analytical column. The solvent system employed was as follows: 2% acetonitrile: water loading solvent, containing 0.1% FA; solvent A: 2% acetonitrile: water, containing 0.1% FA; and solvent B: 100% acetonitrile, containing 0.1% FA. The samples were loaded onto the trap column using loading solvent at a flow rate of 5 µL/min from a temperature controlled auto sampler set at 7°C. Loading was performed for 10 min before the sample was eluted onto the analytical column. Flow rate was set to 350 nL/min and the gradient generated as follows: 2.0% solvent A for 5 min; 2-4% solvent B from 5-10 min; 4-10% from 10-20 min solvent B; and 10-40% solvent B from 20-95 min using Chromeleon non-linear gradient 7; and 40-80% solvent B from 95-100 min. Thereafter, the column was washed for 10 min with 80% solvent B followed by equilibration. Chromatography was performed at 50°C and the outflow delivered to the mass spectrometer through a stainless steel nano-bore emitter.

5.3.9 Mass spectrometry

Mass spectrometry was performed using a Thermo Scientific Fusion mass spectrometer equipped with a Nanospray Flex ionization source. The sample was introduced through a stainless-steel emitter as

mentioned above. Data was collected in positive mode with spray voltage set to 2 kV and ion transfer capillary set to 275°C. Spectra were internally calibrated using polysiloxane ions at m/z = 445.12003 and 371.10024. MS1 scans were performed using the orbitrap detector set at 12 000 resolution over the scan range 350-1650 with AGC target at 3 E5 and maximum injection time of 40 ms. Data was acquired in profile mode.

MS2 acquisitions were performed using monoisotopic precursor selection for ion with charges +2 to +6 with error tolerance set to ±10 ppm. Precursor ions were excluded from fragmentation once for a period of 30 seconds. Precursor ions were selected for fragmentation in HCD mode using the quadrupole mass analyzer with HCD energy set to 35%. Fragment ions were detected in the ion trap mass analyzer using rapid scan rate. The AGC target was set to 1E4 and the maximum injection time to 45 ms. The data was acquired in centroid mode.

5.3.10 Data Analysis

The raw files generated by the mass spectrometers were imported into Proteome Discoverer v1.4 (Thermo Scientific) and processed using the Mascot algorithm (Matrix Science), as well as the SequestHT algorithm included in Proteome Discoverer. Data analysis was structured to allow for methylthio as a fixed modification as well as NQ deamidation (NQ), oxidation (M) and N-terminal acetylation. Peptide validation was performed using the percolator node set to search against a decoy database with FDR less than 1%. An additional analysis, was performed using the X! Tandem Sledgehammer (2013.09.01.1) algorithm using the same settings as before. Database interrogation was performed against the Uniprot green plants with semi-tryptic cleavage allowing for two missed cleavages. The output files generated were combined using Scaffold software version 4.4.3 (Proteomesoftware).

5.3.11 Criteria for protein identification and quantification

Scaffold (version Scaffold_4.7.2, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm (Keller *et al.*, 2002) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99 % probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii *et al.*, 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Quantitation was based on fold change (by sample; n=3) and was performed using total spectra, statistically significant differences were determined by Fisher's exact test using a significance level set to (P<0.05).

5.3.12 Bioinformatics analysis

Functional analysis of proteins identified was conducted using the Blast2GO tool (Conesa and Götz, 2008). FASTA format sequences of the identified and quantified protein set were assigned to gene ontologies using Blast2GO. Blast2GO was initially run to incorporate a sequence description by performing a BLASTp search against the Swissprot database (e-value cut-off of 1×10^{-50}, 20 for the retrieved number of BLAST hits, 33 for the highest scoring pair (HSP) length cut-off). Subsequently, Blast2GO was employed to map the gene ontology (GO), Enzyme Commission (EC) numbers and Interpro terms, and to annotate the sequences (e-value hit filter of 1×10^{-6}, a Hsp-hit coverage cut-off of 0, an annotation cut-off of 55, and a GO weight of 5). The automatic annotation performed by Blast2GO was manually revised to guarantee accurate assignment. The Blast2GO tool was also used to conduct metabolic pathway analyses of identified proteins according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (http://www.genome.jp/kegg/pathway.html).

5.4 Results

5.4.1 Physiological responses to desiccation and storage

The shedding WC of embryonic axes from *A. marina* was *c.* 1.7 g g^{-1}. Following four days of partial dehydration, axis WC dropped to 1 g g^{-1} and germinability declined from 100% on day 0 to ± 50% on day four (data not shown). After 30 d in hydrated storage, the WC of *A. marina* embryonic axes increased from a shedding WC of *c.* 1.8 g g^{-1} to 2.2 g g^{-1}. When seeds were planted in soil at 20 d of storage only ± 50% of the seeds germinated. In contrast, embryonic axes of *T. dregeana* seeds had a shedding WC of *c.* 2.5 g g^{-1}. Following partial dehydration, axis WC dropped to *c.* 0.9 g g^{-1} on day 14 and germinability in these seeds was lost relatively slower than in *A. marina*, from 100% (on day 0) to ±50% on day 14(data not shown). In hydrated storage *c.* 54.2 ± 3.2% of *T. dregeana* seeds germinated after 12 months. The remaining non-germinated seeds were 100% viable but these non-germinated seeds showed a significant ($P < 0.05$) reduction in water content (*c.* 2.0 g g^{-1}) during storage, implying that they were water-stressed during storage.

5.4.2 Proteins identified by LC MS/MS and their functional classification

The total proteome extracted from the embryonic axes of freshly harvested (control), partially dehydrated and hydrated stored *A. marina* and *T. dregeana* seeds was explored using LC MS/MS (Fig. 5.1). Analysis of protein extracts from all samples ($n = 3$) collectively resulted in the identification of 30 proteins (436 spectra) in *A. marina* and 105 proteins (2107 spectra) in *T. dregeana*, when the criteria for identification discussed in section 5.4.1 were applied. Functional classification of identified proteins based on the National Resource for Biotechnology Information (NCBI) putative protein database gene

annotations (gene ontology[GO] level 3) demonstrated that they were associated with a wide range of biological processes, ranging from growth and reproductive processes, to response to stimulus. Based on the GO analysis, of all the processes observed three major biological processes stood out in both *A. marina* (Fig. 5.2a) and *T. dregeana* (Fig. 5.2b) namely: 'cellular processes'; 'response to stimulus' and 'metabolic process'. Most proteins identified in both species were also located mainly in the following cellular components: 'cell part'; 'organelle'; 'organelle part' and 'membrane' (Figs. 5.2c and 5.2d). In terms of molecular function the majority of the proteins identified in both species were associated with 'binding' followed by 'catalytic activity' and then 'antioxidant activity'; the percentage of proteins within these categories was comparable between species (Figs. 5.3a and 5.3b). Since the number of proteins identified in *A. marina* was markedly lower than that identified in *T. dregeana* it is difficult to draw any reliable conclusions on the similarity/dissimilarity between the total proteomes of the two species. Nevertheless, we can say that the number of proteins shared between *A. marina* and *T. dregeana* was relatively comparable between species, irrespective of the treatment (Table 5.1). Thus, the results presented below explore differences in individual protein expression levels between treatments, within species. The comparisons made focus on differences in proteins associated with redox metabolism and primary metabolic activity.

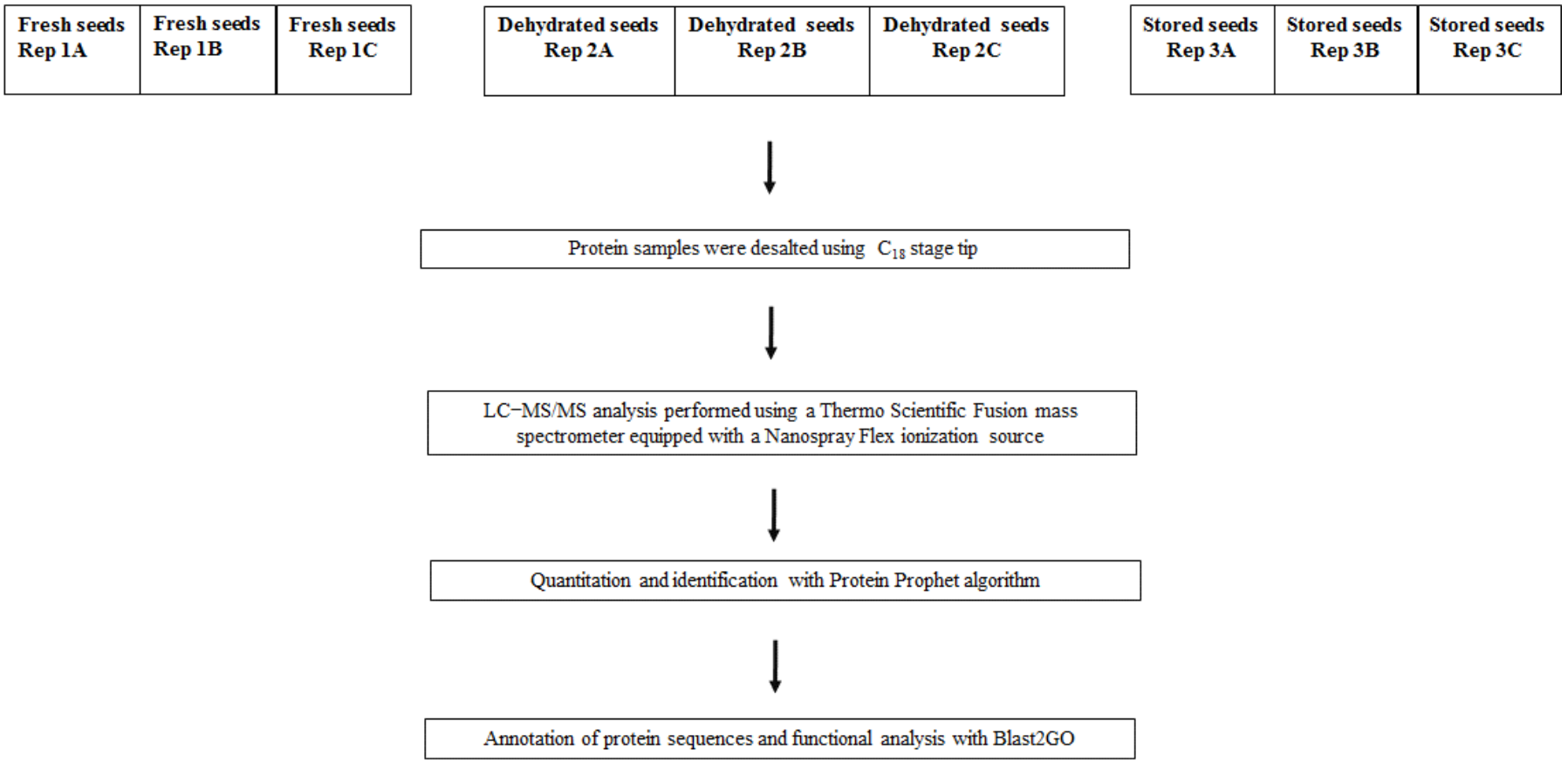

Figure 5.1 Workflow of LC-MS/MS experiments for the fresh, partially dehydrated and stored seeds. For each experiment, the extracted proteins were trypsin-digested. Peptides were desalted on a C_{18} spin column. The sample was then analysed by LC−MS/MS analysis using a Thermo Scientific Fusion mass spectrometer equipped with a Nanospray Flex ionization source. Searches and quantitation was done using Scaffold_4.4.8, Proteome Software. Finally, the identified proteins in the set were annotated and functionally analyzed using the Blast2GO tool based on gene ontology (GO) terms.

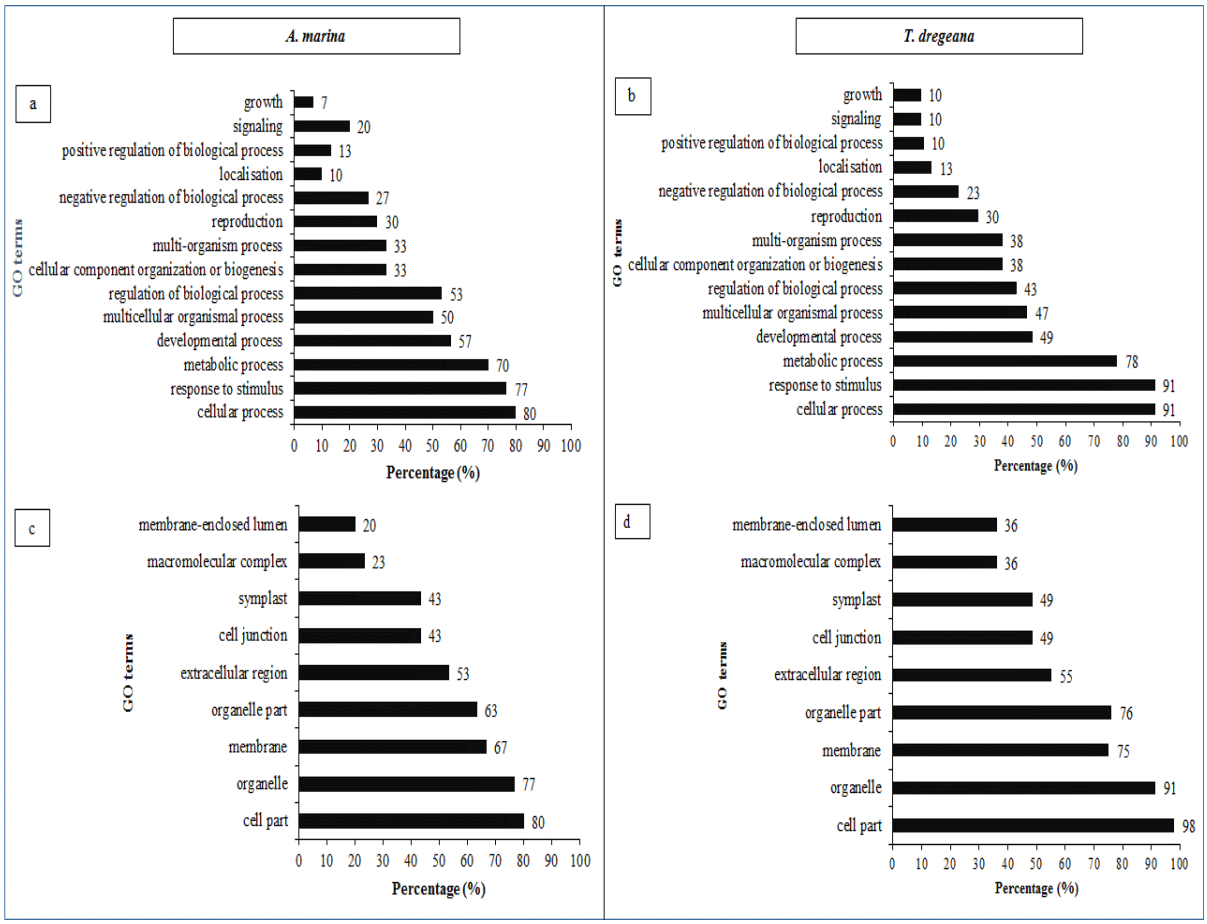

Figure 5.2 Gene ontology annotations for differentially expressed proteins (DEP's) in terms of biological process (a) *A. marina*; (b) *T. dregeana,* cellular components (c) *A. marina* and (d) *T. dregeana* embryonic axes.

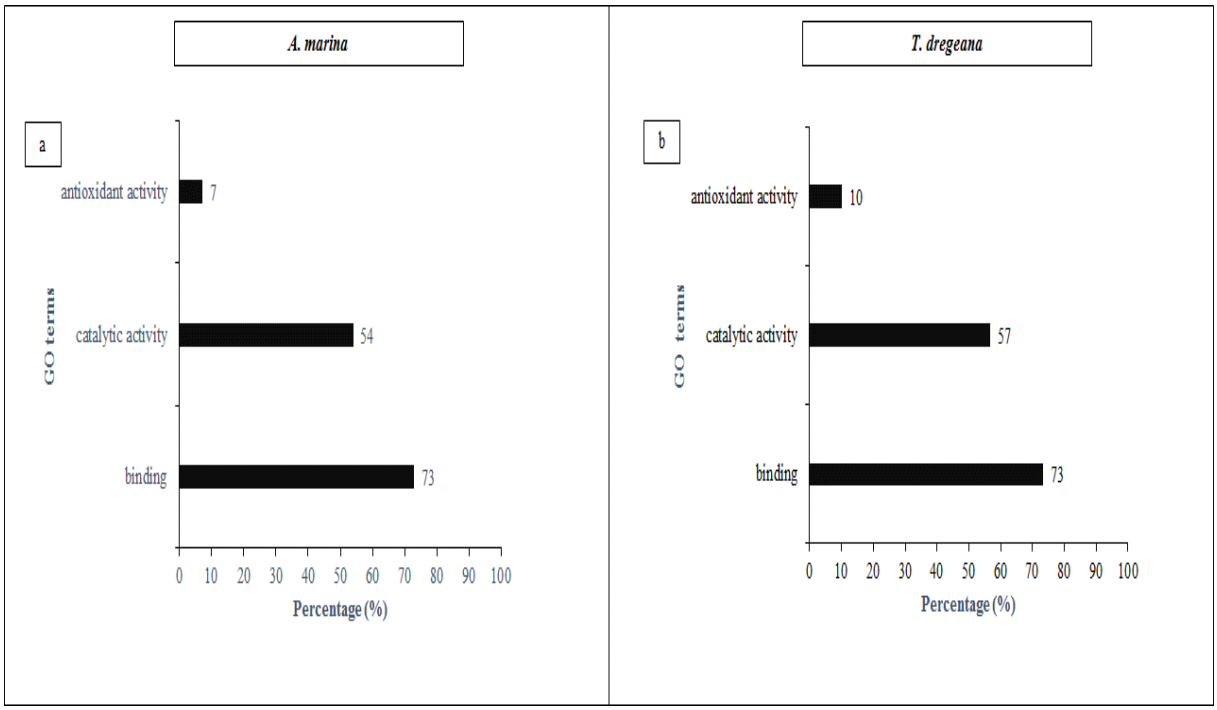

Figure 5.3 Gene ontology annotations for differentially expressed proteins in terms of molecular function in (a) *A. marina* and (b) *T. dregeana* embryonic axes

Table 5.1: Species-specific and shared proteins identified in the axes of freshly harvested (control), hydrated stored and partially dehydrated seeds of *A. marina* and *T. dregeana*.

Treatment	Number of proteins		
	A. marina	*T. dregeana*	Shared
Freshly harvested (control)	17	54	5
Partially dehydrated	7	79	4
Stored	9	86	5

Note: the data represent the total number of proteins identified in each of the treatments within the respective species. Proteins identified in one treatment may also appear in another. Shared refers to the number of proteins found in both species in each of the treatments.

5.4.3 Molecular functional analysis of partially dehydrated A. marina and T. dregeana seeds

Proteins identified in axes of freshly harvested (control) seeds and dehydrated seeds were classified into functional groups based on NCBI gene annotations (GO level 3) for both species (Figs. 5.4a -d). In axes of freshly harvested *A. marina* seeds (control), proteins were categorized according to 12 functional groups (Fig. 5.4a), of which nine were also found in axes of dehydrated seeds (Fig. 5.4c). There was a noticeable increase in the percentage of proteins identified within the following functional groups: isomerase activity (GO: 0016853); lyase activity (GO:0016829); ion binding (GO:0043167); and oxidoreductase activity (GO: 0016491) categories in the embryonic axes of dehydrated seeds of this species compared with the control axes(Figs. 5.4a and c). Conversely, upon dehydration fewer proteins were identified under the following categories relative to control axes: organic cyclic compound binding (GO:0097159); heterocyclic compound binding (GO:1901363); small molecule binding (GO:0036094); and carbohydrate derivative binding (GO:0097367). Proteins identified in the control axes under the categories transferase activity (GO:0016740) and hydrolase activity (GO:0016787) were not present as major functional categories in axes of dehydrated *A. marina* seeds. Alternatively, proteins identified under the categories transferase activity (GO: 0016740), cofactor binding (GO:0048037) and SOD activity (GO:0004784) in the axes of dehydrated seeds were not major functional categories in the control seeds (Figs. 5.4a and c).

Proteins identified in the axes of control and partially dehydrated seeds of *T. dregeana* were categorized under 12 and 10 major functional categories, respectively (Figs 5.4b and d). This was based on the fact that proteins identified under the functional categories peroxidase activity (GO:0004601) and transferase activity (GO: 0016740) in the control did not represent major functional categories in the dehydrated

axes. Unlike seeds of *A. marina*, there was little change in the percentage of proteins identified in the 10 functional categories in the axes of partially dehydrated seeds compared with the axes of the control *T. dregeana* seeds. There was also no marked change in the percentage of proteins under the categories carbohydrate derivative binding (GO: 0097367) and small molecule binding (GO: 0036094) in the axes of control and partially dehydrated seeds (Figs. 5.4 b and d). However, there was a slight increase in the percentage of proteins under the categories oxidoreductase activity (GO:0016491) and ion binding (GO:0043167) in the axes of dehydrated seeds compared with the control (Fig. 5.4d).

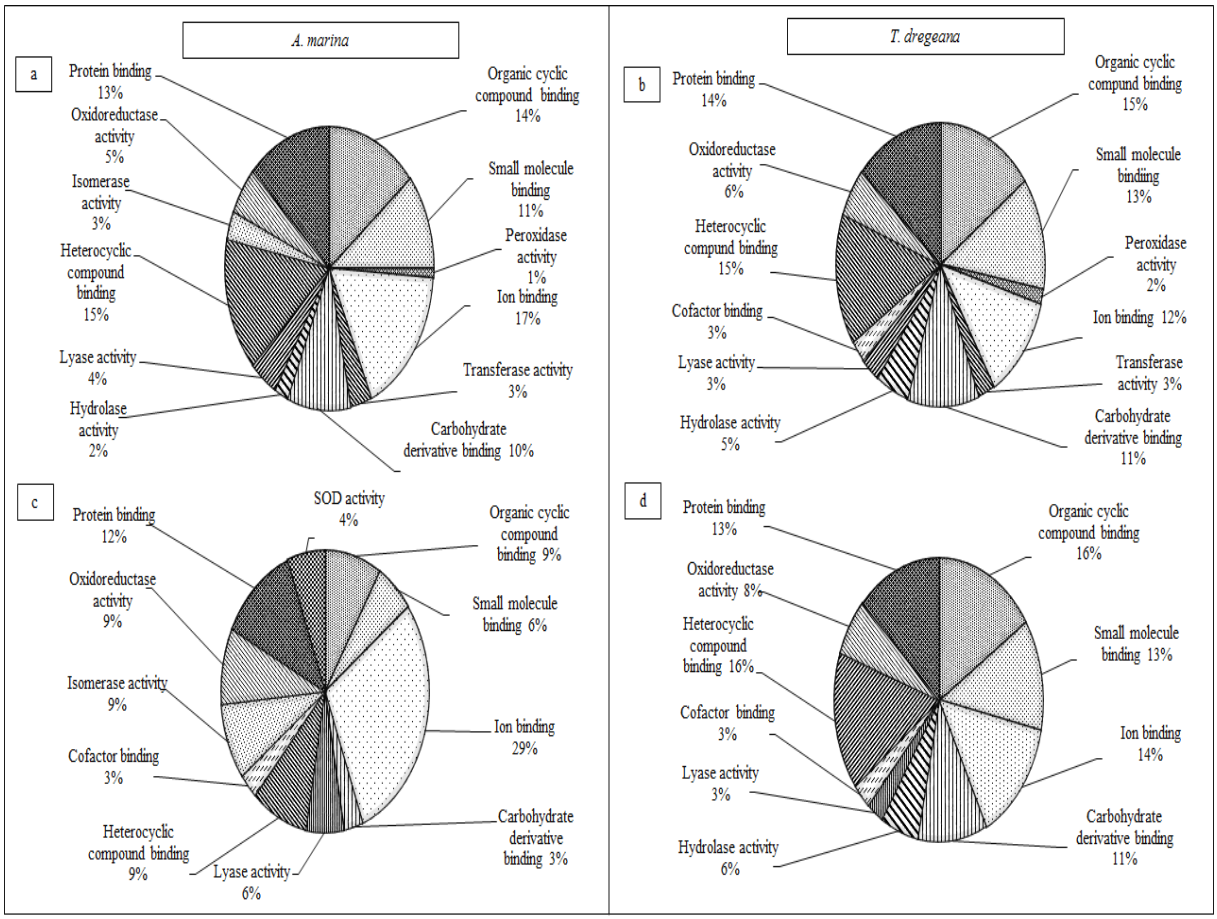

Figure 5.4 Molecular functional groups of proteins in the embryonic axes of *A. marina* (a) control and (c) partially dehydrated, and *T. dregeana* (b) control and (d) partially dehydrated seeds.

5.4.4 Molecular functional analysis of A. marina and T. dregeana seeds in hydrated storage

Proteins identified in the embryonic axes of control and hydrated stored seeds of *A. marina* were grouped under 12 major functional categories (Figs. 5.5 a and c). There was an increase in the percentage of proteins identified under the categories oxidoreductase activity (GO: 0016491) and protein binding (GO: 0005515) in the axes of stored seeds of this species, compared with the control (Figs. 5.5 a and c). There was, however, a decrease in heterocyclic compound binding (GO: 1901363) and no change observed in the percentage of proteins under the functional category small molecule binding (GO: 0036094) in the axes of stored seeds compared with the control. There was a slight increase in the percentage of proteins identified in the remaining categories in the axes of stored seeds relative to the control. Unlike in axes of dehydrated seeds, there were proteins in the category peroxidase activity (GO:000461) that were identified in axes of stored seeds of *A. marina* and compared with the control these increased slightly (1%) with storage (Fig 5.5c). Similar to axes of dehydrated seeds, a percentage of proteins within the functional category SOD activity (GO:0004784) were identified as a major category only in the axes of stored seeds and not in the control (Figs. 5.5 a and c).

In *T. dregeana* seeds proteins identified in the control and stored embryonic axes were grouped under 12 and 10 categories, respectively (Figs. 5.5b and d). The percentage of proteins identified in the control and stored axes were grouped under functional categories that were very similar, with no differences between the control and stored axes in the percentage of protein sequences found within four of these major categories, namely: small molecule binding (GO: 0036094); transferase activity (GO: 0016740); carbohydrate derivative binding (GO: 0097367); and cofactor binding (GO: 0097159). A slightly higher percentage of proteins were grouped under organic cyclic compound binding (GO: 0097159), ion binding (GO: 0043167), oxidoreductase activity (GO: 0016491), heterocyclic compound binding (GO: 1901363) and hydrolase activity (GO: GO:0016787) in the stored axes compared with the control. Alternatively, a slight decrease in the percentage of proteins was observed in terms of protein binding (GO:0005515). Proteins in the control were also grouped under the functional categories peroxidase activity (GO:000461) and lyase activity (GO:0016829) but these groups were absent in the molecular functional graph (up to GO level 3) of proteins in dehydrated *T. dregeana* axes.

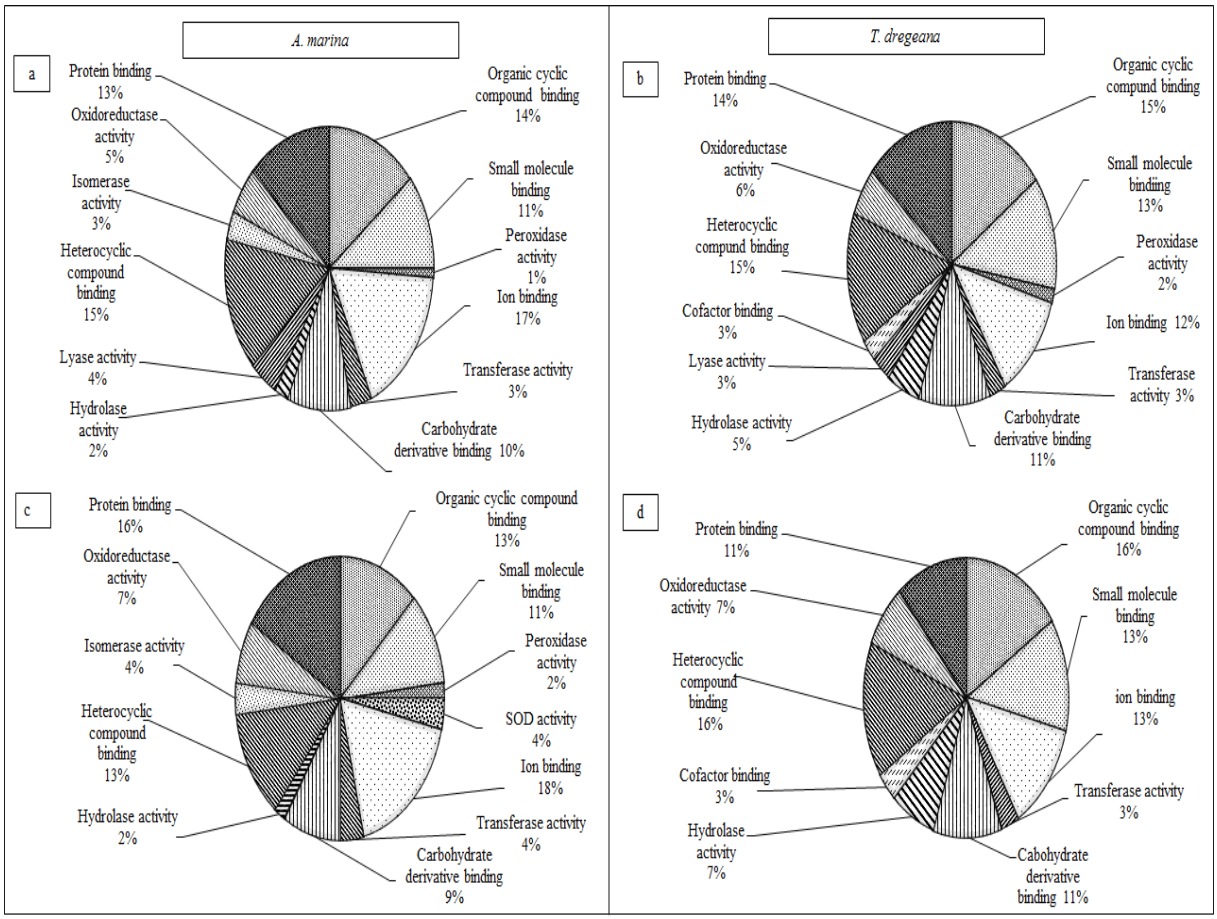

Figure 5.5 Molecular functional groups identified for proteins in the embryonic axes of (a) control and (c) hydrated stored *A. marina*, and (b) control and (d) hydrated stored *T. dregeana* seeds.

5.4.5 Functional analysis of identified proteins in embryonic axes of A. marina and T. dregeana seeds during partial dehydration

In partially dehydrated *A. marina* seeds, proteins identified to be related to the response to stress (GO: 0006950) were grouped under six major categories, namely: cellular response to oxidative stress (GO: 0034599); drought recovery (GO: 0009819); response to salt stress (GO: 009651); cellular response to osmotic stress (GO: 0071470); response to endoplasmic reticulum (ER) stress (GO: 0034976); and response to ROS (GO: 0000302) (Fig. 5.6a). The antioxidant enzyme superoxide dismutase (W1NYZ2; P27082) was identified under the following categories: cellular response to oxidative stress (GO:0034599); cellular response to osmotic stress (GO: 0071470); response to ROS (GO: 0000302); and response to salt stress (GO: 009651) (Table 5.2). Several other proteins were also identified under the response to ROS (GO: 0000302) category and these included an uncharacterised protein (U5DCI6) and glyceraldehyde-3-phosphate dehydrogenase 2 (Q7FAH2) (Table 5.2). Additional proteins identified under the response to salt stress (GO: 009651) category included triose phosphate isomerase (W1PJM9, P48491) and glyceraldehyde-3-phosphate dehydrogenase 2 (Q7FAH2). Only one protein was identified in axes of partially dehydrated *A. marina* seeds under the response to ER stress category (GO: 0034976), which was an uncharacterised protein (U5DCI6) (Table 5.2).

Proteins identified under the response to stress (GO: 0006950) category in axes of dehydrated *T. dregeana* seeds were grouped into two major categories, namely, response to ROS (GO: 0000302) and response to ER (GO: 0034976) (Fig. 5.6b). Under the response to ER stress category (GO: 0034976) there were several proteins, namely, a probable mediator of RNA polymerase II transcription subunit 37c (Q9LHA8), molecular chaperone Hsp90 (Q6UJX6) and nine uncharacterised proteins (M0TS86, M0TXA5, U5DCI6, M0U8Q0, W1PHT9, M0RF29, M0U2M9, W1NRJ1, W1NRE0) (Table 5.3). In the response to ROS (GO: 0000302) category several proteins were identified and included the following: 17.7 kDa heat shock protein (Q39930); alcohol dehydrogenase 1 (P25141); glyceraldehyde-3-phosphate dehydrogenase GAPC2 (Q9FX54, P26518); nucleoside diphosphate kinase (M0SZK9); superoxide dismutase (P35017, M0TEF8, W1NYZ2); chaperone protein ClpB1 (P42730), and uncharacterised proteins (M0TXA5, W1NRJ1, M0TS86, M0S9F7, U5DCI6) (Table 5.3).

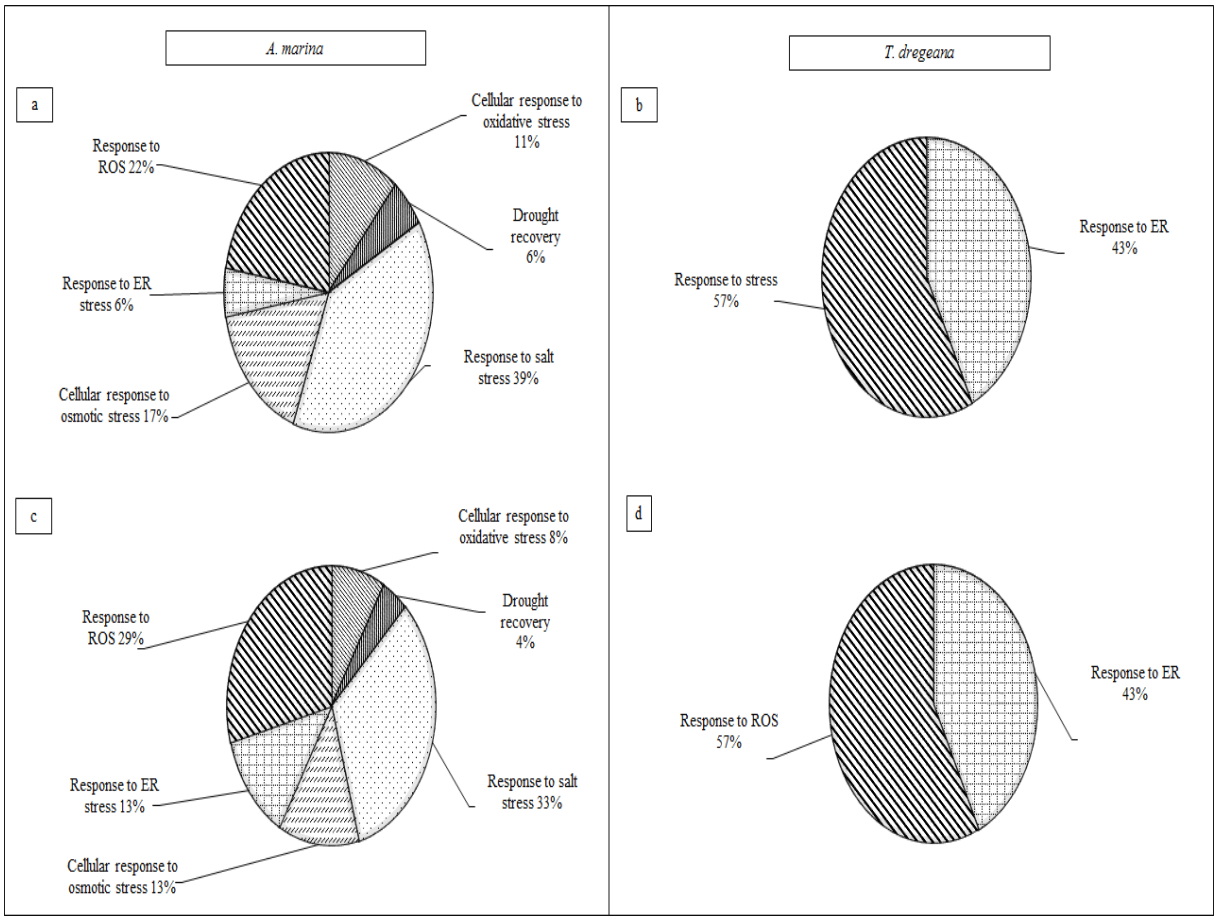

Figure 5.6 Molecular functional groups of stress response proteins in embryonic axes of *A. marina* (a) partially dehydrated & (c) hydrated stored, and *T. dregeana* (b) partially dehydrated & (d) hydrated stored seeds.

Table 5.2: Stress response proteins identified in the embryonic axes of *A. marina* seeds after partial dehydration

Protein name	Accession	Mw (kDa)	SC%	Unique peptides	Species
response to stress (GO: 0006950)					
cellular response to oxidative stress (GO:0034599)					
Superoxide dismutase [Cu-Zn]	W1NYZ2	22	7	1	*Amborella trichopoda*
Superoxide dismutase [Cu-Zn]	P27082	15	9	1	*Nicotiana plumbaginifolia*
response to salt stress (GO:009651)					
Uncharacterized protein	U5DCI6	75	4	2	*Amborella trichopoda*
Triosephosphate isomerase	W1PJM9	27	11	2	*Amborella trichopoda*
Superoxide dismutase [Cu-Zn]	W1NYZ2	22	7	1	*Amborella trichopoda*
Superoxide dismutase [Cu-Zn]	P27082	15	15	2	*Nicotiana plumbaginifolia*
Triosephosphate isomerase	P48491	27	11	2	*Arabidopsis thaliana*
Glyceraldehyde-3-phosphate dehydrogenase 2	Q7FAH2	37	4	1	*Oryza sativa subsp. japonica*
drought recovery (GO:0009819)					
Uncharacterized protein	W1P5L1	19	5	1	*Amborella trichopoda*
cellular response to osmotic stress (GO:0071470)					
Superoxide dismutase [Cu-Zn]	W1NYZ2	22	7	1	*Amborella trichopoda*
Superoxide dismutase [Cu-Zn]	P27082	15	9	1	*Nicotiana plumbaginifolia*

Table 5.2: continued Stress response proteins identified in embryonic axes of *A. marina* seeds after partial dehydration

Protein name	Accession	Mw (kDa)	SC%	Unique peptides	Species
response to endoplasmic reticulum stress (GO:0034976)					
Uncharacterized protein	U5DCI6	75	4	2	*Amborella trichopoda*
response to reactive oxygen species (GO:0000302)					
Uncharacterized protein	U5DCI6	75	4	2	*Amborella trichopoda*
Superoxide dismutase [Cu-Zn]	W1NYZ2	22	7	1	*Amborella trichopoda*
Superoxide dismutase [Cu-Zn]	P27082	15	9	1	*Nicotiana plumbaginifolia*
Glyceraldehyde-3-phosphate dehydrogenase 2	Q7FAH2	37	4	1	*Oryza sativa subsp. japonica*

Table 5.3: Proteins identified in embryonic axes of partially dehydrated seeds of *T. dregeana* in response to stress

Protein name	Accession	Mw (kDa)	SC %	Unique peptides	Species
response to stress (GO: 0006950)					
response to endoplasmic reticulum stress (GO: 0034976)					
Uncharacterized protein	M0TS86	71	25	10	*Musa acuminata subsp. malaccensis*
Uncharacterized protein	M0TXA5	56	25	1	*Musa acuminata subsp. malaccensis*
Uncharacterized protein	U5DCI6	75	6	3	*Amborella trichopoda*
Uncharacterized protein	M0U8Q0	27	10	1	*Musa acuminata subsp. malaccensis*
Uncharacterized protein	W1PHT9	50	9	2	*Amborella trichopoda*
Uncharacterized protein	M0RF29	72	10	6	*Musa acuminata subsp. malaccensis*
Uncharacterized protein	M0U2M9	91	4	2	*Musa acuminata subsp. malaccensis*
Uncharacterized protein	W1NRJ1	72	26	4	*Amborella trichopoda*
Probable mediator of RNA polymerase II transcription subunit 37c	Q9LHA8	71	22	2	*Arabidopsis thaliana*
Molecular chaperone Hsp90	Q6UJX6	80	9	2	*Nicotiana benthamiana*
Uncharacterized protein	W1NRE0	90	6	3	*Amborella trichopoda*
response to reactive oxygen species (GO: 0000302)					
17.7 kDa heat shock protein	Q39930	18	16	2	*Helianthus annuus*
Uncharacterized protein	M0TXA5	56	25	1	*Musa acuminata subsp. malaccensis*

Table 5.3: continued Proteins identified in embryonic axes of partially dehydrated seeds of *T. dregeana* in response to stress

Protein name	Accession	Mw (kDa)	SC %	Unique peptides	Species
Alcohol dehydrogenase 1	P25141	42	9	1	*Petunia hybrida*
Uncharacterized protein	W1NRJ1	72	26	4	*Amborella trichopoda*
Glyceraldehyde-3-phosphate dehydrogenase GAPC2	Q9FX54	37	20	5	*Arabidopsis thaliana*
Nucleoside diphosphate kinase	M0SZK9	16	26	4	*Musa acuminata subsp. malaccensis*
Superoxide dismutase [Mn], mitochondrial	P35017	26	7	1	*Hevea brasiliensis*
Uncharacterized protein	M0TS86	71	25	10	*Musa acuminata subsp. Malaccensis*
Superoxide dismutase [Cu-Zn]	M0TEF8	15	24	4	*Musa acuminata subsp. Malaccensis*
Uncharacterized protein	M0S9F7	94	2	2	*Musa acuminata subsp. Malaccensis*
Uncharacterized protein	U5DCI6	75	6	3	*Amborella trichopoda*
Superoxide dismutase [Cu-Zn]	W1NYZ2	22	13	3	*Amborella trichopoda*
Chaperone protein ClpB1	P42730	101	5	3	*Arabidopsis thaliana*
Glyceraldehyde-3-phosphate dehydrogenase	P26518	37	19	2	*Magnolia liliiflora*
Probable mediator of RNA polymerase II transcription subunit 37c	Q9LHA8	71	22	2	*Arabidopsis thaliana*

5.4.6 Stress response proteins identified in embryonic axes of stored seeds of A. marina and T. dregeana

In *A. marina*, proteins identified in axes of stored seeds were grouped under the same six categories found in axes of dehydrated seeds (Fig 5.6c). As in the dehydrated axes, a 22 kDa and 15 kDa SOD (W1NYZ2, P27082) was found under the following categories: cellular response to oxidative stress (GO:0034599); response to salt stress (GO:009651); cellular response to osmotic stress (GO:0071470) and response to ROS (GO:0000302) (Table 5.4). Other proteins grouped under response to salt stress (GO:009651) included: triosephosphate isomerase (W1PJM9, P48491); ascorbate peroxidase (B2NIX3); putative Hsp90 (B1Q477); glyceraldehyde-3-phosphate dehydrogenase 2(Q7FAH2) and an uncharacterized protein (U5DCI6) (Table 5.4). A glycine-rich protein (A9YWR4) was also found under the cellular response to osmotic stress (GO:0071470) category. In the response to ROS (GO:0000302) category several other proteins were identified: ascorbate peroxidase (B2NIX3); glyceraldehyde-3-phosphate dehydrogenase 2 (Q7FAH2); nucleoside diphosphate kinase (P39207); genomic scaffold (W6L5X4); and an uncharacterized protein (U5DCI6). In the stored axes two additional proteins were also found under the category response to ER stress (GO:0034976) namely, a putative Hsp90 (B1Q477) and genomic scaffold (W6L5X4) (Table 5.4).

Proteins identified in the stored seeds of *T. dregeana* were also grouped into two major categories namely, response to ER stress (GO:0034976) and response to ROS (GO:0000302) (Fig. 5.6d). In response to ER stress (GO:0034976), several proteins such as molecular chaperone Hsp90 (Q6UJX6), probable mediator of RNA polymerase II transcription subunit 37c (Q9LHA8), genomic scaffold (W6L5X4) and 12 uncharacterised proteins (M0T775, M0TXA5, M0U8Q0, M0TZY1, M0RF29, M0U2M9, W1NRJ1, M0TS86, U5DCI6, W1PHT9, M0REB7 and W1NRE0), were grouped under this category (Table 5.5). Stored *T. dregeana* seeds also possessed several additional proteins compared with the dehydrated seeds and these were grouped under the response to ROS (GO:0000302) category. Proteins identified in this category included the following: alcohol dehydrogenase 1(P25141); glutathione peroxidase (W1NIT9); glyceraldehyde-3-phosphate dehydrogenase (P09094; P26518, Q9FX54); 17.7 kDa heat shock protein (Q39930); nucleoside diphosphate kinase (M0SZK9); genomic scaffold (W6L5X4); superoxide dismutase (P35017, M0TEF8, W1NYZ2); ascorbate peroxidase (Q9SXT2); probable mediator of RNA polymerase II transcription subunit 37c (Q9LHA8), and seven uncharacterised proteins (M0T775, M0TXA5, W1NRJ1, W1NKL9, M0TS86, U5DCI6, M0REB7).

Table 5.4: Proteins identified in embryonic axes of stored *A. marina* seeds in response to stress

Protein name	Accession	MW (kDa)	SC%	Unique peptides	Species
response to stress (GO: 0006950) **cellular response to oxidative stress (GO:0034599)**					
Superoxide dismutase [Cu-Zn]	W1NYZ2	22	13	2	*Amborella trichopoda*
Superoxide dismutase [Cu-Zn]	P27082	15	9	1	*Nicotiana plumbaginifolia*
drought recovery (GO:0009819)					
Uncharacterized protein	W1P5L1	19	5	1	*Amborella trichopoda*
response to salt stress (GO:009651)					
Uncharacterized protein	U5DCI6	75	2	1	*Amborella trichopoda*
Triosephosphate isomerase	W1PJM9	27	6	1	*Amborella trichopoda*
Superoxide dismutase [Cu-Zn]	W1NYZ2	22	13	2	*Amborella trichopoda*
Superoxide dismutase [Cu-Zn]	P27082	15	9	1	*Nicotiana plumbaginifolia*
Ascorbate peroxidase (Fragment)	B2NIX3	20	12	2	*Capsicum chinense*
Triosephosphate isomerase	P48491	27	6	1	*Arabidopsis thaliana*
Putative Hsp90-2	B1Q477	80	5	3	*Capsicum chinense*
Glyceraldehyde-3-phosphate dehydrogenase 2	Q7FAH2	37	9	2	*Oryza sativa subsp. japonica*
cellular response to osmotic stress (GO:0071470)					
Superoxide dismutase [Cu-Zn]	W1NYZ2	22	13	2	*Amborella trichopoda*
Superoxide dismutase [Cu-Zn]	P27082	15	15	2	*Nicotiana plumbaginifolia*
Glycine-rich protein	A9YWR4	96	13	5	*Medicago truncatula*

Table 5.4: continued Proteins identified in embryonic axes of stored *A. marina* seeds in response to stress

Protein name	Accession	MW (kDa)	SC%	Unique peptides	Species
Uncharacterized protein	U5DCI6	75	2	1	*Amborella trichopoda*
Putative Hsp90-2	B1Q477	80	5	3	*Capsicum chinense*
Genomic scaffold	W6L5X4	73	9	5	*Phytomonas sp. isolate Hart1*
response to reactive oxygen species (GO:0000302)					
Superoxide dismutase [Cu-Zn]	W1NYZ2	22	13	2	*Amborella trichopoda*
Uncharacterized protein	U5DCI6	75	2	1	*Amborella trichopoda*
Nucleoside diphosphate kinase	P39207	17	21	2	*Arabidopsis thaliana*
Superoxide dismutase [Cu-Zn]	P27082	15	9	1	*Nicotiana plumbaginifolia*
Ascorbate peroxidase (Fragment)	B2NIX3	20	12	2	*Capsicum chinense*
Glyceraldehyde-3-phosphate dehydrogenase 2	Q7FAH2	37	9	2	*Oryza sativa subsp. japonica*
Genomic scaffold	W6L5X4	73	10	5	*Phytomonas sp. isolate Hart1*

Table 5.5: Stress response proteins identified in axes of stored *T. dregeana* seeds

Protein name	Accession:	Mw (kDa)	SC%	Unique peptides	Species
Response to stress (GO: 0006950)					
Response to endoplasmic reticulum stress (GO:0034976)					
Uncharacterized protein	M0T775	50	18	2	*Musa acuminata subsp. malaccensis*
Uncharacterized protein	M0TXA5	56	26	2	*Musa acuminata subsp. malaccensis*
Uncharacterized protein	M0U8Q0	27	14	2	*Musa acuminata subsp. malaccensis*
Uncharacterized protein	M0TZY1	45	7	2	*Musa acuminata subsp. malaccensis*
Uncharacterized protein	M0RF29	72	12	7	*Musa acuminata subsp. malaccensis*
Uncharacterized protein	M0U2M9	91	2	1	*Musa acuminata subsp. malaccensis*
Uncharacterized protein	W1NRJ1	72	27	5	*Amborella trichopoda*
Genomic scaffold	W6L5X4	73	12	3	*Phytomonas sp. isolate Hart1*
Molecular chaperone Hsp90-1	Q6UJX6	80	8	2	*Nicotiana benthamiana*
Uncharacterized protein	M0TS86	71	28	12	*Musa acuminata subsp. malaccensis*
Uncharacterized protein	U5DCI6	75	14	7	*Amborella trichopoda*
Uncharacterized protein	W1PHT9	50	9	2	*Amborella trichopoda*
Uncharacterized protein	M0REB7	53	25	2	*Musa acuminata subsp. malaccensis*
Probable mediator of RNA polymerase II transcription subunit 37c	Q9LHA8	71	24	3	*Arabidopsis thaliana*
Uncharacterized protein	W1NRE0	90	9	5	*Amborella trichopoda*
Response to reactive oxygen species (GO:0000302)					
Uncharacterized protein	M0T775	50	18	2	*Musa acuminata subsp. malaccensis*
17.7 kDa heat shock protein	Q39930	18	17	3	*Helianthus annuus*
Uncharacterized protein	M0TXA5	56	26	2	*Musa acuminata subsp. malaccensis*
Alcohol dehydrogenase 1	P25141	42	12	2	*Petunia hybrida*
Glyceraldehyde-3-phosphate dehydrogenase	P09094	36	18	3	*Nicotiana tabacum*
Glutathione peroxidase	W1NIT9	28	7	2	*Amborella trichopoda*
Uncharacterized protein	W1NRJ1	72	27	5	*Amborella trichopoda*

Table 5.5: continued Stress response proteins identified in axes of stored *T. dregeana* seeds

Protein name	Accession:	Mw (kDa)	SC%	Unique peptides	Species
Glyceraldehyde-3-phosphate dehydrogenase GAPC2	Q9FX54	37	18	5	*Arabidopsis thaliana*
Nucleoside diphosphate kinase	M0SZK9	16	20	3	*Musa acuminata subsp. malaccensis*
Genomic scaffold	W6L5X4	73	12	3	*Phytomonas sp. isolate Hart1*
Superoxide dismutase [Mn]	P35017	26	12	3	*Hevea brasiliensis*
Uncharacterized protein	W1NKL9	28	6	2	*Amborella trichopoda*
Ascorbate peroxidase (Fragment)	Q9SXT2	19	14	2	*Cicer arietinum*
Superoxide dismutase [Cu-Zn]	M0TEF8	15	22	2	*Musa acuminata subsp. malaccensis*
Uncharacterized protein	M0TS86	71	28	12	*Musa acuminata subsp. malaccensis*
Superoxide dismutase [Cu-Zn]	W1NYZ2	22	13	3	*Amborella trichopoda*
Uncharacterized protein	U5DCI6	75	14	7	*Amborella trichopoda*
Glyceraldehyde-3-phosphate dehydrogenase	P26518	37	18	2	*Magnolia liliiflora*
Uncharacterized protein	M0REB7	53	25	2	*Musa acuminata subsp. malaccensis*
Probable mediator of RNA polymerase II transcription subunit 37c	Q9LHA8	71	24	3	*Arabidopsis thaliana*

5.4.7 Identification of differential expressed proteins (DEP's) in embryonic axes of partially dehydrated and stored A. marina and T. dregeana seeds

Twelve proteins in axes of partially dehydrated seeds of *A. marina* and *T. dregeana* seeds were found to be significantly ($P<0.05$) differentially expressed relative to each other (Table 5.6). Of these proteins, proteasome subunit alpha type (MORJE7), triose phosphate isomerase (P48491) and an uncharacterised protein (U5DCI6) were found in greater abundance in dehydrated *A. marina* axes than *T. dregeana* axes. However, the remaining nine proteins which included, adenosylhomocysteinase (Q9LK36), calmodulin (P62201), probable mediator of RNA polymerase II transcription subunit 37c (MED37C), SOD (MOTEF8) and five uncharacterised proteins (MORE63; MOTXA5; MOTS86; W1PYC8; W1NRJ1), exhibited a higher level of expression in axes of dehydrated *T. dregeana* seeds than *A. marina* seeds (Table 5.6).

In the embryonic axes of stored seeds of *A. marina* and *T. dregeana*, 10 proteins were significantly ($P<0.05$) differentially expressed relative each other (Table 5.7). Five of these proteins, namely, nucleoside disphosphate, kinase 1 (P39207), photosystem II reaction center protein (Q85AJ6), triosephosphate isomerase (P48491) and two uncharacterised proteins (W1NN68 and W1P2I9), occurred at higher abundance in axes of stored *A. marina* than *T. dregeana* seeds. In contrast, the remaining five proteins which included, glyceraldehyde-3-phosphate dehydrogenase (Q9FX54), probable mediator of RNA polymerase II transcription subunit 37c (Q9LHA8) and three uncharacterised proteins (MORE63; MOTXA5; W1PYC8), had a higher abundance in axes of stored *T. dregeana* than *A. marina* seeds (Table 5.7).

Table 5.6: Quantitative analysis of proteins found in embryonic axes of partially dehydrated seeds of *A. marina* and *T. dregeana* using Fisher's exact test (*P*<0.05)

	Accession	Mw (kDa)	Dehydrated *A. marina*	Dehydrated *T. dregeana*	*P*-value
Adenosylhomocysteinase	Q9LK36	53	Low	high	0.015
Calmodulin	P62201	17	Low	high	0.0034
Glyceraldehyde-3-phosphate dehydrogenase	P26518	37	Same	same	0.56
Proteasome subunit alpha type	M0RJE7	26	High	Low	0.012
Probable mediator of RNA polymerase II transcription subunit 37c	MED37C	71	low	high	0.0062
Triose phosphate isomerase	P48491	27	high	Low	0.0001
Superoxide dismutase [Cu-Zn]	MOTEF8	15	low	high	0.0037
Uncharacterised protein	MORE63	17	low	high	0.0009
Uncharacterised protein	MOTXA5	56	low	high	0.016
Uncharacterised protein	MOTS86	71	low	high	0.0015
Uncharacterised protein	U5DCI6	75	high	low	0.0012
Uncharacterised protein	W1PYC8	48	low	high	0.0001
Uncharacterised protein	W1NRJ1	72	low	high	0.0009

Note:

High represents a higher abundance of the individual protein relative to the abundance of that protein in dehydrated seeds of the other species
Low represents a lower abundance of the individual protein relative to the abundance of that protein in dehydrated seeds of the other species

Table 5.7: Quantitative analysis of proteins found in embryonic axes of stored seeds of *A. marina* and *T. dregeana* using Fisher's exact test (*P* <0.05)

	Accession	Mw (kDa)	Stored seeds *A. marina*	Stored seeds *T. dregeana*	*P*-value
Glyceraldehyde-3-phosphate dehydrogenase	Q9FX54	37	low	high	0.036
Nucleoside diphosphate kinase 1	P39207	17	high	low	0.011
Proteasome subunit alpha type	M0RJE7	26	low	high	0.67
Photosystem II reaction center protein	Q85AJ6	3	high	low	0.011
Superoxide dismutase [Cu-Zn]	MOTEF8	15	same	same	0.49
Probable mediator of RNA polymerase II transcription subunit 37c	Q9LHA8	71	low	high	0.028
Triosephosphate isomerase	P48491	27	high	low	0.0001
Uncharacterised protein	MORE63	17	low	high	0.0001
Uncharacterised protein	MOTXA5	56	low	high	0.036
Uncharacterised protein	W1NN68	17	high	-	0.0001
Uncharacterised protein	W1PYC8	48	low	high	0.0001
Uncharacterised protein	W1P2I9	106	high	low	0.012

Note:

High represents a higher abundance of the individual protein relative to the abundance of that protein in stored seeds of the other species
Low represents a lower abundance of the individual protein relative to the abundance of that protein in stored seeds of the other species

5.4.8 Pathway analysis of proteins identified in embryonic axes of stored and partially dehydrated A. marina and T. dregeana seeds

In addition to categorizing identified protein sequences based on biochemical function in both species, sequences were assigned to metabolic pathways via KEGG (Kanehisa *et al.*, 2015) using enzyme commission (Ec) numbers as the basis for assignment. Identified proteins in both axes of control and stored *A. marina* seeds were grouped under 13 different pathways: glycolysis/ gluconeogenesis; carbon fixation in photosynthetic organisms; fructose and mannose metabolism; inositol phosphate metabolism; pentose phosphate pathway and methane metabolism; purine metabolism; phenylpropanoid biosynthesis; glutathione metabolism; ascorbate and aldarate metabolism; pentose phosphate pathway; pyrimidine metabolism; and thiamine metabolism (Table 5.8). However, proteins identified in the axes of dehydrated seeds of *A. marina* could only be grouped under six of these categories: purine metabolism; phenylpropanoid biosynthesis; glutathione metabolism; ascorbate and aldarate metabolism; pentose phosphate pathway; pyrimidine metabolism; and thiamine metabolism (Table 5.8).

Proteins identified in axes of control seeds of *T. dregeana* were grouped into 12 pathways: glycolysis/gluconeogenesis; methane metabolism, thiamine metabolism; fructose and mannose metabolism; ascorbate and aldrate metabolism; alanine; aspartate and glutamate; metabolism; glutathione metabolism; galactose metabolism; amino sugar and nucleotide sugar metabolism; pentose and glucuronate interconversions; butanoate metabolism; and beta-alanine metabolism (Table 5.9). When the identified proteins in the axes of the control were compared with the dehydrated and stored axes, the proteins identified in the treatments were grouped under 11 additional pathways: carbon fixation in photosynthetic organisms; purine metabolism; pyruvate metabolism; cysteine and methionine metabolism; glyoxylate and dicarboxylate metabolism; citrate cycle (TCA cycle); pentose phosphate pathway; phenylpropanoid biosynthesis; glycine, serine and threonine metabolism; fatty acid degradation; and starch and sucrose metabolism (Table 5.9).

Table 5.8: KEGG pathway analysis of proteins identified in embryonic axes of dehydrated and stored seeds of *A. marina*

Pathways	Control		Dehydrated		Stored	
	No. of sequences	No. of enzymes	No. of sequences	No. of enzymes	No. of sequences	No. of enzymes
Glycolysis/ gluconeogenesis	7	7	5	6	3	4
Carbon fixation in photosynthetic organisms	6	6	4	4	3	3
Fructose and mannose metabolism	3	2	3	2	2	1
Inositol phosphate metabolism	2	1	2	1	2	1
Pentose phosphate pathway	2	2	2	2	1	1
Methane metabolism	3	2	2	2	0	0
Purine metabolism	3	3	0	0	2	3
Phenylpropanoid biosynthesis	1	1	0	0	1	1
Glutathione metabolism	1	1	0	0	1	1
Ascorbate and aldarate metabolism	1	1	0	0	1	1
Pentose phosphate pathway	2	2	0	0	1	1
Pyrimidine metabolism	2	1	0	0	1	1
Thiamine metabolism	1	1	0	0	1	1

Table 5.9: KEGG pathway analysis of proteins identified in embryonic axes of dehydrated and stored seeds of *T. dregeana*

Pathways	Control		Dehydrated		Stored	
	No. of sequences	No. of enzymes	No. of sequences	No. of enzymes	No. of sequences	No. of enzymes
Glycolysis/ gluconeogenesis	12	12	18	12	14	12
Carbon fixation in photosynthetic organisms	0	0	18	11	14	9
Purine metabolism	0	0	13	5	15	5
Pyruvate metabolism	0	0	11	9	7	8
Methane metabolism	6	3	11	3	7	3
Thiamine metabolism	8	1	10	1	12	1
Cysteine and methionine metabolism	0	0	10	6	7	5
Glyoxylate and dicarboxylate metabolism	0	0	8	4	4	2
Citrate cycle (TCA cycle)	0	0	8	5	6	5
Pentose phosphate pathway	0	0	7	3	7	3
Phenylpropanoid biosynthesis	0	0	5	2	6	2
Fructose and mannose metabolism	4	2	5	2	5	2
Glycine, serine and threonine metabolism	0	0	4	4	2	2
Fatty acid degradation	0	0	4	2	2	2
Ascorbate and aldrate metabolism	3	3	4	3	4	3
Starch and sucrose metabolism	0	0	3	3	4	4
Alanine, aspartate and glutamate metabolism	2	2	3	5	2	2
Glutathione metabolism	2	3	2	2	4	4
Galactose metabolism	2	3	2	3	2	3
Amino sugar and nucleotide sugar metabolism	2	3	3	4	3	4
Pentose and glucuronate interconversions	2	3	7	3	7	3
Butanoate metabolism	2	2	2	2	2	2
Beta-alanine metabolism	2	3	3	3	2	3

5.5 Discussion

Moothoo-Padayachie *et al*. (2016) (as shown in Chapter 3), showed through assessments of both recalcitrant-seeded species in hydrated storage that as in other studies (Farrant *et al.*, 1989, 1992b) *A. marina* seeds are extremely short-lived (16-21 days [d], Farrant *et al.*, 1997, Calistru *et al.*, 2000) compared with *T. dregeana* seeds which can be successfully stored for months (Goveia *et al.*, 2004). Although, recalcitrant seeds are maintained under conditions of saturated relative humidity in hydrated storage (Berjak and Pammenter, 2013, FAO, 2013), the seeds eventually germinate or lose viability (Farrant *et al.*, 1989, Farrant *et al.*, 1996, Moothoo-Padayachie *et al.*, 2016). Physiological and ultrastructural studies have shown that the embryonic axes of recalcitrant seeds undergo germinative development in hydrated storage (Farrant *et al.*, 1986a, Pammenter *et al.*, 1994, Berjak and Pammenter, 2000, Moothoo-Padayachie *et al.*, 2016).

Furthermore, these changes imply that the seeds require additional water which if not supplied exposes the seeds to a water stress even under hydrated storage conditions (Farrant *et al.*, 1986a, Moothoo-Padayachie *et al.*, 2016). Berjak *et al*. (1989) suggested that the rate at which seeds germinated in storage was dependent on how the seeds germinated naturally. Findings by Moothoo-Padayachie *et al*. (2016) support this suggestion as the rate of water uptake and germination was far more rapid in seeds of *A. marina* than in *T. dregeana*. Germination in seeds of *A. marina* was also associated with an earlier spike in ROS production, and reduced levels of antioxidant activity compared with seeds of *T. dregeana* during hydrated storage.

In the present study, a label-free proteomic LC-MS/MS analysis of highly (*A. marina*) and moderately (*T. dregeana*) recalcitrant seeds exposed to partial dehydration and storage was conducted. A considerably higher number of proteins was identified in seeds of *T. dregeana* (105 proteins) compared with *A. marina* (30 proteins). The number of proteins identified in embryonic axes of *T. dregeana* seeds is comparable to other recalcitrant species such as in *Araucaria angustifolia* in which 96 proteins were identified (Balbuena *et al.*, 2009). The lower number of proteins identified in *A. marina* axes may be due to a combination of factors which include the low protein content of these seeds which has been previously reported and is not a general feature of desiccation sensitive seeds (Farrant *et al.*, 1992a). Proteomic studies of recalcitrant seeds in general are also difficult because they contain many interfering compounds (e.g. polyphenolic compounds) which present challenges in key steps namely, protein extraction and solubilisation (Balbuena *et al.*, 2011). Seeds of *A. marina* are high in polyphenolic compounds (Farrant *et al.*, 1992a, Anguelova-Merhar *et al.*, 2003), which are known to combine reversibly with proteins via covalent condensation reactions (Carpentier *et al.*, 2005). Although, a lower number of proteins were identified in axes of *A. marina* seeds compared with axes of *T. dregeana* seeds,

the number of proteins identified in *A. marina* axes (30) was still comparable to that of recalcitrant species such as *Camellia sinensis* in which 34 individual proteins were identified. Due to the large discrepancy in number of proteins identified in each of the species, an interspecies comparison based on the number of proteins identified was not possible thus the study focuses instead on determining interspecies differences based on differential expression of individual proteins/functional categories found in both species.

Differential expression of proteins was evident from the total number of proteins identified within treatments within species (Table 5.1). In seeds of *A. marina*, a higher number of proteins were identified in the axes of the control in comparison to axes of partially dehydrated and stored seeds. However, because of the relatively lower yield it is difficult to determine if the lower expression is a consequence of the treatments or a result of challenges with protein extraction as mentioned above. In contrast, a lower number of proteins were identified in the axes of the control *T. dregeana* seeds and a higher number of proteins were identified in the axes of partially dehydrated and stored seeds. These results suggest that there is definitely an up-regulation/increase in expression of proteins in response to partial dehydration and storage in *T. dregeana* seeds, which will be discussed in greater detail below.

The major functional categories that differed between the proteome of the control (freshly harvested seeds) and the treatments in terms of presence or absence were related to transferase activity, SOD activity and peroxidase activity (Fig. 5.4-5.5). Studies have shown that both SOD and peroxidase activity are induced during desiccation but decrease with prolonged desiccation treatment (Bai *et al.*, 2011, Chen *et al.*, 2011). Interestingly, Song *et al*. (2004) showed that SOD and ascorbate peroxidase (APX) activities were compromised in dehydrated *T. dregeana* seeds resulting in elevated levels of ROS. In this study peroxidase activity was identified as a functional category in control seeds of *T. dregeana* (Fig. 5.4b and 5.5b) but was absent as a functional category in dehydration and storage treatments, further suggesting that peroxidase activity may be compromised during these treatments.

In the axes of dehydrated and stored *A. marina* seeds (Fig. 5.6 a and c) six major categories were evident which included the following: response to ROS; response to ER; cellular response to osmotic stress; cellular response to oxidative stress; drought recovery and response to salt stress. A higher percentage of proteins were classified under the functional categories response to ROS and response to ER in axes of stored *A. marina* seeds compared with dehydrated *A. marina* seeds (Fig. 5.6a and c). These results further substantiate findings by Moothoo-Padayachie *et al*. (2016) that the poor storage longevity of *A. marina* seeds may be due to increased levels of ROS that trigger germination in *A. marina* seeds during storage. A lower percentage of proteins were classified under the following functional categories: cellular response to osmotic stress; cellular response to oxidative stress; drought recovery; and response

to salt stress in the axes of stored seeds of *A. marina* compared with axes of dehydrated *A. marina* seeds. A possible reason for the identification of proteins related to salt stress in *A. marina* seeds when a stress involving partial dehydration or mild dehydration in storage was applied in this study (Pammenter *et al.*, 1994) can be explained by the cross-tolerance phenomenon. Biotic and abiotic stress responses use common signals, pathways and triggers making it possible for one type of stress to activate plant responses that facilitate tolerance to several other types of stress (Wang *et al.*, 2012, Foyer *et al.*, 2016). This allows plants to withstand multiple mild and severe environmental stresses simultaneously, using the same mechanisms.

In terms of response to stress, two major functional categories were evident based on NCBI gene annotations to a GO level 3 in both treatments for *T. dregeana* seeds namely, response to ROS and response to ER stress (Fig. 5.6b and d). Proteins identified within the response to ROS category in both treatments confirm previous findings for recalcitrant seeds: that partial dehydration (Hendry *et al.*, 1992, Varghese and Naithani, 2002) and storage (Tommasi *et al.*, 2006) is accompanied by increased potential for oxidative stress, which necessitates heightened enzymic antioxidants (Pukacka and Ratajczak, 2006). Earlier ultrastructural studies, suggested that there was a probable onset of metabolic stress in embryonic axes of recalcitrant seeds during drying and that the ER played a pivotal role during dehydration and storage (Motete *et al.*, 1997, Berjak and Pammenter, 2000, Kioko *et al.*, 2006). For example, Wesley-Smith (2001) found that the ER cisternae in axes dried slowly often became rearranged into concentric rings which was also associated with cessation of growth in drought-stressed mustard seeds (Bergfeld and Schopfer, 1984), and with the inhibition of protein synthesis in pollen tubes subjected to heat shock (Kandasamy and Kristen, 1989). Thus, the results in this study confirm on a molecular level what was observed in terms of ultrastructural changes in previous studies.

In response to ROS, a 15 and 22 kDa SOD (P27082, W1NYZ2) was identified in the embryonic axes of both dehydrated and stored *A. marina* seeds (Table 5.4). However, interestingly a 15 kDa SOD (MOTEF8) was found in higher abundance in the axes of *T. dregeana* than *A. marina* seeds, following partial dehydration (Table 5.6). SOD is an enzyme that can regulate concentrations of both intracellular superoxide and peroxide efficiently in order to prevent formation of hydroxyl radicals through the Fenton reaction (Bowler *et al.*, 1994). Thus, this result may in part provide reasons for why *T. dregeana* seeds were able to survive took longer (14 d) to reach ±50% viability loss, compared with *A. marina* which exhibited similar levels of mortality in a relatively shorter period of time (4 d). Certain 'housekeeping' antioxidants may be up-regulated in seeds of recalcitrant species during oxidative stress; Chen *et al.* (2011) for example, found that desiccation initially induced antioxidant enzymes such as SOD in *Camellia sinensis* seeds but with prolonged desiccation these activities declined.

In *T. dregeana*, a 17.7 kDa heat shock protein (Q39930) was identified in axes of both dehydrated and stored seeds in response to ROS (Fig. 5.2-5). A study by Wehmeyer and Vierling (2000) has shown a correlation between the decline in small heat shock proteins (sHSPs) and desiccation intolerance in *Arabidopsis* seeds, suggesting that sHSPs play a role in desiccation tolerance. This phenomenon has also been shown during development and germination of maize embryos (Huang *et al.*, 2012). In Chapter 4 (section 4.5.5) a 16.9 kDa heat shock protein was also identified in *T. dregeana* and was shown to be down-regulated during dehydration and storage. In the present study, no significant differences ($P > 0.05$) in the abundance of the 17.7 kDa heat shock protein was evident between the treatments and the control (data not shown). However, in response to ER stress a probable mediator of RNA polymerase II transcription subunit 37c (MED37C), also known as heat shock 70 kDa protein 4, was found to have a higher abundance in axes of dehydrated *T. dregeana* than axes of dehydrated *A. marina* seeds (Table 5.6). This 70 kDa heat shock protein (MED37C) was also found to be in greater abundance in axes of stored *T. dregeana* than *A. marina* seeds (Fig. 5.7). Heat shock proteins have been suggested to play an important role as "molecular chaperones" to prevent the aggregation or promote the proper refolding of denatured proteins (Parsell and Lindquist, 1993). In doing so, HSPs play an important role in maintaining cellular homeostasis and proper biogenesis (Lin *et al.*, 2001). Although it was shown in Chapter 4 that HSPs may be down-regulated during desiccation in *T. dregeana* seeds, the comparatively higher abundance of this protein compared with dehydrated *A. marina* seeds may be a contributing factor to its relatively greater 'tolerance' to dehydration and longer storage longevity.

Another interesting protein, proteasome alpha subunit type (M0RJE7), was found to be slightly higher in abundance in axes of dehydrated *A. marina* seeds than axes of dehydrated *T. dregeana* seeds (Table 5.6). In plants, the 26S proteasome is essential for protein quality control because it degrades misfolded and denatured proteins during normal plant development and under adverse conditions (Kurepa *et al.*, 2009). With a lower abundance of HSPs, as mentioned above for axes of dehydrated *A. marina* seeds, more proteasome alpha subunit may be required than in *T. dregeana* axes which have a higher abundance of HSPs (Table 5.6), promoting the proper refolding of denatured proteins (Parsell and Lindquist, 1993). During storage, the proteasome alpha subunit occurred at a higher abundance in axes of *T. dregeana* than *A. marina* seeds but this difference was not significant ($P > 0.05$) (Table 5.7). However, a decrease in proteasome activity has been reported to accompany ageing in recalcitrant *Shorea robusta* seeds (Parkhey *et al.*, 2015).

Studies have likened the recalcitrant seeds of species like *A. marina* and *T. dregeana* to developing seedlings, since their chlorophyllous cotyledons and axes show signs of being photosynthetically active leading up to, and following germination (Berjak *et al.*, 1984, Farrant *et al.*, 1986b, Ramlall *et al.*, 2015). Interestingly, a photosystem II reaction center protein (Q85AJ6) occurred in higher abundance in axes

of stored *A. marina* seeds than in *T. dregeana* seeds (Table 5.7). This result provides further evidence for the comparatively higher metabolic and faster germination rate of *A. marina* in storage, relative to *T. dregeana* seeds and confirms on a molecular level what other authors have suggested based on ultrastructural and physiological observations (Berjak *et al.*, 1984, Farrant *et al.*, 1986b, 1993b). Furthermore, a nucleoside diphosphate kinase (NDP) (P39207) was identified in higher abundance in axes of stored seeds of *A. marina* relative to axes of stored *T. dregeana* seeds (Table 5.7). Nucleoside diphosphate kinase (NDP) is a ubiquitous housekeeping enzyme that catalyses the transfer of γ-phosphate from ATP to NDP through autophosphorylation (Parks and Aganwal, 1973). In a study by Pan *et al*. (2000), reversed genetics were used to suppress NDP kinase gene expression in *Oryza sativa* L. These transgenic plants exhibited developmental abnormalities, in particular suppression of cell elongation processes. Cell elongation is a key requirement for germination (Obroucheva, 2008). Thus, a higher abundance of this enzyme in axes of stored *A. marina* compared with stored *T. dregeana* seeds may also contribute to the faster germination rate in the former (as discussed in Chapter 3, section 3.4.2).

Calmodulin (CaM) (P62201) a calcium (Ca^{2+}) sensing protein was found in higher abundance in axes of dehydrated *T. dregeana* than *A. marina* seeds (Table 5.6). The calmodulin family is a major class of calcium sensor proteins which collectively play an important role in cellular signalling cascades through the regulation of numerous target proteins. Calcium (Ca^{2+}) plays a key role in the structural integrity of the cell wall and membrane system and has been shown to act as an intracellular regulator in plant growth and development including stress responses (Reddy, 2001, Sanders *et al.*, 2002, Shabala *et al.*, 2006). Osmotic stress can induce a series of responses at the molecular and cellular levels, one of which is an increase in cytosolic Ca^{2+} concentration and subsequent transduction of Ca^{2+} signals that promote appropriate cellular responses in an effort to alleviate potential damage (Xiong and Zhu, 2002, Xu *et al.*, 2011). In a study by Xu *et al*. (2011), expression of a novel calmodulin-like protein $OsMSR_2$ (*Oryza sativa* L. Multi-Stress-Responsive gene 2) was found to confer enhanced salt and drought tolerance in *Arabidopsis thaliana* accompanied by altered expression of stress/ABA-responsive genes. These results suggest an additional reason for the improved tolerance of *T. dregeana* to desiccation compared with *A. marina* seeds. Interestingly, maize cytosolic Hsp70 was identified to bind to CaM in the presence of Ca^{2+} induced by heat shock and could inhibit the activity of CaM-dependent NADK in a concentration-dependent manner, but its role in desiccation stress response has not been reported. As mentioned earlier, a 70 kDa HSP (MED37C) was also found at a higher abundance in axes of dehydrated *T. dregeana* than *A. marina* seeds (Sun *et al.*, 2000).

The KEGG pathway analysis of proteins performed for both *A. marina* and *T. dregeana* seeds revealed that during partial dehydration and storage, most proteins identified were associated with primary metabolic processes such as glycolysis and carbon fixation in photosynthetic organisms (Tables 5.8-

5.9). A greater number of identified sequences were related to specific metabolic pathways in the treated seeds than in the control seeds, suggesting that both dehydration and storage leads to increased expression of metabolically-related proteins (Tables 5.8 – 5.9). In both treatments of *A. marina* and *T. dregeana* seeds the majority of proteins within the energy metabolism category were related to glycolysis (Tables 5.8 - 5.9), illustrating the high energy demand for cell survival during stress. Interestingly, the glycolytic enzyme, triose phosphate isomerase (P48491) was relatively more abundant in axes of *A. marina* than *T. dregeana* seeds during dehydration and storage (Tables 5.6 – 5.7). This suggests a higher energy requirement in *A. marina* seeds compared with *T. dregeana* seeds, which again can be related to the fact that *A. marina* seeds possess higher metabolic activity (Farrant *et al.*, 1992b) and a faster germination rate (Moothoo-Padayachie *et al.*, 2016) than *T. dregeana* seeds (Chapter 3, section 3.4.2). Triose phosphate isomerase has also been reported to show increased abundance following desiccation in *C. sinensis* seeds (Chen *et al.*, 2011). Another glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (Q9FX54) was found in higher abundance in stored *T. dregeana* than stored *A. marina* seeds (Table 5.7). These results suggest that the retention of functional metabolically-related proteins such as glyceraldehyde-3-phosphate dehydrogenase (Q9FX54) during hydrated storage in *T. dregeana* seeds, may be a contributing factor their longer storage lifespan relative to those of *A. marina*.

Interestingly, another metabolism-related enzyme, adenosylhomocysteinase (Q9LK36), occurred at significantly ($P<0.05$) higher abundance in axes of dehydrated *T. dregeana* than dehydrated *A. marina* seeds (Table 5.6). The abundance of this enzyme was also relatively higher in axes of stored *T. dregeana* than *A. marina* seeds but this difference in abundance was not significant ($P > 0.05$) (data not shown). Adenosylhomocysteinase plays a major role in the synthesis of the one-carbon carrier S-adenosylmethionine (SAM) from methionine which is a major methyl donor in methylation reactions in plants (Rocha *et al.*, 2005). Adenosylhomocysteinase catalyses the conversion of adenosylhomocysteine to homocysteine. Adenosylhomocysteine is a competitive inhibitor of S-adenosylmethionine dependent methyl transferase reactions. Therefore, adenosylhomocysteinase (Q9LK36) during dehydration, particularly in *T. dregeana* seeds, may play a major role in the control of DNA or other substrates that require methylation via the regulation of the intracellular concentration of adenosylhomocysteine. In a study by Rocha *et al*. (2005) Arabidopsis hog1-1 mutant plants were shown to have reduced adenosylhomocysteinase activity. The hog1 mutant plants also grew slowly, had low fertility and reduced germination. Complementation of the hog1-1point mutation with a T-DNA containing the gene coding for adenosylhomocysteinase restored DNA methylation, fast growth and normal seed viability. Adenosylhomocysteinase has also been identified in the recalcitrant species *A. angustifolia* during seed development (Balbuena *et al.*, 2009). Therefore, low abundance of adenosylhomocysteinase in *A. marina* seeds may represent one of the reasons for its higher desiccation

sensitivity and shorter storage lifespan than *T. dregeana* seeds. It seems likely that desiccation (be it via physical dehydration or storage) leads to reduced expression of adenosylhomocysteinase which by interfering with DNA methylation reactions compromises viability/germinability in recalcitrant seeds.

5.6 Concluding remarks and recommendations

Although considerable research effort has been devoted towards characterising the physiological and biochemical responses of recalcitrant seeds to desiccation and storage, the molecular mechanisms underlying desiccation sensitivity in these seeds are still poorly understood. In this regard, proteomics which is fast becoming a powerful tool for functional analysis in plants, can be used to understand the molecular basis of desiccation sensitivity in recalcitrant seeds (Bai *et al.*, 2011, Chen *et al.*, 2011). However, labelling of proteins in the presence of interfering compounds can be challenging, especially in recalcitrant-seeded species. Analyses of the total proteome extracted from the embryonic axes of *A. marina* seeds was not possible using iTRAQ due to the presence of interfering compounds in particular a high amount of phenolic compounds (despite several attempts at optimisation of the protocol for its removal; unpublished findings). Therefore, in this study, a label-free proteomics method was employed to compare the protein profiles of *A. marina* and *T. dregeana* seeds exposed to partial dehydration and during storage.

The nature and degree of responses of recalcitrant seeds to desiccation stress are species-specific. This was evident from the present study by the differential seed viability and proteomic responses of *A. marina* (highly recalcitrant) and *T. dregeana* (moderately recalcitrant) seeds to partial dehydration (intense desiccation stress, over a short duration) and hydrated storage (mild desiccation stress, over a prolonged duration). Despite these differences, both stresses (partial dehydration and storage) perturbed parts of the proteome responsible for protein synthesis, maintenance of redox status, stress tolerance and provision of energy for cell survival significantly. Proteins found in higher abundance in the embryonic axes of *A. marina* seeds compared with *T. dregeana* seeds included triose phosphate isomerase, photosystem II reaction center protein and nucleoside diphosphate kinase, and are thought to contribute to the faster germination rate and hence, shorter storage lifespan of *A. marina* seeds. In the axes of dehydrated and stored *T. dregeana* seeds, proteins in higher abundance compared with *A. marina* seeds included SOD, a 70 kDa HSP, calmodulin, glyceraldehyde dehydrogenase and adenosylhomocysteinase all of which are believed to have contributed to the lower levels of desiccation sensitivity and longer storage lifespan of *T. dregeana* seeds. Pammenter and Berjak (1999) suggested that seed recalcitrance was a consequence of the absence and/or poor expression of some of the mechanisms involved in desiccation tolerance in orthodox seeds. The proteomic responses to partial dehydration and storage identified in the two recalcitrant-seeded species investigated here now allow us to narrow these potential

mechanisms down to proteins involved in a few key metabolic processes, e.g. protein synthesis, maintenance of redox status, stress tolerance and provision of energy for cell survival. Future research should focus on comparing expression levels of some of the functionally important proteins identified here between recalcitrant and orthodox seeds. Additionally, the difference in protein expression patterns between *A. marina* and *T. dregeana* uncovered here should be used to explore the continuum of seed desiccation sensitivity proposed by Berjak *et al*. (2008). Such studies will provide further mechanistic clues to the factors underlying desiccation sensitivity in recalcitrant seeds.

CHAPTER 6: Concluding Remarks and Recommendations

This study provides mechanistic insight into the desiccation sensitivity and reduced storage longevity of recalcitrant seeds and its findings can be used to improve on the design of short, medium- and long- term seed germplasm conservation protocols. The key conclusions of this study and how they relate to previous findings are discussed below. Recommendations for future avenues for research on recalcitrant seeds and the phenomenon of desiccation sensitivity/tolerance are also provided.

Comparison of *A. marina* and *T. dregeana* seed responses to partial dehydration and storage in terms of viability, ROS production and glutathione redox capacity, led to the confirmation that *A. marina* seeds are much more desiccation sensitive and have a shorter lifespan in hydrated storage compared with *T. dregeana* seeds. During partial dehydration *A. marina* seeds lost water at a much faster rate than those of *T. dregeana* seeds which substantiated earlier findings (Farrant *et al.*, 1989, Farrant *et al.*, 1997) regarding inter-species variability in drying rates. Partial dehydration studies also revealed that ROS play a deleterious role in the embryonic axes of dehydrated *A. marina* seeds as evidenced by a spike in ROS production ($\cdot O_2^-$ and H_2O_2) which coincided with 50% viability loss. However, in dehydrated *T. dregeana* seeds ROS levels in embryonic axes declined and were found to be highly reduced compared with the control, which supports previous suggestions that a dampening of the ROS-based trigger for germination during dehydration can lead to reduced germinability (Varghese *et al.*, 2011). This ROS-based trigger for germination manifested in hydrated stored seeds of both *A. marina* and *T. dregeana* as well and given that extra water was not provided to these seeds for the completion of germination they lost viability in storage. Interestingly, this trigger for germination occurred much earlier in *A. marina* seeds than *T. dregeana* seeds. The level of glutathione in *A. marina* seeds compared with *T. dregeana* seeds also suggested that *T. dregeana* seeds may have more prolonged/higher antioxidant protection than *A. marina* seeds. These results suggest that the mechanisms underlying desiccation-induced seed viability loss may differ across recalcitrant-seeded species based on the rate and extent to which they lose water during partial drying and storage. Although, recalcitrant seed desiccation sensitivity and storage longevity is modulated by redox metabolism, the specific ROS and antioxidants that contribute to this control may differ across species.

Through an assessment of the relationship between germination rate and storage lifespan in these two recalcitrant-seeded species of contrasting storage longevity it was confirmed that the shorter-lived *A. marina* seeds have a faster germination rate compared with *T. dregeana* seeds. Rapid

germination in *A. marina* was found to be associated with earlier spikes in ROS production and reduced antioxidant activity as germination was approached. In contrast, in slower germinating *T. dregeana* seeds, there was a delay in this spike in ROS production and these seeds had sustained antioxidant activity. These findings suggests that differences in the timing and intensity of the ROS-based trigger for germination may account for differences in storage longevity in recalcitrant seeds. These results were found to support the hypothesis suggested by Berjak *et al*. (1989), that the hydrated storage lifespan of recalcitrant seeds is dependent on the rate at which these seeds germinate naturally. Assessing germination in both recalcitrant-seeded species in terms of its physiology and biochemistry did present some challenges though, as unlike orthodox seeds recalcitrant seed post-harvest development is unabbreviated and even within the same harvest seeds can vary in terms of their developmental stage. Inter- and intra- seasonal variation in seed quality also meant that related parameters had to measured on the same batch of seeds – this can be extremely time consuming and logistically very challenging.

Assessment of the proteomic responses of *T. dregeana* seeds to partial dehydration and hydrated storage using isobaric tags for relative and absolute quantitation (iTRAQ) revealed that despite the differences in the intensity and duration of the desiccation stress imposed during partial dehydration (intense, short stress) and hydrated storage (less intense, prolonged stress), both treatments led to proteomic changes related to cellular redox imbalance and increased cell energy demands. Most importantly, key antioxidant proteins such as 2-cysperoxiredoxin were up-regulated in stored *T. dregeana* seeds which points towards their importance in protecting recalcirant seeds against oxidative stress during hydrated storage and possibly suppressing the biochemical trigger for germination during storage, as in *T. dregeana* seeds (Moothoo-Padayachie *et al*., 2016).

To compare the proteomic responses of *A. marina* and *T. dregeana* seeds to partial dehydration and hydrated storage, a label-free proteomics LC-MS/MS method was employed as proteins in *A. marina* protein extracts (from freshly harvested, partially dehydrated or stored seeds) could not be identified due to problems encountered with peptide labelling because of high amounts of interfering compounds, in particular phenolic compounds. Again, despite differences in the intensity and duration of the desiccation stress drying and storage key metabolic functions such as cellular redox balance and cell energy demand were compromised in both species during both treatments. The relatively longer storage lifespan of *T. dregeana* seeds in storage appears to be a consequence of specific proteins occurring at higher levels than in *A. marina* seeds; these include superoxide dismutase (SOD), adenosylhomocysteinase and calmodulin proteins. LEA proteins which are associated with desiccation tolerance (Galau *et al.*, 1986) were not found in in both species, using iTRAQ or label-free LC-MS/MS analysis. This result supports the argument that the degree of

desiccation sensitivity in recalcitrant seeds in part is due to the lack of accumulation/absence of LEA proteins (Han *et al.*, 1997, Delahaie *et al.*, 2013). The proteomic profile of recalcitrant- seeds is not well characterised compared with orthodox types. The proteomic data presented here therefore provides part of the proteomic footprint for desiccation sensitivity in seeds but more comparative proteomic studies are needed.

In this study, a ROS-based trigger for germination was found to play a key role in both desiccation sensitivity and storage longevity in both species; this trigger was also shown to be under the control of antioxidants. Since the timing of the ROS-based trigger for germination may determine the storage longevity of recalcitrant seeds, future studies should look at manipulating the timing of this ROS-based trigger by possibly using ROS blocking agents such as DPI and DMTU and/or adding an exogenous supply of antioxidants such as glutathione in order to extend the storage lifespan of recalcitrant seeds. Since changes in the proteome were evident in both recalcitrant species during dehydration and storage. Given the similarities in proteomic responses of both species to partial dehydration and hydrated storage investigated here it is clear that that proteins related to cellular redox metabolism, redox metabolism and protein synthesis (amongst others) play a key role in desiccation tolerance and deserve further investigation in this context. Differential protein expression patterns between *A. marina* and *T. dregeana* uncovered should also be used to design studies that explore the continuum of seed desiccation sensitivity suggested by Berjak *et al*. (2008) and others. These future studies may provide further mechanistic clues to the factors underlying desiccation sensitivity in recalcitrant seeds. Protein data for recalcitrant-seeded species are also very limited and even rare when one considers global protein databases (Balbuena *et al.*, 2011); more research on the proteomic profile of recalcitrant seeds is therefore needed.

In conclusion, this study adopted a multidisciplinary approach to understand the phenomenon of seed recalcitrance and more broadly desiccation sensitivity. The strength of its findings and more importantly value of the questions these findings have in turn generated, validate this multidisciplinary approach to recalcitrant seed biology. The results from this study have provided a basis for many new avenues of research on recalcitrant seeds and will be extremely useful in designing/improving protocols for the short-, medium- and long- term storage of recalcitrant seeds.

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