Some effects of drying rate and wet storage on aspects of the physiology and biochemistry of embryonic axes from desiccation-sensitive seeds

by

Tobias M. Ntuli

BSc, BSc (Hons), MSc *cum laude* (Natal)

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Foreword

Those who matter most to us are taken away too soon,

To my late dad, Thembinkosi Alson (1940- 1987),

Your spirit lives on!

"I do not have the pointer on the truth."

"I hope to live long enough to see us return to the vigorous debates where we argue who is right and wrong, not who is good and bad. My experience is most people I have known in this work are good people who love their country desperately and I am profoundly grateful for a brief period I had a chance to be one of them."

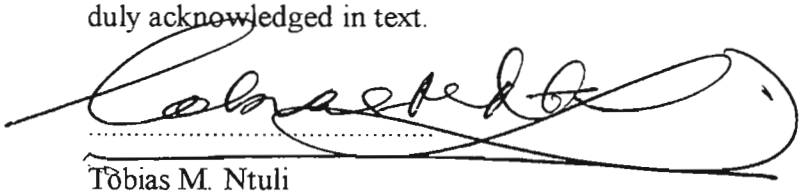
"I did it for the weakest possible reason. I can."

William Jefferson Clinton, 42nd President of the United States of America (1993-2000)

Preface

The experimental work described in this dissertation was carried out in the Plant Cell Biology and Plant Physiology Research Laboratories and Biochemistry Laboratory in the Division of Biological Sciences, School of Life and Environmental Sciences, University of Natal, Durban, South Africa and the Seed Science Research Laboratory in the Department of Crop and Weed Science, Horticulture Research International, Wellesbourne, United Kingdom under the supervision of Professors Patricia Berjak, Norman W. Pammenter and Michael T. Smith.

The present study represents original work by the author and has not been submitted in any form to another university. Where use was made of the work of others, it has been duly acknowledged in text.



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Abstract

Desiccation-sensitive seeds show differential viability characteristics during drying at different rates. A number of studies have demonstrated that rapid dehydration permits survival to lower water contents than does slower desiccation.

The aim and objective of the present study was to test the hypothesis which states that rapid drying of desiccation-sensitive seeds removes water sufficiently fast to reduce the accumulation of metabolic damage. In addition, the hypothesis that wet storage subjects desiccation-sensitive seeds to mild, but increasingly severe, water stress causing oxidative damage if additional water is not supplied, was tested.

In the present study, axes of germinating orthodox seeds of *Pisum sativum* and newly-shed recalcitrant counterparts of *Quercus robur*, *Strychnos madagascariensis*, *Trichilia emetica*, *Trichilia dregeana* and *Avicennia marina* were subjected to rapid or slow drying or wet storage. For those species where more than one harvest was investigated, differences were observed in water contents at shedding. For all the species studied, the dehydration rate could be described by an exponential and a modified inverse function for both desiccation regimes, and the water content remained constant with wet storage.

The level of tetrazolium staining and germination percentage of axes decreased sharply drying and hydrated storage such that the marked decline took place at lower water contents upon rapid than slow dehydration.

The conductivity of electrolyte leachate increased progressively during desiccation and moist storage of axes of all species investigated. Greater membrane leakage occurred upon slow, than rapid dehydration in axes of all species studied.

Activities of respiratory enzymes which have a potentially regulatory role in glycolysis, phosphofructokinase (PFK), or the tricarboxylic acid cycle, malate dehydrogenase (MDH), and levels of the oxidized form of the coenzyme, nicotinamide adenine dinucleotide (NAD), of the enzymes of the electron transport chain, NADH dehydrogenases of NADH-ubiquinone (coenzyme Q) reductase (complex I) and NADH-cytochrome *c* reductase (complex IV), were monitored in the present investigation.

In addition, the role of free radical activity in the form of lipid peroxidation, which has been implicated in loss of viability in seeds, was examined by assaying the levels of hydroperoxides. The involvement of the free radical processing enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR), and the antioxidant, ascorbic acid (AsA), was also ascertained.

The activity of PFK in axes of *P. sativum* remained constant during drying and wet storage. However, PFK activity increased as rapid dehydration and hydrated storage of *Q. robur* axes proceeded. In contrast, the activity of PFK in axes of *Q. robur* decreased during slow desiccation. Similarly, PFK activity was reduced upon drying, and moist storage, of *T. dregeana* axes such that higher activity of PFK was seen during rapid than slow dehydration. The activity of PFK in *A. marina* axes also declined upon desiccation.

The activity of MDH in axes of *P. sativum* was also unchanged during drying and wet storage. However, an increase in MDH activity was recorded in *Q. robur* axes during dehydration and hydrated storage such that the activity of MDH was higher upon slow than rapid desiccation. In contrast, MDH activity in axes of *T. dregeana* decreased as drying proceeded. Similarly, the activity of MDH declined during dehydration and moist storage of *A. marina* axes.

An increase in the level of NAD occurred in axes of *P. sativum* during drying. In contrast, a decrease in NAD levels was seen upon dehydration and wet storage of *Q. robur* axes such that the level of NAD was higher upon rapid than slow desiccation. There was an enhancement of the level of NAD in axes of *T. dregeana* during hydrated storage. Conversely, NAD levels declined during drying of *A. marina* axes.

A decrease in the level of hydroperoxides in axes of *P. sativum* was seen as rapid drying proceeded. In contrast, hydroperoxide levels increased during wet storage of *P. sativum* axes. Similarly, the levels of hydroperoxides were enhanced upon dehydration and hydrated storage of *Q. robur* axes such that they were higher in axes during slow desiccation compared to those dried rapidly. Conversely, the hydroperoxide level in axes of *T. dregeana* was reduced upon rapid dehydration. In contrast, an elevation of the level of hydroperoxides was observed during moist storage. The levels of hydroperoxides remained constant as desiccation and wet storage of *A. marina* axes proceeded.

The activity of SOD in axes of *P. sativum* decreased during rapid drying. In contrast, SOD activity increased upon slow dehydration and wet storage of *P. sativum* axes. There was a decline in the activity of SOD in *Q. robur* axes during slow desiccation. Similarly, SOD activity was diminished upon drying of axes of *T. dregeana*. The activity of SOD in *T. dregeana* axes was enhanced during hydrated storage. An elevation in SOD activity also took place during rapid dehydration and moist storage of axes of *A. marina*.

The activity of CAT did not change during drying of axes of *P. sativum*. However, a decrease in CAT activity in *Q. robur* axes was seen upon slow dehydration and wet storage. Similarly, the activity of CAT declined as desiccation of axes of *T. dregeana* proceeded. In contrast, CAT activity in *A. marina* axes increased during slow drying.

Whereas the activity of GR in axes of *P. sativum* increased during drying and wet storage, GR activity decreased in *A. marina* axes upon all treatments such that the activity of GR was higher during rapid than slow dehydration. GR activity also declined upon slow desiccation and hydrated storage of axes of *Q. robur*. Similarly, the activity of GR in *T. dregeana* axes was reduced during moist storage.

Finally, a decrease in the level of AsA in axes of *P. sativum* took place during drying. Nonetheless, dehydration and wet storage of *Q. robur* axes were associated with no siginificant change in AsA levels. There was also a decline in the level of AsA in axes of *T. dregeana* as rapid desiccation proceeded. Similarly, a reduction in AsA level occurred upon slow drying of axes of *A. marina*.

The results presented here are consistent with the observation that drying and wet storage adversely affected the respiratory enzymes, PFK, MDH and NADH dehydrogenase. It is suggested that the resultant metabolic imbalance led to more leakage of electrons from the mitochondrial electron transport chain than normal, and through lipid peroxidation increased levels of hydroperoxides. In addition, dehydration and hydrated storage may depress the activities of free radical processing enzymes, SOD, CAT and GR and levels of antioxidant, AsA. This phenomenon was less pronounced during rapid, in comparison to slow, desiccation and moist storage. However, it appears that the above biochemical events are overtaken by physical damage at higher water contents in the highly recalcitrant seeds. It was concluded that the differential effects of

the drying rate and wet storage on responses of desiccation-sensitive seeds varies with tissue, harvest, species and the degree of desiccation sensitivity.

Key to symbols and abbreviations

ABA	= abscisic acid
ACC	= 1-aminoacylpropane 1-carboxylic acid
AFM	= atomic force microscopy
ANOVA	= analysis of variance
APOD	= ascorbate peroxidase
AsA	= ascorbic acid (ascorbate)
CAT	= catalase
CSI	= chemical shift imaging
dm	= dry matter
DPPC	= dipalmitoylphosphatidylcholine
DSC	= differential scanning calorimetry
EP/SR(I)	= electron paramagnetic/spin resonance (imaging)
ENDOR	= electron nuclear double resonance
FFA	= free fatty acid
FTIR	= Fourier transform infrared
G6PDH	= glucose-6-phosphate dehydrogenase
GPOD	= guaicol peroxidase
g g^{-1} dm	= g H_2O / g dry matter
GR	= glutathione reductase
IBPGR	= International Board for Plant Genetic Resources
IPGRI	= International Plant Genetic Resources Institute
LEA	= late embryogenesis abundant (proteins)
LTSEM	= low-temperature scanning electron microscopy
MDH	= malate dehydrogenase
MIP	= major intrinsic protein
NMR(I)	= nuclear magnetic resonance (imaging)
PGR	= plant growth regulator
PFK	= phosphofructokinase

PIP	= plasmalemma intrinsic protein
PL(s)	= phospholipid(s)
POD	= peroxidase
PV(P)P	= polyvinyl(poly)pyrolidone
RH	= relative humidity
ROS	= reactive oxygen species
RWC	= relative water content
SOD	= superoxide dismutase
TOC	= tocopherol
T_g	= glass to liquid transition temperature
TIP	= tonoplast intrinsic protein
T_m	= membrane phase transition temperature
TZ	= tetrazolium
V_{50}	= 50% of the original viability
wc	= water content
ZR	= zeatin riboside

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Chapter 1

General introduction

1.1 General background

Seeds are the source of man's most important staple foods. For example, cereal grains alone, which comprise *c.* 90% of all cultivated seeds, contribute up to half the global per capita energy intake (Bewley, 1997; Bradford and Cohn, 1998).

In addition to providing humans with a direct and indirect source of food and animal feed, seeds are used in a vast array of industrial and commercial practices such as the production of oils, fibres, flavourings and drugs. As a result, the trade in seeds on the international market forms a substantial part of the global economy. For instance, it was estimated that the value of genetically manipulated seed, alone, could reach about US$30 and 60 billion by 2005 and 2015, respectively (Anonymous, 1997; Bradford and Cohn, 1998).

Furthermore, seeds, in the form of seed banks, play a critical role in the conservation of biodiversity. In this regard, it is noteworthy that the United Nations considers seed storage as the cheapest form of conservation of plant genetic resources (International Board for Plant Genetic Resources [IBPGR], 1976).

1.2 Seed classification

Traditionally, seeds are described as orthodox or recalcitrant on the basis of their ability, or lack thereof, to tolerate desiccation (a highly desirable trait in agriculture and conservation of biodiversity in seeds of flowering plants) and depending on their responses to chilling and their longevity in storage (Roberts, 1973). Characteristically, orthodox seeds usually undergo a period of maturation drying on the parent plant towards the end of their pre-shedding development, during which their water contents fall to very low levels and, finally, come to equilibrium with ambient relative humidity (RH). However, in some species, there is continued physiological maturation after drying and before shedding (e.g. cotton [Hughes and Galau, 1991]). Also, some orthodox seed species undergo dehydration after rather than before shedding (e.g. tomato [Berry and Bewley, 1991]). Irrespective of when drying occurs, orthodox

seeds tolerate dehydration to low water contents (5% or less, wet mass basis [Ellis *et al.*, 1990b]) and are amenable to cooling to low temperatures (-18°C or less [IBPGR, 1976]). Under these conditions, they can survive in hermetic storage for long periods (up to 100 years or longer [IBPGR, 1976]). Their survival period in storage can be predicted from their water content and temperature by the formula:

$$\nu = K_i - p / (10^{exp K_E - C_w \log_{10} m - C_H t - C_Q t^2})$$

where: ν = probit percentage viability

p = storage period (days)

m = water content (%, wet basis)

t = temperature (°C)

K_i = seed lot constant

K_E, C_W, C_H and C_Q = species viability constants (Ellis and Roberts, 1980; Ellis and Hong, 1996). The equation is subject to both low and high water content limits (Roberts and Ellis, 1989). Beyond the upper limit, seed longevity in hermetic storage is no longer reduced with further increases in water content. Similarly, no increases in survival accompany continued reduction in water content below the lower limit (Ellis *et al.*, 1990b).

In contrast to orthodox seeds, recalcitrant types are shed at relatively high water contents, apparently undergoing little or no maturation drying on the parent plant. In addition, they show variability in their sensitivities to desiccation and chilling and in storage lifespans and hence have been categorised as minimally, moderately and highly recalcitrant (Farrant *et al.*, 1988). Their longevity in moist storage is frequently brief and only rarely exceeds a few months (reviewed in Chin and Roberts, 1980; Bewley and Black, 1994; Smith and Berjak, 1995; Vertucci and Farrant, 1995; Berjak and Pammenter, 1997a,b; Pammenter and Berjak, 1999).

Recently, a third category of seeds - intermediate - has been identified (Ellis *et al.*, 1990a; 1991a,b,c). They tolerate dehydration to low water contents (though not as low as orthodox seeds). However, if tropical, they are sensitive to low temperatures in the desiccated state (Hong and Ellis, 1998).

Berjak *et al.* (1989) used the term poikilohydrous to refer to orthodox seeds, on one hand, as many undergo maturation drying on the host plant, are shed metabolically quiescent and come to equilibrium with ambient humidity (Farrant *et al.*, 1993a,b). On

the other hand, recalcitrant seeds have been said to be homoiohydrous (Berjak *et al.*, 1989; 1990; Farrant *et al.*, 1992a) as they are shed at high water contents whilst they are relatively metabolically active and die if they come into equilibrium with ambient humidity (Roberts and King, 1980). Nonetheless, recalcitrant seeds do reach that stage even if it is after extended periods of exposure (discussed in Dickie and Pritchard, 2002). It has been suggested that their curtailed lifespans are a consequence of the initiation of germination on, or shortly after, shedding (Pammenter *et al.*, 1984) and in many cases additional water is required for the process to go to completion (Farrant *et al.*, 1986).

Plants that produce recalcitrant seeds generally occur in habitats conducive to relatively rapid, if not immediate, seedling establishment, such as wetlands, aquatic environments and tropical rainforests, usually where there is no temperature constraint (reviewed by Roberts and King, 1980; Berjak *et al.*, 1989; von Teichman and van Wyk, 1994; Hong and Ellis, 1997; 1998; Hong *et al.*, 1998; Farnsworth, 2000; Hay *et al.*, 2000; Pammenter and Berjak, 2000; Dickie and Pritchard, 2002). In such environments, there can be little selective advantage to maturation drying and dormancy.

Oliver *et al.* (2000) argued that desiccation-tolerance was the ancestral state for early land plants (liverworts, hornworts and mosses), but that this trait was lost early in the evolution of tracheophytes, possibly beginning in Silurian (from 439 mya). In this regard, it is interesting to note that none of the vegetative tissues of extant gymnosperms are known to tolerate desiccation (Gaff, 1980; reviewed by Proctor and Pence, 2002). Farnsworth (2000) and Oliver (2000) have suggested that the most parsimonious explanation of the current distribution of species seed desiccation sensitivity is by convergent loss of tolerance from tolerant ancestors. Recalcitrance may be a derived trait evolved through neoteny and is probably associated with large seeds and trees (reviewed by Dickie and Pritchard, 2002). Alternatively, systematic analyses, mainly based on primitive seed structures, have led to the proposal that seed desiccation sensitivity is the ancestral state, with tolerance evolving early, and several times independently (von Teichman and van Wyk, 1991; 1994; Pammenter and Berjak, 2000).

In conclusion, it is noteworthy that Berjak and Pammenter (1994; 1997a,b; 2001; 2004) and Pammenter and Berjak (1999) and Dussert *et al.* (1999) and Sun (1999)

contend that natural populations show a continuum of responses to desiccation subtended by orthodoxy, on one hand, and extreme recalcitrance, on the other, unlike highly inbred crop species. This view is in contrast to that of Walters (1999) who proposes five discrete levels of desiccation-tolerance. Thus, Berjak and Pammenter (1994) propose that when reporting on recalcitrance, it is important to give as much information about the species concerned as possible. These data include: (1) natural habitat of the species, (2) time to reach 50% of the original viability (V_{50}) during storage under fully hydrated conditions at specified temperature, (3) mean water content of both embryonic axes and storage tissues for V_{50} on dehydration at specified drying rates, (4) water activity corresponding to V_{50}, (5) temperature for V_{50}, (6) low-temperature sensitivity of dehydrated seeds, (7) sensitivity to temperature of dehydration, (8) dormancy and (9) taxonomic status.

1.3 Seed development (Embryogenesis)

Farrant *et al.* (1993b) suggested that the differences in post-shedding behaviour among seed types arise as a result of differences in pre-shedding development. However, there are few studies on the development of recalcitrant seeds [e.g. *Quercus alba* L. (Bonner, 1976), *Guilfoylia monostylis* (Benth) F. Muell. (Nkang and Chandler, 1986), *Podocarpus henkelii* Stapf. (Dodd *et al.*, 1989), *Acer pseudoplatanus* L. (Hong and Ellis, 1990) and *Quercus robur* L. (Finch-Savage, 1992; Finch-Savage *et al.*, 1992; Grange and Finch-Savage, 1992) in contrast to orthodox seeds. Consequently, comparison of the acquisition or non-acquisition of desiccation-tolerance between the two seed types in terms of their development is presently difficult but important in elucidating the basis of desiccation-tolerance (reviewed by Farrant *et al.*, 1993b; Finch-Savage, 1996; Kermode and Finch-Savage, 2002).

Much of the evidence on the characteristics of recalcitrant seeds comes from a study on the development and behaviour of the highly recalcitrant seeds of the white mangrove, *Avicennia marina* (Berjak *et al.*, 1984; Farrant *et al.*, 1985; 1992a,b; 1993a,b,c). It may be expected that other recalcitrant seeds as well as intermediate seeds will have characteristics between those of *A. marina* and orthodox seeds. Mutants of *Zea mays* and *Arabdopsis thaliana* which produce recalcitrant seeds (Neill *et al.*, 1986 and Koorneef *et al.*, 1989, respectively) are also useful for comparative purposes.

The embryological development in *A. marina* is similar to that occurring in other dicotyledonous angiosperm seeds (Farrant *et al.*, 1992a). In addition, it appears to be under the same plant growth regulator (PGR) control (Farrant *et al.*, 1993c).

However, embryo formation occurs outside of the ovular tissue, unlike the situation in most seed types (Farrant *et al.*, 1992a). As a result, there is no integument-derived seed coat. Thus, the embryo is bounded by the pericarp. Such behaviour, called cryptoviviparity, is thought to be related to the mangrove habit (Tomlinson, 1986) and is not a general rule of desiccation-sensitive seeds (Farrant *et al.*, 1993b).

The development of orthodox seeds can be conveniently divided into three confluent stages. The single-celled zygote undergoes extensive mitotic division during histodifferentiation. The resultant cells differentiate to form the embryo (axis and cotyledons). Concurrently, there is the formation of the triploid endosperm or haploid megagametophyte. Thereafter, cell division ceases and seeds grow by laying down reserve material. Finally, the development of orthodox seeds is terminated by some degree of drying called maturation drying which results in a gradual reduction in metabolic activity as water is lost from seed tissues. As a result, the embryo passes into a metabolically inactive or quiescent state.

1.3.1 Histodifferentiation

Farrant *et al.* (1993b) maintained that the process of histodifferentiation is essentially similar in the seed types (reviewed by Kermode and Finch-Savage, 2002). It is also under the same PGR control. Indole-3-acetic acid, the main auxin occurring naturally in plants, cytokinins and gibberellic acid are thought to promote the formation of embryonic tissues.

1.3.2 Seed growth

Recalcitrant seeds undergo the process of growth by cell expansion and reserve deposition in common with their orthodox counterparts (reviewed by Farrant *et al.*, 1993b; Kermode and Finch-Savage, 2002). The nature and quantity of the reserves accumulated during seed development have been related to germination characteristics, such as the germination rate, which, in turn, has been related to the degree of recalcitrance and the natural habitat (Berjak *et al.*, 1989; Farrant *et al.*, 1989). It is, therefore, not surprising that recalcitrant seeds show variability in the nature and quantity of reserves amassed. While *A. marina* acquires carbohydrates mainly in the form of soluble oligosaccharides, with starch as the only form of complex reserve accumulated (Farrant *et al.*, 1992a), other recalcitrant seeds (e.g. *Quercus alba* [Bonner, 1976], *Camellia sinensis* O. Kuntze [Devey *et al.*, 1987], *Podocarpus henkelii* [Dodd *et al.*, 1989] and *Landolphia kirkii* Dyer [Berjak *et al.*, 1992] accumulate various complex reserves similarly to orthodox seeds.

A difference between the two seed types, as regards reserve accumulation, concerns the levels of abscisic acid (ABA). This PGR is widely believed to play an important role in reserve deposition, particularly in that of proteins, in orthodox seeds (e.g. Quatrano, 1987, Kermode, 1990). Recalcitrant seeds show variability in the amounts of ABA during development. While embryonic axes of *A. marina* show insignificantly low levels of this PGR, together with lack of storage proteins (Farrant *et al.*, 1992a; 1993c), cotyledons of *Quercus robur* maintain high quantities of ABA almost until shedding (Finch-Savage *et al.*, 1992). However, peak ABA levels have subsequently been shown to be significantly lower than those reported for many orthodox seeds (Finch-Savage and Farrant, 1997).

1.3.3 Reserve utilisation

Farrant *et al.* (1993c) observed a decline in the levels of ZR towards the end of development in seeds of *A. marina*. Such a decline is thought to promote reserve utilisation in the metabolically active seeds (Farrant *et al.*, 1992a).

1.3.4 Acquisition of germinability

It has been shown that isolation of the embryo from covering tissues allows germination to occur precociously for many orthodox seeds (reviewed by Farrant *et al.*, 1993b; Finch-Savage, 1996; Kermode and Finch-Savage, 2002). Such an event has been shown for a variety of seeds (Ihle and Dure, 1972; Quebedeaux *et al.*, 1976; Long *et al.*, 1981; Ackerson, 1984; Bartels *et al.*, 1988; Bochicchio *et al.*, 1988; Kermode and Bewley; 1988; Rasyad *et al.*, 1990).

However, application of ABA prevents the germination of excised embryos (Crouch and Sussex, 1981; Long *et al.*, 1981; Quatrano *et al.*; 1983; Eisenberg and Masceranhas, 1985; Xu *et al.*, 1990). High levels of ABA and/or high osmolality are held to promote the development of the embryo (Finkelstein *et al.*, 1985; Quatrano, 1987; Welbaum and Bradford, 1988; Kermode, 1990; Xu *et al.*, 1990).

Seeds of *A. marina* appear to show a similar behaviour to orthodox seeds with respect to the control of precocious germination, although its ABA is located extra-embryonically, in the pericarp (Farrant *et al.*, 1993b). The levels of ABA decline during maturation drying in non-dormant orthodox seeds (King, 1976; Kermode, 1990). The desiccated state prevents germination.

The acquisition of germination is similar in both seed types (Farrant *et al.*, 1993b). It is thought that most orthodox seeds are able to achieve normal post-germinative growth once they have reached a stage of near-completion of reserve accumulation, or at, or just after, the onset of maturation drying (Obendorf *et al.*, 1980; Dasgupta *et al.*, 1982; Kermode and Bewley, 1985, Rosenberg and Rinne, 1986; Ellis *et al.*, 1987; Kermode, 1990; Rasyad *et al.*, 1990). In addition, gibberellins and/or other PGRs are thought to be a requirement for seedling establishment in orthodox seeds. It is noteworthy that the competence to respond to, or for the synthesis of, these PGRs might be achieved only after some desiccation in orthodox seeds (Kermode and Bewley, 1985; 1986; Kermode, 1990).

1.3.5 Acquisition of desiccation-tolerance

Orthodox seeds do not tolerate desiccation during early development. They undergo a transition to a desiccation-tolerant state approximately midway through development (reviewed by Kermode, 1990; 1995; Kermode and Finch-Savage, 2002).

The rate of drying during early development is critical. Whole seeds of several legumes (Adams *et al.*, 1983; Ellis *et al.*, 1987) and castor bean (*Ricinus communis*) (Kermode and Bewley, 1985) are unable to withstand rapid drying at early stages of development. In contrast, full germination is evident at the same stage in seeds dried slowly. Tolerance of rapid dehydration generally occurs only at or near the completion of reserve deposition just after the onset of maturation drying (Rogerson and Matthews, 1977; Kermode and Bewley, 1985; Ellis *et al.*, 1987). It is thought that gradual water loss may allow protective changes to occur.

Tolerance to desiccation increases throughout reserve accumulation in most recalcitrant seeds as in their orthodox counterparts (Hong and Ellis, 1990; Farrant *et al.*, 1997). However, recalcitrant seeds are shed before full desiccation-tolerance is acquired. Nonetheless, there appears to be no clear end-point to development in this seed type. Thus, development in recalcitrant seeds is said to be indeterminate (Finch-Savage and Blake, 1994).

1.3.6 Maturation drying

One of the fundamental differences between orthodox and recalcitrant seeds is that recalcitrant seeds do not undergo maturation drying *sensu stricto*. Although some recalcitrant seeds may undergo a considerable reduction in embryo water content during their development, hydration levels at shedding are nevertheless high (Berjak *et al.*, 1992; Finch-Savage *et al.*, 1992). Unlike orthodox seeds, recalcitrant seeds remain hydrated and metabolically active throughout development and do not become tolerant of a significant degree of desiccation at any stage (Berjak *et al.*, 1989; Farrant *et al.*, 1989; 1993a). It is worth noting that not all orthodox seeds undergo full maturation drying (e.g. tomato [Berry and Bewley, 1991]).

1.4 Mechanisms of desiccation-tolerance

Part of the puzzle of desiccation-tolerance in plants is that it is very uncommon but nearly universal (reviewed by Vertucci and Farrant, 1995; Alpert, 2000; Alpert and Oliver, 2002). The relative biomass of desiccation-tolerant plants in all but the most arid or frigid habitats is very low and fewer than one in a thousand species of flowering plants is known to tolerate desiccation.

In addition, desiccation-tolerance appears common, though not universal in bryophytes (e. g. Richardson, 1981; Proctor, 1990), common in lichens (Kappen and Valladeres, 1999), uncommon in pteridophytes, rare in angiosperms and no gymnosperms are known to tolerate desiccation (Gaff, 1980; reviewed by Dickie and Pritchard, 2002) even though gymnosperms may have desiccation-tolerant seeds or pollen (Hoekstra, 2002; Kermode and Finch-Savage, 2002). Desiccation-tolerance also occurs in non-lichenised fungi, cyanobacteria and algae (Mazur, 1968; Schonbeck and Norton, 1978; Potts, 1994; 1999; Doods *et al.*, 1995) but little is known about its extent. It must be very common in free-living algae and bacteria that grow on surface of plants or soil, where they are probably subject to desiccation (Alpert and Oliver, 2002). It is also common in seeds of flowering plants, but tolerance to desiccation is not an exclusive preserve of orthodox seeds. It is the rule rather than the exception in other propagating structures such as pollen and spores of lower plant and fungi, and buds and somatic embryos of at least some spermatophytes also possess this unusual feature.

It was thought that the mechanism of this phenomenon were purely mechanical until recently when Bewley (1979) suggested that desiccation-tolerance is primarily protoplasmic in nature. Much of our understanding of cellular mechanisms of desiccation-tolerance comes from studies on orthodox seeds (reviewed by Bewley and Black, 1994). Desiccation-tolerance in lower orders such as mosses and algae is held to be based on repair rather than protection, as is hypothesised for seeds (Bewley and Oliver, 1992).

Despite its widespread occurrence, the mechanisms of desiccation-tolerance are not yet fully understood (reviewed by Vertucci and Farrant, 1995; Alpert and Oliver, 2002). It may be concluded that desiccation-tolerance is difficult to achieve and is energetically costly, because not all tissues possess this ability. There is probably a trade-off between this trait and growth. Alternatively, tolerance to desiccation is lost

in plants that are not exposed to it due to lack of selection pressure to maintain this characteristic.

Desiccation-tolerance is acquired during development and is lost after germination in seeds (reviewed by Vertucci and Farrant, 1995; Finch-Savage, 1996; Berjak and Pammenter, 1997a,b; Kermode and Finch-Savage, 2002). Many investigators have shown that embryos become more tolerant as they mature and less tolerant as they germinate for both recalcitrant and orthodox seeds (Rogerson and Matthews, 1977; Bewley, 1979; Long *et al.*, 1981; Sargent *et al.*, 1981; Dasgupta *et al.*, 1982; Adams *et al.*, 1983; Kermode and Bewley, 1985; Farrant *et al.*, 1986; 1988; 1989; Rosenberg and Rinne, 1986; Fischer *et al.*, 1988; Berjak *et al.*, 1989; 1992; 1993; Welbaum and Bradford, 1989; Hong and Ellis, 1990; 1992; Berry and Bewley, 1991; Pritchard, 1991; Finch-Savage, 1992; Sun and Leopold, 1993; Tompsett and Pritchard, 1993; Farrant *et al.*, 1997). In contrast, Berjak *et al.* (1992) showed that axes from seeds of *Landolphia kirkii* became more desiccation-sensitive as they matured. However, it is noteworthy that in that study desiccation sensitivity was assessed in terms of electrolyte leachate conductivity rather than germinability and that the axes from immature seeds were ungerminable. Furthermore, no change in desiccation sensitivity was observed in seeds of *Avicennia marina* after they had become germinable (Farrant *et al.*, 1993a).

Desiccation-tolerance differs greatly among species within genera, provenances, harvests and individuals within species and tissues within individuals in seeds (reviewed by Dickie and Pritchard, 2002). In addition, tolerance to desiccation varies highly with temperature of drying conditions (Berjak *et al.*, 1994; Ntuli *et al.*, 1997; reviewed in Song *et al.*, 2003) and rate, as well as conditions upon rehydration (reviewed by Alpert and Oliver, 2002; Pammenter *et al.*, 2002; Song *et al.*, 2003).

A number of mechanisms have been implicated in desiccation-tolerance. However, none of them, alone, seems to explain the phenomenon fully. Presently, it appears that the presence of at least some of these mechanisms and possibly some yet to be identified is necessary for the attainment of desiccation-tolerance.

1.4.1 Desiccation protectants

a. The role of sucrose, oligosaccharides and cyclitols

The water replacement hypothesis is one of the earliest theories attempting to account for desiccation-tolerance in living tissues (Clegg, 1986; Crowe and Crowe, 1986). It proposes that non-reducing sugars replace water on macromolecular surfaces during dehydration, thus enabling stabilisation of membranes in the desiccated state (reviewed by Crowe *et al.*, 1992).

High levels of sugars, particularly the disaccharide, sucrose, and oligosaccharides, raffinose and stachyose, have been suggested to afford desiccation-tolerance in orthodox seeds (Leopold and Vertucci, 1986; Koster and Leopold, 1988; Chen and Burris, 1990; Leprince *et al.*, 1990a; Blackman *et al.*, 1992). Obendorf and co-workers have recently implicated free and galactosyl sugar alcohols or cyclitols in a similar role (Horbowicz and Obendorf, 1994; Obendorf, 1997).

Drying induces lipid phase transitions from the lamellar liquid-crystalline phase to the solid gel phase (reviewed by Crowe *et al.*, 1992; 1997). A rise in the membrane phase transition temperature (T_m) commences with the loss of the last 10-12 water molecules per phospholipid (PL) molecule at water contents below 0.2 to 0.3 g g^{-1}. Sugars depress the T_m by interacting with the polar head groups of PLs and replace the water molecules (e.g. Hoekstra *et al.*, 1991).

However, the aforementioned observations are confounded by the fact that high levels of soluble sugars, particularly stachyose, occur in the highly recalcitrant seeds of *A. marina*. In addition, sucrose comprises 45% of the total sugar content of the moderately recalcitrant *Camellia sinensis* (tea) seeds, consisting of 11% of the dry matter of these seeds (reviewed by Berjak *et al.*, 1989).

Dehydration-induced damage occurs at hydration levels far higher than those at which water would be removed from membrane surfaces in *C. sinensis* and other recalcitrant seeds (Berjak *et al.*, 1989; 1992; Pammenter *et al.*, 1991). In this regard, Leopold and collaborators have postulated that vitrification (or glass formation) is perhaps the major mechanism by which desiccation-tolerance is achieved in orthodox seeds (Koster and Leopold, 1988; Williams and Leopold, 1989; Koster, 1991). There is evidence to suggest that the metastable glass (vitrified) state is promoted at low water contents, when sucrose and certain oligosaccharides and/or galactosyl cyclitols form high high-viscosity amorphous super-saturated solutions (Obendorf, 1997). The

prevalence of glasses imposes a stasis on metabolic and deleterious reactions (Leopold *et al.*, 1994).

The presence of the glassy state depends on three factors: (1) water content, (2) temperature and (3) chemical composition. A decrease in the water content of the tissue results in an increased glass transition temperature (T_g). The value of T_g is also dependent on the composition of the amorphous state. T_g is known to vary with molecular weight (Slade and Levine, 1991). For instance, a sugar of high molecular weight, like stachyose, exhibits a higher T_g over the entire range of water contents than a small molecular weight sugar such as glucose.

However, it is unlikely that achievement of the glassy state occurs at the high water contents at which recalcitrant seeds die, except perhaps when excised axes survive very rapid dehydration to appropriate water contents (Pammenter *et al.*, 1993). In this regard, it has been suggested that a significant proportion of sugars may be tightly associated with late embryogenesis abundant (LEA) proteins (Walters *et al.*, 1997; Wolkers *et al.*, 1998c). Such complexes are held to control and optimise the rate of water loss during dehydration of orthodox seeds.

b. The role of late embryogenesis abundant (LEA) proteins

A variety of orthodox seeds accumulate a set of dehydration- and/or ABA-inducible hydrophilic, and heat-stable (with the exception of Group 5) proteins at the time of acquisition of desiccation-tolerance during late stages of embryogenesis (Cuming and Lane, 1979; Aspart *et al.*, 1984; Williamson *et al.*, 1985; Galau *et al.*, 1986; 1987; 1991; 1993; Galau and Hughes, 1987; Bartels *et al.*, 1988; Bochicchio *et al.*, 1988; Goday *et al.*, 1988; Mundy and Chua, 1988; Rosenberg and Rinne, 1988; 1989; Blackman *et al.*, 1991; Hughes and Galau, 1987; 1989; 1991; Bradford and Chandler, 1992; Ried and Walker-Simmons, 1993; Roberts *et al.*, 1993; Mao *et al.*, 1995; Wolkers et al., 1998c). LEA proteins have been linked to desiccation-tolerance (Close *et al.*, 1989; 1993; Kermode, 1990; Blackman *et al.*, 1991; Bewley and Olivier, 1992; Bradford and Chandler, 1992; Mao *et al.*, 1995; Wolkers et al., 1998c).

A number of mechanisms by which LEA proteins may protect cellular constituents have been suggested. It has been hypothesised that they bind to macromolecular surfaces in much a similar way as postulated for sucrose and oligosaccharides (Dure *et al.*, 1989). Many LEA proteins have extensive regions of random coiling which have

been proposed to promote the binding of water, as a result helping to maintain a minimum water requirement (Ingram and Bartels, 1996). In addition, Barker *et al.* (1988) suggested that the random coil nature of some of the LEA proteins may allow them to conform to the shape of cellular constituents thus helping to maintain their solvation state by virtue of their hydroxyl groups when water is removed. Furthermore, those authors proposed that Group 2 LEA proteins (dehydrins) provide surfaces that would sequester ions by virtue of their amphipathic helical repeats. LEA proteins may also act as anchors in the structural network that stabilises cytoplasmic components during drying and in the desiccated state (Walters *et al.*, 1997; Wolkers et al., 1998c).

The genes that encode LEA proteins in developing cotton seed are comprised of two distinct classes whose regulation is co-ordinated (Galau *et al.*, 1986; 1987; 1991; Galau and Hughes, 1987; Hughes and Galau, 1991). One class contains six different *lea* transcripts which appear relatively early in development and reach maximum level about three days before the onset of maturation drying. The other class contains 12 transcripts which appear late in maturation and reach maximum expression just before and during maturation drying.

LEA proteins fall into five groups by virtue of sequence similarities (Dure *et al.*, 1989; Ingram and Bartels, 1996; Cumming, 1999). Group 1 LEA proteins are characterised by a 20-amino acid motif (Cumming and Lane, 1979). Similarly, Group 2 LEA proteins (dehydrins) share a characteristic 15-amino acid motif, the K-segment, which is a stretch of serine residues and a conserved motif near the N-terminus of the protein (Close, 1996; 1997). Dehydrins are the most widespread and studied. Group 3 LEA proteins are characterised by a 11-amino acid motif (Dure *et al.*, 1989) which is predicted to form an amphipathic α-helix. Group 4 and 5 LEA proteins are more hydrophobic than other LEA proteins and are not resistant to high temperature (Dure *et al.*, 1993b; Galau *et al.*, 1993).

The evidence for the involvement of LEA proteins in desiccation-tolerance is circumstantial but compelling (reviewed by Alpert and Oliver, 2002). For instance, ABA-deficient and ABA-insensitive double-mutants of *Arabidopsis thaliana* seeds do not dry on the parent plant, do not tolerate desiccation and lack several LEA proteins (Koorneef *et al.*, 1989; Meurs *et al.*, 1992). In addition, the observation that seeds of *A. marina* do not produce these particular proteins (Farrant *et al.*, 1992b) supports the

hypothesis that lack of such proteins might be an inherent feature of desiccation sensitivity (Bradford and Chandler, 1992).

In contrast, Finch-Savage *et al.* (1994b), Farrant *et al.* (1996) and Han *et al.* (1997) have shown LEA proteins in several recalcitrant seeds of temperate and sub-/tropical trees, respectively. Furthermore, Blackman *et al.* (1991) showed that LEA proteins accumulated in soybeans before desiccation-tolerance developed.

c. The role of small heat-shock proteins (sHSPs)

Heat-shock proteins (HSPs) have recently been associated with desiccation-tolerance. In contrast to those of other eukaryotes, the most prominent HSPs of plants are small heat-shock proteins (sHSPs). They have monomeric molecular masses of 15-42kDa, but assemble into oligomers of nine to over 20 subunits, depending on the protein (Waters *et al.*, 1996).

Expression of sHSPs has been observed before and after maturation drying in developing seeds and in resurrection plants (Almoguera and Jordano, 1992; Coca *et al.*, 1994; DeRocher and Vierling, 1994; Alamillo *et* al., 1995; Wehmeyer *et al.*, 1996; Collada *et* al., 1997; Wehmeyer and Vierling, 2000). While sHSPs are relatively abundant during the first few days of germination, they decline rapidly as it progresses (reviewed by Alpert and Oliver, 2002; Buitink *et al.*, 2002).

sHSPs are molecular chaperones. They interact with other proteins which help to maintain protein structure under denaturing conditions (Waters *et al.*, 1996; Gething, 1997, Feder and Hofmann, 1999; Soto *et al.*, 1999; Sales *et al.*, 2000).

d. The role of major intrinsic proteins (MIPs)

Major intrinsic proteins (MIPs) are a family of channel proteins that are mainly represented by aquaporins in plants. They are generally divided into tonoplast intrinsic proteins (TIPs) and plasmalemma intrinsic proteins (PIPs) according to their subcellular localisation (reviewed by Maurel *et al.*, 1997).

For instance, the vacuolar membrane protein, α-TIP, a water channel protein accumulates during seed maturation in parenchyma cells of seed storage organs. Synthesis of this integral membrane protein does not appear to be related, in a quantitative manner, to storage protein deposition. A role in seed desiccation,

cytoplasmic osmoregulation and/or seed rehydration has been suggested (Johnson *et al.*, 1989).

The water-channel activity can be regulated by phosphorylation. The protein assembly as a 60 Å X 60 Å square in which each subunit is formed by a heart-shaped ring comprised of α-helices (Daniels *et al.*, 1999).

Homologues to PIPs and TIPs are controlled by dehydration and ABA in desiccation-tolerant resurrection plant *Craterostigma plantagineum* (Mariaux *et al.*, 1998). Members of a subset of PIPs (PIPa) are regulated by ABA-dependent and ABA-independent pathways.

e. The role of osmolytes

Many plants and microorganisms accumulate organic compounds of low molecular weight known as osmolytes or compatible solutes in response to environmental stresses that cause cellular dehydration, such as drought, freezing and osmotic shock (Amuti and Pollard, 1977; Zhang *et al.*, 1982; Hoekstra *et al.*, 1992b; Saranga *et* al., 1992; Takagi *et* al., 1997; Hare *et* al., 1998; Strom, 1998). Their accumulation correlates with improved stress tolerance. Among such compatible solutes are proline, serine, glutamate, glycine-betaine, carnitine, mannitol, sorbitol, fructans, polyols, trehalose, sucrose and oligosaccharides.

It has been shown in model experiments that these substances stabilise protein structure and activity by keeping macromolecules preferentially hydrated thus preventing them from unfolding (Arakawa and Timasheff, 1985; Rudolph and Crowe, 1985; Anchordoguy *et* al., 1987; 1988; Carpenter and Crowe, 1988, Carpenter *et al.*, 1990; 1992). When bulk water is removed, preferential hydration fails (Crowe *et al.*, 1990). In addition, osmolytes are thought to maintain turgor (reviewed in Walters *et al.*, 2002).

f. Deployment of amphipathic/amphiphilic molecules

Cells may contain various cytoplasmic amphiphilic metabolites. Recently, Hoekstra and co-workers have suggested that endogenous amphipathic substances such as flavonoids and antioxidants may partition from the aqueous polar cytoplasm into the lipid phase, such as membranes and lipid bodies, with loss of water (Hoekstra *et al.*, 1997; 1999; Golovina *et al.*, 1998; Hoekstra and Golovina, 1999; 2000; Buitink *et* al., 2002; Golovina and Hoekstra, 2002).

On one hand, such partitioning into membranes could seriously perturb membrane structure with adverse effects on permeability properties of membranes (Herbette *et al.*, 1983; Takahashi *et al.*, 1998). Thus, partitioning into membranes has been used to explain transient leakage of cytoplasmic solutes from rehydrating anhydrobiotes commonly termed imbibitional damage. On the other hand, the presence of amphiphiles in membranes could obviate the formation of the gel phase in lipid bilayers by promoting fluidisation. Furthermore, partitioning into membranes might be extremely effective at inserting amphiphilic antioxidants such as tocopherol/vitamin E into membranes upon dehydration, thus promoting desiccation-tolerance and extending storage longevity.

In desiccation-tolerant organisms, the mobility of amphiphiles decreases on further drying. In contrast, it remains high in desiccation-sensitive organisms until almost all water is lost. For partitioning to be beneficial, it must be controlled to confer desiccation-tolerance and minimise its detrimental effects on membrane function.

1.4.2 Intracellular physical characteristics

a. Reserve deposition and degree of vacuolation

It has been suggested that the accumulation of complex reserves and the consequent low level of vacuolation might limit the physical disruption caused by dehydration and so contribute to tolerance of desiccation (Iljin, 1957; Kermode and Bewley, 1986; Kermode, 1990; Farrant *et al.*, 1997). This argument is supported by the reverse scenario in *A. marina* seeds, which remain highly desiccation-sensitive. These seeds accumulate predominantly soluble sugars and remain highly vacuolated (Farrant *et al.*, 1992a). Many recalcitrant seeds, however, do accumulate large quantities of complex reserves. These species include, among others, *Quercus alba* (Bonner, 1976), *Araucaria angustifolia* (Farrant *et al.*, 1989), *Podocarpus henkelii* (Dodd *et al.*, 1989), *Camellia sinensis* (Berjak *et al.*, 1991) and *Landolphia kirkii* (Berjak *et al.*, 1992). While these seeds appear to tolerate a relatively greater decrease in water content than those of *A. marina* (Farrant *et al.*, 1985; 1986; 1993a), all are notably desiccation-sensitive.

b. Intracellular de-differentiation

In orthodox seeds, intracellular structures are simplified and minimised at the onset of maturation drying (Klein and Pollock, 1968; Bewley, 1979; Dasgupta *et al.*, 1982; Galau *et al.*, 1991; Vertucci and Farrant, 1995; Farrant *et al.*, 1997). It should be remembered that membranes and cytoskeletal elements are vulnerable to dehydration (Berjak and Pammenter, 1997a,b; 2001; 2004; Pammenter and Berjak, 1999).

In this regard, Farrant *et al.* (1997) reported a substantially higher proportion of cell volume occupied by mitochondria in *A. marina* seeds, which are highly recalcitrant, than those of *A. hippocastenum*, which are less recalcitrant. In contrast, in *P. vulgaris* seeds, which are orthodox, mitochondria occupied a significantly smaller proportion of the cell volume, even prior to maturation drying. Furthermore, mitochondria of recalcitrant seeds showed well-developed cristae, typical of an active hydrated condition. Interestingly, mitochondria of *P. vulgaris* seeds were almost completely de-differentiated at comparable water contents to their recalcitrant counterparts.

c. Integrity of cyto- and nucleoskeletons

The cyto- and nucleoskeletons provide internal support to cells. Additionally, they impose organisation of the cytoplasm and nucleus, respectively. Whilst microtubules consist of polymerised α- and β-tubulin, microfilaments are composed of F-actin, a polymer of G-actin.

In the hydrated state, there is an extensive microfilamentous network in the root tip cells of embryonic axes of *Q. robur*. Following dehydration, this network is dismantled. At damagingly low water contents, the ability to re-assemble on rehydration is lost (reviewed by Berjak and Pammenter, 1997a,b; 2001; 2004; Berjak *et* al., 1999; Pammenter and Berjak, 1999, Mycock *et al.*, 2000). In contrast, orderly re-assembly of cytoskeletal elements on imbibition was reported in orthodox seeds.

d. DNA integrity and chromatin condensation

The retention of DNA integrity is vital to survival upon dehydration and subsequent rehydration (reviewed by Osborne and Boubriak, 1994; 1997). *In vivo*, DNA can assume different conformations depending on the water activity, the base sequence and the presence of specific binding proteins (Osborne and Boubriak, 1994; 1997). It has been shown that DNA integrity was retained in embryos of orthodox seeds and in pollen, during drying (reviewed by Osborne and Boubriak, 1994; 1997).

Chromatin structure changes as a function of hydration. In the desiccation-tolerant phase of developing and germinating orthodox seeds, chromatin is in the condensed state (Osborne and Boubriak, 1994; 1997). During germination, resumption of DNA replication is associated with both chromatin decondensation and loss of desiccation-tolerance (Deltour, 1985; Leprince *et al.*, 1995a).

e. The possible role of oleosins

Oleosins are layers of unique protein that surround lipid droplets (Huang, 1992). They consist of a central hydrophobic domain, which interacts with the lipid, and an amphipathic N-terminal which, with the C-terminal domain, facilitates interaction with the aqeous cytromatrix.

Leprince *et al.* (1998) recorded lack (or inadequate amount) of oleosin in desiccation-sensitive seeds. On rehydration, those tissues showed loss of stability of

lipid bodies. It is worth noting that coalescence of lipid bodies is a common abnormality accompanying deterioration of both orthodox and recalcitrant seeds.

1.4.3 Repression of metabolism

Rogerson and Matthews (1977) observed a sharp decline in the levels of sugars, which preceded a fall in the respiratory rate, prior to the acquisition of desiccation-tolerance in developing seeds of *Pisum sativum* (garden pea). Those authors suggested that such an event facilitated desiccation-tolerance in these tissues by, presumably, obviating metabolic damage.

Furthermore, Brunori (1967) showed that cell cycling was arrested at G1 phase during maturation drying in orthodox seeds. Following imbibition, the first round of S-phase replication occurred during G2 phase. As soon as cells enter G2M, during which mitosis takes place, desiccation-tolerance is lost (Sen and Osborne, 1974).

In contrast, seeds of *A. marina* showed transient arrest of DNA replication lasting no more than 24 h around shedding (Boubriak *et al.*, 2000). Sacandé *et al.* (1997) reported a situation where there was hardly any arrest of cell cycling in recalcitrant/intermediate/sub-orthodox seeds of *Azadirachta indica* (neem). Ongoing cell cycling was associated with loss of the ability to synthesise and repair DNA (Osborne and Boubriak, 1994; 1997; Boubriak *et al.*, 1997; 2000).

1.4.4 The role of free radical processing systems

Antioxidant systems have been suggested to play a role in desiccation-tolerance. Evidence for their involvement comes from a number of studies which have shown that susceptibility to peroxidation may increase with dehydration (Rockland, 1969; Bewley, 1979; McKersie *et al.*, 1988; Leprince *et al.*, 1990b; 1992; Dhindsa, 1991; Hendry *et al.*, 1992; Chaitanya and Naithani; 1994; Chandel *et al.*, 1995; Finch-Savage *et al.*, 1994a; 1996; Li and Sun; 1999; Tommasi *et al.*, 1999; Greggains *et al.*, 2001).

Among the studies conducted in this area, there appears to be a correlation between the nature and efficacy of antioxidants and desiccation-tolerance. For example, the level of tocopherol (vitamin E), a lipid-soluble compound that slows the initiation of autoxidation of lipids, is about ten times higher in orthodox embryos of maize and soybean than in the recalcitrant counterparts of *Q. robur* (compare Priestley *et al.*,

1980 and Leprince *et al.*, 1990b with Hendry *et al.*, 1992). The efficiency of antioxidative systems in developing embryos depends on the species, tissue type, their water potentials and the developmental status of the embryo (Leprince *et al.*, 1990b; Arrigoni *et al.*, 1992; Hendry *et al.*, 1992; Cakmak *et al.*, 1993; Chandel *et al.*, 1995; Li and Sun, 1999; Greggains *et al.*, 2001).

1.4.5 Repair (Damage restitution)

Storage of orthodox seeds at high temperatures and water contents causes damage which decreases vigour and brings about viability loss. Before the onset of viability loss, diminished vigour is manifested as an increasing time lag between seed imbibition and radicle extension. During this period, intracellular repair mechanisms become operational and repair must be effected before germination can occur (Osborne, 1983). Repair during this phase in orthodox seeds occurs at the level of protein macromolecules (Mudgett *et al.*, 1997), membranes (Berjak and Villiers, 1972a) and nucleic acids (Elder *et al.*, 1987). Indeed, the efficacy of osmopriming of low-vigour orthodox seeds is attributed to the repair processes which occur while the seeds are held at water potentials that allow this metabolism, but preclude germination (Bray, 1995).

There are very few studies of repair by damaged recalcitrant seeds (reviewed by Berjak and Pammenter, 2004). As an example, no DNA repair was possible following rehydration of the highly-recalcitrant seeds of *Avicennia marina* once 22% of the originally-present water had been lost (Boubriak *et al.*, 2000). In addition, there is accumulating evidence that free radical processing systems failed during dehydration of recalcitrant seeds and were assumed to remain ineffective on rehydration (e.g. Chaitanya and Naithani, 1994; Chandel *et al.*, 1995; Finch-Savage *et al.*, 1996; Li and Sun, 1999; Greggains *et al.*, 2001) Thus, it appears that the repair mechanisms of recalcitrant seeds are as sensitive to water loss as are all other processes.

1.5 Dormancy

Despite apparent adaptations for rapid germination, a few recalcitrant seeds of temperate species are dormant at shedding, such as *Acer pseudoplatanus* (Hong and Ellis, 1990), *Aesculus hippocastanum* (Tompsett and Pritchard, 1993) and *Zizania palustris* (Kovach and Bradford, 1992a). In contrast, viviparous germination is a common event in recalcitrant seeds of tropical species such as *Avicennia marina* (Farrant *et al.*, 1993b) and *Telfaria occidentalis* (Akoroda, 1986).

Many authors have identified seed dormancy with the absence of a germination response under conditions that should facilitate this process (e.g. Harper, 1959; Vleeshouwers *et al.*, 1995; Simpson, 1990; Murdoch and Ellis, 1992). Recently, however, Vleeshouwers *et al.* (1995) have proposed that a distinction should be made between dormancy release and germination elevation as well as between induction of dormancy and inhibition of germination. Those authors define dormancy as a seed characteristic, the degree of which defines what conditions should be met to make the seed germinate. Dormancy, unlike the absence of a germination response, is seen not as an all-or-nothing property.

Dormancy occurs in, at least, three forms: (1) physiological dormancy (reviewed by Cohn, 1987; Baskin and Baskin, 1989; 1998), (2) dormancy caused by a hard seed coat and (3) dormancy caused by underdevelopment of the embryo (Simpson, 1990; Vleeshouwers *et al.*, 1995). Breaking of dormancy caused by underdevelopment is sometimes referred to as after-ripening (Simpson, 1990).

Dormancy can also be classified as primary or secondary (Crocker, 1916; Vleeshouwers *et al.*, 1995; Karssen, 1982; reviewed by Hilhorst, 1995; 1998). Primary dormancy is the dormancy state of the freshly shed seed. If primary dormancy is relieved but suitable conditions are not present and germination does not occur, secondary dormancy may develop.

It has frequently been suggested that the primary function of dormancy is survival during prolonged unfavourable conditions (reviewed by Vleeshouwers *et al.*, 1995). In contrast, those authors contend that dormancy is a device for surviving short periods of favourable conditions. They argue that during unfavourable conditions, the lack of germination-elevating factors will prevent germination on one hand. Dormancy, on the other hand, prevents germination during transient favourable conditions when it is likely that the seedling that originates from the seed will not survive.

Hilhorst (1993; 1995; 1998) has presented a hypothetical model for the regulation of dormancy and stimulation of germination in seeds. In that model, it is proposed that environmental factors of light and temperature and internal factors such as GA play a pivotal role in the release of dormancy and stimulation of germination in seeds. In addition, Karssen (1982) has implicated ABA in the induction of dormancy and inhibition of germination. Nonetheless, Vleeshouwers *et al.* (1995) caution that as yet, there is only circumstantial evidence to support the model. Those authors contend, though, that the model structures and integrates a large number of observations on dormancy and germination in a concise and comprehensible way.

In conclusion, Cohn (1996) has reviewed a number of chemical mechanisms of breaking seed dormancy and concluded that such mechanisms are useful for the attainment of at least two objectives. Firstly, they serve as molecular probes of the mechanisms involved in the transition from developmental arrest to growth. Secondly, they can increase the efficacy of weed control and crop establishment. However, successful field applications have been limited due to insufficient potency of available chemicals and poor understanding of the mechanisms of chemical action.

1.6 Germination

As mentioned above, germination in orthodox seeds is under PGR control. For example, ABA is thought to prevent precocious germination prior to maturation drying and might promote further development (Long *et al.*, 1981; King, 1982; Quatrano *et al.*, 1983; Bray and Beachy, 1985; Eisenberg and Mascarenhas, 1985; Finkelstein *et al.*, 1985; DeLisle and Crouch, 1989; Kermode, 1990).

Maturation drying appears to cause a decline in and/or negation of the effect of ABA (Kermode, 1990; Bewley and Oliver, 1992). It is associated with metabolic quiescence (Kermode, 1990) and acts as a punctuation between development and germination, thus enabling seeds to tolerate adverse environmental conditions. However, this pattern of events may not be universal. For instance, seeds of cotton and tomato may not need this switch (Hughes and Galau, 1991 and Berry and Bewley, 1991, respectively).

Upon imbibition, non-dormant seeds undergo re-activation of their metabolic processes. These events include, among others, production of catabolic and anabolic

enzymes which mobilise reserves and synthesise structural components, respectively. The process is completed with the elongation of the radicle.

In contrast, the situation is considerably different in recalcitrant seeds. Such seeds show neither maturation drying, nor quiescence and there is no definite punctuation between development and germination, although in many, there is a definite period of low metabolism. For example, seeds of *A. marina* initiate reserve utilisation prior to abscission (Farrant *et al.*, 1992a). This phenomenon has been observed in other recalcitrant seeds (e.g. *C. sinensis* [Berjak *et al.*, 1991], *L. kirkii* [Berjak *et al.*, 1992] and *Q. robur* [Finch-Savage *et al.*, 1992]).

Nonetheless, seeds of *A. marina* show an increase in metabolism upon shedding (Farrant *et al.*, 1992a,b) akin to that observed in orthodox seeds. However, these events are not accompanied by qualitative changes in protein synthesis (at least in *A. marina* [Farrant *et al.*, 1992b]), even though cell division occurs. These observations have led those authors to conclude that germination, in the sense defined by Côme and Corbineau (1990), does not actually occur.

Côme and Corbineau (1990) have proposed that germination *sensu stricto* involves only the activation of the embryo and is complete by the onset of root elongation. For instance, it is difficult to identify any metabolic process that could be considered to be associated specifically with germination in *A. marina*. Development appears to grade imperceptibly into germination (Farrant *et al.*, 1992a). However, in many recalcitrant species, there is a more pronounced switch from development to germination, and this event may occur even occur in storage.

1.7 Deteriorative changes associated with loss of viability in seeds

It might seem of little relevance to discuss deteriorative mechanisms of orthodox seeds in storage in the present treatise. However, this has been done in view of the marked similarities between deteriorative changes in orthodox seeds in storage and those in recalcitrant seeds during dehydration and in wet storage particularly, at the biochemical and ultrastructural levels (see Smith and Berjak, 1995). Moreover, our understanding of desiccation sensitivity could benefit from studies on maturing and germinating orthodox seeds. During maturation and germination, orthodox seeds have yet to attain or have lost, respectively, full desiccation-tolerance.

1.7.1 Desiccation-tolerant (orthodox) seeds

a. Factors influencing viability

As early as 1972, Heydecker identified at least four distinct but interacting determinants of longevity of orthodox seeds in storage. They are: (1) genetic factors, (2) pre-harvest and maturational effects, (3) mechanical factors and (4) storage environment, particularly RH and temperature.

i. Genetic factors: It may be expected that seed longevity has a genetic basis. This phenomenon was demonstrated for maize some 50 years ago by Lindstrom (1942) (Smith and Berjak, 1995). However, the genetic differences among species and cultivars could be masked or amplified by environmental factors during development and those operating during harvest and storage. The study of Lindstrom (1942) has been borne out by more recent studies (Scott, 1981; Bewley and Black, 1982; Moreno Martinez *et al.*, 1988; Ramamoorthy *et al.*, 1989; Diojode, 1990). Those studies have equated genetic differences to biochemical parameters, such as levels of polyamines, putrescine and spermidine (Lozano and Leopold, 1988; Lozano *et al.*, 1989; Matilla, 1996) and morphological characteristics, such as hard, impermeable coats (Harrington, 1972; Flood and Sinclair, 1981; Ohlrogge and Kernan, 1982).

ii. Pre-harvest and maturational effects: Environmental factors such as temperature, rainfall, photoperiod, soil and atmospheric moisture and soil mineral status not only influence the storage reserves of developing seeds but their vigour as well (reviewed by Smith and Berjak, 1995). This phenomenon is probably the basis for the differences in vigour characteristics among harvests of the same cultivar. It is interesting to note that while many studies have shown that heavier or larger seeds generally show superior vigour and longevity characteristics, Olaridan and Mumford (1990) have shown that smaller seeds of *Amaranthus* species showed superior germination and storage characteristics.

iii. Mechanical factors: The advantages of mechanisation in commercial practices during harvesting and threshing are self-evident. These practices, however, may inflict damage on seeds, especially those that are excessively dry. Such mechanical damage may contribute to loss of viability in storage.

iv. Storage environment: Harrington (1972) proposed a generalisation that was to become a central dogma in orthodox seed storage. It states that seed storage life is decreased by increases in storage temperature and RH. As a "rule of thumb", it was

suggested that storage life is halved for every 5 °C increase in storage temperature and for every 1% increase in relative humidity. When both factors come into play, they are additive. Recently, Smith and Berjak (1995) have proposed a two-stage model of seed ageing. A seminal feature of that model is the realisation that both enzymatic and non-enzymatic reactions are substantially influenced by the extent and nature of water binding in seeds. Three zones (I, II and III) of hydration are distinguished following the convention of Fennema (1976) and correspond to equilibrium RH ranges (0-25%, 25-80% and 80-99%). It is postulated that certain molecular events can be associated with each hydration level.

The hypothesis states that at high temperatures and RHs, lipid peroxidation is likely to increase exponentially and the induction period will be a matter of a few days. At the other extreme (i.e. low temperatures and RH), deteriorative damage is likely to take of the order of years before being manifested. While the hypothesis suggests that at very low seed water content, seed longevity would be maximal, there is evidence to suggest that below a critical level, a further decrease in water content adds no advantage to seed longevity (Roberts, 1991) and, in fact, may even actually contribute to seed deterioration (Vertucci and Roos, 1990).

b. Changes associated with viability loss

Many events have been suggested to be basic to, or associated with, loss of viability in seeds. Such changes are: (1) biochemical or (2) ultrastructural. However, these changes can be detected only on imbibition.

i. Biochemical changes: Many biochemical changes occur in deteriorating seeds. However, it is presently difficult to discriminate between primary and secondary events (Smith and Berjak, 1995). Those authors argue that such a situation may be largely due to the limited number of studies carried out so far. Additionally, investigators have studied diverse aspects of deterioration using different techniques at various stages of degeneration for a wide range of seeds.

They include, *inter alia*,: (1) changes in DNA status and metabolism, (2) changes in RNA metabolism and protein synthesis, (3) changes in enzyme activities, (4) changes in reserves, (5) changes in respiratory and energy metabolism and (6) changes in free radical processing systems.

(1) Changes in DNA status and metabolism: Osborne and co-workers have shown that molecular dysfunction at the DNA level can be correlated with declining viability (Roberts and Osborne, 1973; Osborne, 1983; Osborne and Boubriak, 1994; 1997; Coello and Vázquez-Ramos, 1996; Boubriak *et al.*, 1997; 2000). Repair enzymes may also suffer damage during seed storage (Elder and Osborne, 1993; Osborne and Boubriak, 1994; 1997; Coello and Vázquez-Ramos 1996; Boubriak *et al.*, 1997; 2000). Consequently, repair during a pre-germination lag phase may be evident before full recovery (Vázquez *et al.*, 1991). Inadequate repair mechanisms could lead to nonsense information or DNA molecules with impaired function. Eventually, damage reaches a stage at which gross chromosomal aberrations become evident and repair is impossible. This event is followed by cell death.

(2) Changes in RNA metabolism and protein synthesis: As physiological expressions of seed deterioration, such as reduced germinability and seedling growth, suggest low rates of synthesis, it is not surprising that attention has been directed towards the functioning and possible relationships of RNA metabolism and protein synthesis in the deteriorative process. Studies which have investigated the role of RNA and protein synthesis in the deteriorative process, reveal that there exists a differential stability among the different species of RNA (Roberts and Osborne, 1973; Bray and Chow, 1976; Dell'Aquilla *et al.*, 1976). They show that while both r- and m-RNAs are affected, tRNA is particularly resistant to deterioration.

As far as protein synthesis is concerned, Roberts *et al.* (1973) have shown progressive degradation of ribosomes. Those authors also reported loss of and reduction in activities of elongation factors 1 and 2, respectively.

(3) Changes in enzyme activities: A number of changes in enzyme activities during the deterioration of seeds have been documented in the literature. They include: (1) increased free fatty acid (FFA) production as a result of lipolytic activity, (2) increased hydrolysis of phytin by phosphatases and (3) increased proteolysis by proteases.

(4) Changes in reserves: Changes in enzyme activities and increased protein crosslinking ([including enzymes], Ching and Schoolcraft, 1968) and carbonyl-amine reaction ([including the Maillard reaction], Feeney *et al.*, 1975) between carbohydrates and amino acids and proteins lead to changes in the nature and levels of storage carbohydrates, lipids and proteins.

(5) Changes in respiratory and energy metabolism: Since germination involves energy-dependent cell division and growth, it is not surprising that many investigators have examined respiratory activity. Changes in this regard include reduced coupling, greater oxygen consumption and a reduced P:O ratio (Abu-Shakra and Ching, 1967).

As far as ATP production is concerned, Smith and Berjak contend that the picture that emerges is far from clear. Studies which have shown a correlation between ATP content and viability in seeds of unrelated species (Ching and Danielson, 1972; Ching, 1973; Lunn and Madsen, 1981) have been confounded by those that have indicated that ATP levels are not good indicators of viability (Styler *et al.*, 1980; Mazor *et al.*, 1984).

(6) Changes in free radical processing systems: Recently, Bailly *et al.* (1996; 1997; 1998) have shown that the activities of free radical scavenging enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) were adversely affected by accelerated ageing in sunflower seeds.

ii. Ultrastructural changes: They include: (1) chromosome aberrations, (2) membrane changes, (3) general cytoplasmic and nuclear deterioration.

(1) Chromosome aberrations: The idea that chromosome aberrations underlie seed deterioration was the first to be suggested (Navashin, 1933). There is a good correlation between viability loss and chromosome damage (Roberts, 1972a,b; Villiers, 1974). Such chromosome damage represents gross damage to DNA. When a critical proportion of aberrant dividing cells occurs, growth ceases and death follows.

(2) Membrane changes: There are several ultrastructural studies that clearly show that membranes undergo deteriorative changes with increasing seed age which are manifested at imbibition (Berjak and Villiers, 1972a,b,c; Hallam, 1973; Simola, 1974; 1976; van Staden *et al.*, 1975; Berjak *et al.*, 1986; Dawidowicz-Grzegorzewska and Podstolski, 1992). They include: (1) abnormalities of mitochondrial and plastid inner and outer membranes, (2) lobing of the nuclear envelope, (3) fragmentation and loss of the endoplasmic reticulum, (4) dissolution of the bounding membranes of vacuoles and protein bodies, (5) fusion of lipid bodies to form larger bodies or irregular pools, (6) discontinuities in the plasmalemma and (7) the occasional appearance of floccular material in the extra-protoplasmic space (Smith and Berjak, 1995).

(3) General cytoplasmic and nuclear deterioration: Apart from the changes mentioned above, there are other ultrastructural changes which are consistently associated with seed ageing. They include, among others, chromatin clumping, cytoskeletal and nucleolar abnormalities, and loss of ribosomes (including polysomes) (Smith and Berjak, 1995). It is noteworthy that such changes would impact on the DNA, RNA and protein synthetic systems and consequently on repair systems.

1.7.2 Desiccation-sensitive (recalcitrant) seeds

a. Changes associated with desiccation

Water plays a plethora of roles in cells including: (1) providing cell structure, (2) providing the medium for the diffusion of substrates to the active sites of enzymes, (3) the stabilisation of macromolecular conformations through hydrophylic and hydrophobic interactions, (4) the sequestration of cellular constituents and (5) acting as a reactant and product in many important reactions (reviewed by Vertucci and Farrant, 1995; Walters *et al.*, 2002).

Removal of water from cells leads to changes in their physical and physiological properties (reviewed by Vertucci and Farrant, 1995; Walters *et al.*, 2002). It may be expected, therefore, that this process could have adverse consequences.

i. Physical damage: At a cellular level, the first sign of water-stress is loss of turgor (Levitt, 1980b). This event occurs at water potentials of –1 to –2 MPa and may lead to cell collapse. A number of factors, which influence the effects of physical stress during water loss, have been suggested. They include: (1) the size of the cell, (2) vacuolar space (Levitt, 1980a,b), (3) plasma membrane PL composition (Uemura and Steponkus, 1989) and (4) organellar geometry (Bewley, 1979; Levitt, 1980b; Bergstrom *et al.*, 1982; Kaiser, 1982; Oliver and Bewley, 1984; Oertli, 1986).

At lower water potentials, cells shrink as they lose water (Merryman, 1974; Steponkus, 1979; Steponkus and Lynch, 1989; Steponkus *et al.* 1995). Osmotic adjustments lessen the water potential difference between cells and the environment and augment the amount of dry matter in cells and, thus, can prevent loss of water and cell contraction (Jones and Gorman, 1983). However, they are ineffective at reducing strains when cells are exposed to even lower water contents (Wolfe and Bryant, 1999).

When cells that have not acclimatised to water-stress shrink by 50-80%, they burst when returned to the original water potential. This observation led to the concept of

the 'critical cell volume', which describes the limit to which a cell can contract in an osmotic excursion or reversible contraction-expansion cycle when cooled and subsequently warmed (Merryman, 1974).

Differences in the degree to which cell walls contract compared with the protoplasm may cause mechanical stress and damage to the plasmalemma during drying. The tight attachment of the plasmalemma to the cell wall creates tension to the cell membrane in shrinking cells (Murai and Yoshida, 1998a). This phenomenon is most profound at the cell wall-plasmalemma attachments near the plasmodesmata (Iljin, 1957; Bewley and Krochko, 1982; Walters *et al.*, 2002). Plasmolysis, during which the plasma membrane separates from the cell wall, mitigates damage to the whole cell during severe water-stress (Murai and Yoshida, 1998b). There is evidence to suggest that cells in desiccation-tolerant seeds are slightly plasmolysed (Perner, 1965; reviewed in Walters *et al.*, 2002).

However, observations of plasmolysis may be an artefact of the aqueous fixatives used to study dry organisms (Öpik, 1985; Platt *et al.*, 1997; Wesley-Smith, 2001). In studies using anhydrous chemical fixation (Öpik, 1985) and freeze substitution (Wesley-Smith, 2001; Wesley-Smith *et al.*, 2001), the plasma membrane remained closely appressed to the cell wall, and both the cell wall and plasmalemma became highly convoluted during desiccation of tolerant cells.

It is noteworthy that Öpik (1985) showed that the plasmalemma separated from the cell wall during rehydration as a result of differential swelling of the cell wall-plasmalemma association caused by detergents such as dimethylsulphoxide. The mechanical properties of the cell wall, which include: (1) elasticity, (2) ability to fold and (3) association with plamsodesmata, influence the degree to which plasma membrane disruption occurs as a consequence of contraction or expansion (Webb and Arnott, 1982; Öpik, 1985; Murai and Yoshida, 1998b; Vicré *et al.*, 1999).

Cell membranes must fold and/or vesiculate to accommodate the volume changes during cell contractions. Conservation of membrane surface area is critical for successful rehydration. If the surface area is reduced too much, cells burst upon rehydration. This observation suggests there is a critical minimum surface area, rather than a critical minimum volume, down to which cells can survive (Steponkus, 1979; Steponkus and Lynch, 1989; Steponkus *et al.*, 1995).

It is interesting to note that cells which are not acclimatised to cold, which generally respond like their desiccation-sensitive counterparts to water-stress, contract through invaginations of the plasma membrane which eventually form endocytotic vesicles that can not be reincorporated into the plasmalemma upon warming (Steponkus and Lynch, 1989; Steponkus *et al.*, 1995). In contrast, the plasmalemma from protoplasts tolerant of water-stress contract through exocytotic extrusions, which remain continuous with the plasma membrane and, as a result, help conserve the membrane surface area (Steponkus and Lynch, 1989; Steponkus *et al.*, 1995).

It appears that high PL:sterol ratios and high amounts of diunsaturated fatty acids in the plasmalemma facilitate exocytotic folding in shrinking protoplasts and greater elasticity of expanding membranes (Steponkus and Lynch, 1989; Steponkus *et al.*, 1995). Protoplasts with these properties tend to survive to lower water potentials.

Most orthodox and recalcitrant seeds, except for those with highly vacuolated cells (e.g. *Avicennia marina* [Farrant *et al.*, 1992a; 1993a,b]), are fairly tolerant of water-stress to water potentials of –12 MPa or higher, although recalcitrant seeds will tolerate this stress only in the short term. Nonetheless, drying results in some degree of cell contraction, which is mostly completed when the water potential of the cell is reduced to –12 MPa. In cells that survive water potentials of –12 MPa but not lower, both endo- and exocytotic vesicles have been observed (reviwed in Walters *et al.*, 2002).

In severely dried cells of fully desiccation-tolerant seeds, the plasmalemma stays intact and closely attached to the cell wall. This observation suggests that membrane surface area remains relatively constant (Öpik, 1985).

Some membrane constituents may be removed during cell contraction as evidenced by whorls of membranes close to the plasmalemma in seed cells (Webster and Leopold, 1977; Öpik, 1985; Wesley-Smith *et al.*, 2001) and circular membrane structures and plastoglobuli within chloroplasts in sections of leaf tissue from desiccation-tolerant angiosperms (Farrant *et al.*, 1999). These membrane bodies have been proposed to provide additional membrane reserves upon rehydration (Webster and Leopold, 1977; Farrant *et al.*, 1999).

Mechanisms by which such structures would be reinstated are not clear. Furthermore, their very existence could be artefacts of aqeous fixation. Alternatively, these membrane abnormalities may arise from other organelles such as endoplasmic

reticulum and may participate in autophagy or vacuole formation (Wesley-Smith *et al.*, 2001).

The shapes of nuclei, mitochondria and plastids in dried cells of desiccation-tolerant seeds are irregular and convoluted (Öpik, 1985). This observation suggests that the surface area of the membranes of these organelles is also conserved by folding.

The membranes of cell vacuoles experienced tensions similar to those described for protoplasts during reversible contraction-expansion cycles following exposure to water potentials of –2.5 to –5 MPa (Murai and Yoshida, 1998b). As a result, they are prone to rupture with lethal consequences. Highly vacuolated cells of immature seeds (Berjak *et al.*, 1984; 1994; Farrant *et al.*, 1989) and desiccation-sensitive tissue (Farrant and Sherwin, 1997; Farrant, 2000) are particularly sensitive to tonoplast dissolution.

Replacing the water in vacuoles with solid material reduces the degree to which vacuoles must contract, thereby lessening the tension on tonoplast membranes during drying. Dry matter reserves naturally accumulate during embryogenesis in orthodox and some recalcitrant seeds. This phenomenon may explain the progressive tolerance to desiccation in developing seeds (Farrant *et al.*, 1997; Farrant and Walters, 1998). There is also accumulation of dry matter in vacuoles of vegetative tissues in many of the desiccation-tolerant angiosperm species during acclimatisation to water-stress.

In addition to protection by filling cells with dry matter, the consequences of cell contraction can be alleviated by initial high surface area-to-volume ratios of cells and vacuoles (reviewed in Iljin, 1957; Bewley, 1979; Walters *et al.*, 2002). This phenomenon may explain why cells from non-vascular plants, which usually have small vacuoles, do not suffer physical damage (reviewed by Bewley and Krochko, 1982).

Severe water-stress is associated with dismantling of mitochondria. For instance, mitochondria in mature orthodox seeds lack defined cristae (Bergtrom *et al.*, 1982; Thompson and Platt-Aloia, 1984; Farrant *et al.*, 1997) and mitochondrial proteins are easily extractable from dried pollen (Hoekstra and Roekel, 1983). Conversely, mitochondria from recalcitrant and immature orthodox seeds are more defined. This condition has been linked to greater sensitivity to desiccation (Farrant *et al.*, 1997).

Chloroplast structure is also degraded during dehydration. For example, dried leaves of desiccation-tolerant grasses *Borya nitida* and *Xerophyta humilis* become yellow concurrent with the loss of grana stacks in chloroplasts (Gaff and Hallam, 1974; Farrant, 2000). Furthermore, there was a decline in photosynthetic activity, as measured by efficiency of photosytem II, at water potentials between –3 and –4 MPa (Wiltens *et al.*, 1978; Hetherington *et al.*, 1982b; Sherwin and Farrant, 1998; Tuba *et al.*, 1998; Csintalan *et al.*, 1999).

This decrease could be a consequence of photochemical damage. However, Demmig-Adams and Adams (1992) and Farrant (2000) contend that it is more likely a reflection of protective dismantling of photosystem II. Indeed, the dismantling of photosynthetic apparatus during drying of *B. nitida* and *X. humilis* is required for survival in those species (Gaff and Hallam, 1974; Farrant, 2000). This phenomenon is evidenced by the fact that plants dried too rapidly stay green and do not recover.

Slight water-stress at water potential between –1 and –3 MPa enhances protein synthesis, which is held to be important for conferring tolerance (Ried and Walker-Simmons, 1993; Ingram and Bartels, 1996; Oliver *et al.*, 1998; Mundree *et al.*, 2000; Whittaker *et al.*, 2001). However, further desiccation reduces the rate of protein synthesis in both tolerant and sensitive cells (Salmen-Espindola *et al.*, 1994; Ingram and Bartels, 1996; Oliver *et al.*, 1998; Mundree *et al.*, 2000; Whittaker *et al.*, 2001). It is thought that this event is a result of dismantling of endoplasmic reticulum, dictyosomes and polysomes (Webster and Leopold, 1977; Thompson and Platt-Aloia, 1984; Farrant *et al.*, 1997; Wesley-Smith *et al.*, 2001).

The cytoskeleton of cells from recalcitrant seeds is disrupted at fairly high water potentials during dehydration (e.g. –3.8 MPa for *Trichilia dregeana* [Berjak *et al.*, 1999] and –3.5 MPa for *Quercus robur* [Mycock *et al.*, 2000]). Nonetheless, cytoskeletal disassembly also occurs in cells of desiccation-tolerant seeds and vegetative tissues during dehydration. In this regard, Mycock *et al.* (2000) contend that the distinguishing factor between the two tissue types is the failure to reconstitute in desiccation-sensitive material.

At the molecular level, intermolecular associations of polar lipids are intrinsically linked to the water content of the medium (reviewed in Walters *et al.*, 2002). Under aqueous conditions, polar lipids spontaneously align to form micelles or bilayer structures depending on the polar head group of the lipid. Acyl chains within bilayers

are mobile. This situation gives considerable fluidity to the structure. It also allows proteins and other constituents to be inserted.

Drying brings membrane bilayers into close proximity, thus, causing membrane constituents to separate laterally into different domains enriched with particular lipid classes or proteins (Lis *et al.*, 1982; Bryant and Wolfe, 1989; 1992; Rand and Parsegian, 1989; Bryant *et al.*, 1992; Crowe and Crowe, 1992; Steponkus *et al.*, 1995; Hoekstra and Golovina, 1999). The closer packing between membranes and membrane constituents results in greater rigidity of the fatty acid domain within the bilayer. Molecular remix occurs upon rehydration. However, the reactions that occurred during the desiccated state may have irreversible consequences (reviewed by Crowe *et al.*, 1992; 1997).

Two mechanisms have been suggested to explain why fatty acid domains become more rigid based on either intra- on interlamellar events. One hypothesis states that the associated fatty acids compress because of increased van der Waals attractions when water molecules are removed from between adjacent polar head groups (Crowe *et al.*, 1990; Crowe and Crowe, 1992; Hoekstra and Golovina, 1999). The other states that strong repulsive hydration forces keep different bilayers separate as they come into close apposition, but create isotropic tensions which lead to lateral compression within the acyl domain (Lis *et al.*, 1982; Wolfe, 1987; Rand and Parsegian, 1989; Bryant and Wolfe, 1992; Wolfe and Bryant, 1999).

Increased rigidity of the acyl domain eventually leads to lipid phase transitions within the membrane from a lamellar liquid-crystalline to solid gel state (Ladbrooke and Chapman, 1969; Cullis and de Kruijff, 1979). While these phase transitions are reversible, they interfere with the semi-permeable properties of membranes. Permanent damage comes from permanent exclusion of proteins from parts of the bilayer (Rand and Parsegian, 1989; Bryant and Wolfe, 1992; Crowe and Crowe, 1992; Hoekstra and Golovina, 1999).

Transient damage also occurs upon rehydration; the rush of water on to an inelastic membrane may cause it to rupture (Murphy and Noland, 1982; Steponkus *et al.*, 1995; Hoekstra *et al.*, 1999) or imperfect packing among different domains may cause leakage of cellular constituents (Crowe and Crowe, 1992; Hoekstra *et al.*, 1999).

The close approach of membrane systems and the lateral demixing of membrane constituents can lead to even greater problems to membrane integrity than lamellar

liquid-crystalline to solid gel phase transitions. Membranes may fuse together, thus causing complete loss of compartmentation within the cell (Crowe and Crowe, 1992; Steponkus *et al.*, 1995). The mechanism that causes polar lipids to cross over to a different bilayer is unclear.

In principle, four factors are thought to allow the formation of inverted micelles within closely appressed bilayers. They include: (1) the hydration characteristics of individual lipids and lipids in a mixture, (2) the intrinsic curvature of different head groups, (3) the water content and (4) the temperature (Cullis and de Kruijff, 1979; Crowe *et al.*, 1986; Steponkus *et al.*, 1995).

The polar head groups coalesce into rings and the acyl chains extend radially outward in what is called the hexagonal phase in domains enriched with non-bilayer-forming lipids such as phosphatidylethanolamine-diglycerides or monogalactosyl-diglycerides (Cullis and de Kruijff, 1979; Siegel *et al.*, 1994; Steponkus *et al.*, 1995). Membrane fusion via hexagonal phase changes is rare in native membranes (reviewed in Walters *et al.*, 2002). However, it has been shown in cells from non-acclimatised leaves that were lethally cooled (Steponkus *et al.*, 1995) and more frequently in animal cells (Cullis and de Kruijff, 1979; Crowe and Crowe, 1992).

Membrane fusion is common in desiccation-damaged cells, protoplasts and liposomes (e.g. Crowe *et al.*, 1986; Steponkus *et al.*, 1995). Nonetheless, this damage does not occur via hexagonal phase changes. For instance, fusion of the plasmalemma and endomembranes was demonstrated in cold-acclimatised rye and oat leaves at temperatures and water potentials between –10 and –40 °C and –12 and –48MPa, respectively, depending on the level of cold tolerance achieved (Steponkus *et al.*, 1995). Upon rehydration, fused membranes produce vesicles that exclude constituents or are combinations of different membrane systems. Because the osmotic balance inside and outside the cell is completely disrupted, vesicles from membrane fusions are unable to expand during rehydration (Steponkus *et al.*, 1995).

The water contents and temperatures at which phase transitions of prepared membrane systems occur, depend on two factors (Ladbrooke and Chapman, 1969; Cullis and de Kruijff, 1979; Crowe *et al.*, 1989a,b; Steponkus *et al.*, 1995). They are: (1) the saturation of the acyl chains and (2) the presence of non-PLs. A water potential of about –12 MPa is often cited as critical. It has been suggested that structural water needed for proper spacing of polar head groups is removed at water potentials below

that level (Ladbrooke and Chapman, 1969; Crowe *et* al., 1990). Also, potentially deforming hydration forces result from the close approach of molecules at a water potential of about –12 MPa (Wolfe, 1987).

Changes in bilayer spacing or membrane lamellar liquid-crystalline to solid gel lipid phase transitions have been detected in both desiccation-tolerant and -sensitive plant cells during desiccation, with little difference observed with degree of tolerance (McKersie and Stinson, 1980; Seewaldt *et al.*, 1981; Priestley and de Kruijff, 1982; Singh *et al.*, 1984; Kerhoas *et al.*, 1987; Crowe *et al.*, 1989a,b; Hoekstra *et al.*, 1991; 1992a; Sun *et al.*, 1994; Hoekstra and Golovina, 1999). For example, Seewaldt *et al.* (1981) observed a membrane lipid phase transition in tolerant soybean cotyledons when seeds were dried to less than 0.2 g g^{-1} dm which correspond to a water potential of –12 MPa (Vertucci and Roos, 1990).

However, water potentials between –10 and –15 MPa also mark the survival limit of recalcitrant seeds. Nonetheless, a membrane-mediated mechanism is often invoked to explain damage in desiccation-sensitive seeds and pollen because the membrane integrity of their cells is compromised upon rehydration (McKersie and Stinson, 1980; Berjak *et al.*, 1992; 1993; Poulsen and Eriksen, 1992; Sun and Leopold, 1993; Sun *et al.*, 1994; Ntuli *et al.*, 1997; Wolkers *et al.*, 1998b).

Protein structure is conserved during drying to extremely low levels (Schneider and Schneider, 1972; Kuntz and Kauzman, 1974; Ruegg and Hani, 1975; Fujita and Noda, 1978; Careci *et al.*, 1980; Takahashi *et al.*, 1980; Jaenicke, 1981; Rupley *et al.*, 1983; Walters *et al.*, 2002). Some proteins even maintain functional activity, albeit at low levels, when dry (Acker, 1969; Potthast, 1978; Labuza, 1980; Rupley *et al.*, 1983).

Secondary structure of cytoplasmic proteins from desiccation-tolerant pollen was also conserved upon drying in the absence of protectant sugars. This observation demonstrated the innate stability of the secondary structure of proteins as a result of the high degree of α-helical structures (Wolkers and Hoekstra, 1995). Furthermore, the reversibility of sorption-desorption isotherms of numerous proteins supported the idea that conformational changes of proteins during hydration were slight and reversible (Bull, 1944; D'Arcy and Watt, 1970; reviewed by Walters *et al.*, 2002).

Slight and reversible changes in protein structure, particularly secondary structure, have been attributed to volumetric changes from loss of water rather than to the native structure of proteins. These changes occur at low water contents and water potentials

of between 0.2 and 0.1 g g^{-1} dm and –70 to –200 MPa, respectively (Ruegg and Hani, 1975; Griebenow and Klibanov, 1995).

In fact, drying stabilises protein structures, thus making them particularly resistant to ageing (Franks *et al.*, 1991; Constantino *et al.*, 1998) and heat denaturation (Echigo *et al.*, 1966; Ruegg *et al.*, 1975; Fujita and Noda, 1978; Takahashi *et al.*, 1980; Jaenicke, 1981; Leopold and Vertucci, 1986; Wolkers and Hoekstra, 1997). The extreme stability of protein structure with low hydration may be attributed to stronger intramolecular associations compared with the situation of polar lipids. Such interactions would reduce the need for hydrogen bonding with water to maintain structural integrity, thus preventing the need for water replacement, as suggested by Crowe and Crowe (1992) and/or provide mechanical strength that resists deformation when molecules are compressed, thus obviating the need for mechanical barriers, as proposed by Wolfe and Bryant (1999).

However, the conformations of some proteins are irreversibly damaged by drying or freeze-drying in the absence of protectants (Hanafusa, 1969; Carpenter *et al.*, 1987; 1990; Franks *et al.*, 1991; Prestrelski *et al.*, 1993). For instance, enzymes such as lactate dehydrogenase and polypeptides such as poly-L-lysine are particularly labile (Prestrelski *et al.*, 1993) and damage is exacerbated if molecules are freeze-dried rather than air-dried (Franks *et al.*, 1991). In this regard, rate of drying also has a large effect on the conservation of protein structure (Wolkers *et al.*, 1998a,b). It appears greater preservation is achieved by rapid drying conditions.

The structure and activity of proteins are compromised if they are stored under extremely dry condition of approximately 0.1 g g^{-1} dm or –200 MPa or less (Kuntz and Kauzmann, 1974; Luscher-Mattli and Ruegg, 1982; Sanches *et al.*, 1986; Labrude *et al.*, 1987). Substantial deterioration of the lattice of protein crystals was attributed to the refolding of polypeptide chains to increase packing efficiency (Kuntz and Kauzmann, 1974). Other studies have shown that severe drying exposes haem groups on proteins, thus promoting free radical production (Sanches *et al.*, 1986; Labrude *et al.*, 1987). At such low contents, proton exchanges among charged amino acids could be measured, suggesting that these sites were exposed (Careri *et al.*, 1980; Rupley *et al.*, 1983).

Many mechanisms have been suggested to cause damage to proteins at low water contents. They include: (1) exposure of the reactive sites, (2) increased relaxation as

they fill voids left by water and (3) relaxation of the glassy matrix which embeds proteins. Such mechanisms are responsible for the deterioration of stored seeds and pollen. Nonetheless, protein structure is stable in seeds stored at 30% RH (Golovina *et al.*, 1997). Furthermore, increased ageing rates in seeds and pollen stored below the 'critical water content' have been attributed to reduced viscosity of the aqueous medium in cells that are almost completely dry (Buitink *et al.*, 1998a).

The same destabilising forces that perturb lipid and protein structures may also affect nucleic acids upon dehydration (Rau *et al.*, 1984). DNA is a particularly stable molecule (Wayne *et al.*, 1999). Its structure is maintained in the absence of water and it reversibly unfolds at high temperatures (Bonner and Klibanov, 2000). The intermolecular distances of dehydrating DNA strands are comparable to those of condensed DNA in hydrated nuclei (Rau *et al.*, 1984). This observation suggests that DNA structures are resistant to perturbation resulting from dense packing.

When DNA is replicating during germination and, therefore, is decondensed the cells concomitantly become susceptible to desiccation injury (Deltour and Jacqmard, 1974; Crèvecoeur *et al.*, 1988). Also, rapidly dividing cells during embryogenesis are sensitive to drying (Myers *et al.*, 1992). Additionally, desiccation did not affect the structure of condensed and decondensed chromatin in desiccation-tolerant or sensitive maize embryos, respectively (Leprince *et al.*, 1995a). However, in those studies, the chelation of Ca^{2+} and other divalent cations by ethylenediamine tetra-acetic acid present in the medium used for chromatin spreading, may have relaxed previously condensed chromatin, thus accounting for the reportedly similar results in desiccation-tolerant and sensitive material (reviewed by Pammenter and Berjak, 1999).

ii. Metabolic damage: Removal of water from cells results in an increased concentration of solutes and an increase in the viscosity of the aqueous medium. These events precipitate a number of changes in metabolic pathways of plant cells (reviewed by Walters *et al.*, 2002). For example, assimilation of CO_2 in photosynthetic tissue and growth are impaired although growth is much more sensitive to water-stress than CO_2 assimilation.

Particular metabolic activities are believed to occur at specific moisture levels (Clegg, 1978; Leopold and Vertucci, 1989). In this regard, it is important to note that cells may be sensitive to the rate of desiccation in addition to loss of water *per se* (Farrant *et al.*, 1985; Pammenter *et al.*, 1991; Pritchard, 1991; Berjak *et al*, 1993).

Often, protein synthesis is temporarily stimulated during mild dehydration (reviewed by Vertucci and Farrant, 1995; Ingram and Bartels, 1996; Oliver *et al.*, 1998). This event is thought to lead to the production of proteins with putative protection characteristics. Observations of increased occurrence of polysomes and endoplasmic reticulum in slightly water-stressed recalcitrant seeds suggest that certain, and possibly similar, metabolic pathways may also be induced in seeds that do not acquire full desiccation-tolerance (Berjak *et al.*, 1984; Farrant *et al.*, 1989; Pammenter *et al.*, 1998).

However, these changes in metabolism do not indicate that cells have already experienced damage; when briefly water-stressed, most organisms resume normal metabolism once the stress is relieved. Nonetheless, prolonged mild stress, which could be considered akin to drought, is deleterious to both vegetative and embryonic tissues.

Metabolism slows down at water potentials less than –2MPa. However, not all reactions are affected by dehydration in the same way. For instance, whilst protein synthesis slows down at relatively high water potentials (reviewed by Bewley and Krochko, 1982; Clegg, 1986; Salmen-Espindola *et al.*, 1994; Ingrams and Bartels, 1996; Mundree *et* al., 2000 Whittaker *et* al., 2001), respiration continues to much lower levels (Vertucci and Leopold, 1984; Vertucci and Roos, 1990; Salmen-Espindola *et al.*, 1994; Leprince and Hoekstra, 1998; Leprince *et al.*, 1999; Farrant, 2000; Walters *et al.*, 2001).

Various reactions within photosynthetic (Wiltens *et al.*, 1978; Hetherington *et al.*, 1982a; Vertucci *et al.*, 1985; Vertucci and Leopold, 1986; Farrant, 2000) and respiratory (Vertucci and Leopold, 1986; Leprince and Hoekstra, 1998; Leprince *et al.*, 2000) pathways respond differently to low water contents. The differing responses to water-stress among and within metabolic pathways lead to imbalances in metabolism. Furthermore, metabolic imbalances may be confounded by the respiration of fungi, which occur at water potentials as low as –20MPa in orthodox and recalcitrant seeds (Mycock and Berjak, 1990; Goodman, 1994; Calistru *et al.*, 2000).

Damage by metabolic stress is most pronounced at water potentials between –2 and –5 MPa and diminishes in effect as cells are dried to –12MPa (Leprince *et al.*, 2000; Walters *et al.*, 2001). In this regard, both desiccation-sensitive and tolerant organisms

are damaged when stored at intermediate water potentials, though the time-dependency of the damage varies considerably among species and tissues (Walters *et al.*, 2001).

A by-product of continued respiration and photosynthesis when other metabolic processes are shut off is the accumulation of high-energy intermediates that leak out of mitochondria and plastids and form reactive oxygen species (ROS) and free radicals (Puntarulo, 1991; Dean *et al.*, 1993; Hendry, 1993; Leprince *et al.*, 1993a; 1994; 1995b; Smirnoff, 1993; Foyer *et al.*, 1994; Halliwell and Gutteridge, 1999). ROSs and free radicals react with proteins, lipids and nucleic acids, thus causing permanent damage to enzymes (Wolff *et al.*, 1986; Dean *et al.*, 1993; Halliwell and Gutteridge, 1999), membranes (Senaratna and McKersie, 1983; 1986; Chan, 1987; McKersie *et al.*, 1988; 1989; Finch-Savage *et al.*, 1996; Halliwell and Gutteridge, 1999; Leprince *et al.*, 2000) and chromosomes (Dizdaroglu, 1994). Peroxidation of lipids decreases the fluidity within membranes (McKersie *et al.*, 1988; 1989), thus interfering with their selective permeability upon rehydration.

High levels of free radicals have been detected in desiccation-sensitive seeds upon dehydration (Senaratna and McKersie, 1983; 1986; McKersie *et al.*, 1988; Hendry *et al.*, 1992; Leprince *et al.*, 1993a; 1994; 1995b; 1999; 2000). The origin and sequence of the events following the appearance of these toxic compounds remains unclear. They may be produced by the water-stressed cell (Leprince *et al.*, 1993a; 1994; 1995b; 1999; 2000; Leprince and Hoekstra, 1998) or as a result of the associated fungi (Goodman, 1994; Finch-Savage, 1999). Additionally, they may precede or precipitate damage (Finch-Savage *et al.*, 1996; Leprince *et al.*, 2000) or arise after the cell has already died (Finch-Savage, 1999).

There are several ways in which cells can protect themselves from metabolic imbalance and ROS-mediated damage. At higher moisture levels, free-radical-processing enzymes efficiently detoxify ROS (Bewley, 1979; Dhindsa, 1987; Hendry, 1993; Smirnoff, 1993; Foyer *et al.*, 1994; Kranner and Grill, 1997; Sherwin and Farrant, 1998; Farrant, 2000). Nonetheless, these enzymes appear ineffective at low water contents and antioxidants, such as tocopherol (TOC) and ascorbic acid/ascorbate may be more effective (reviewed by McKersie *et al.*, 1988; Pammenter and Berjak, 1999).

Amphipathic molecules such as TOC can partition between aqueous and lipid domains according to the water content of the cell and the polarity of the molecule (Golovina *et al.*, 1998). In addition, a controlled repression of metabolism upon drying may also mitigate the consequences of unbalanced metabolism (reviewed by Leprince *et al.*, 1993b; Vertucci and Farrant, 1995; Pammenter and Barjak, 1999).

Cells with more organelles and greater definition of organelle structure appear more sensitive to desiccation (Bewley, 1979; Hetherington, 1982a; Gaff, 1989; Berjak *et al.*, 1990; Farrant *et al.*, 1997; Farrant and Walters, 1998; Farrant, 2000). This effect may be a result of the requirement to protect more membranes or greater ROS production because of higher metabolism.

In this regard, it is noteworthy that conditions that reduce metabolism such as low temperature (Leprince *et al.*, 1995b) or highly complex substrates (Leprince *et al.*, 1990a) reduce sensitivity to desiccation. Additionally, desiccation-sensitive cells respire at comparatively greater rates than their tolerant counterparts at the same water content (Farrant *et al.*, 1997; Leprince *et al.*, 1999; Walters *et al.*, 2001). This phenomenon may reflect the properties of the mitochondria themselves or the cellular matrix.

Leprince and Hoekstra (1998) have suggested that changes in viscosity with dehydration in desiccation-sensitive cells are not as marked as in their tolerant counterparts. Consequently, metabolism is not as restricted in desiccation-sensitive as in tolerant cells. Furthermore, it has been suggested that the packaging of macromolecules in desiccation-sensitive cells is not as dense (Wolkers *et al.*, 1998a,d). Thus, diffusion of oxygen through the cell matrix is facilitated.

iii. Desiccation damage: Removal of water that is intimately associated with surfaces of macromolecules can be considered desiccation damage *sensu stricto* (reviewed by Pammenter and Berjak, 1999; Walters *et al.*, 2001). Those authors contend that desiccation-tolerant tissues can survive removal of most (but not all) of bound water.

Membrane structures appear more prone to desiccation damage *sensu stricto* than do proteins or DNA, perhaps because of the intense hydrogen bonding within proteins and nucleic acid structures (reviewed in Walters *et al.*, 2002). Protection from damage often lies in the ability of the structure or the surrounding medium to offer mechanical resistance to the stress or to accommodate the stress through enhanced elasticity.

b. Changes associated with hydrated storage

i. Cellular events: There may be no clear marker event between the end of development and initiation of germination in some recalcitrant seeds (Farrant *et al.*, 1993b). Furthermore, seeds of all the tropical and subtropical species so far examined and *Quercus robur* initiate germination in hydrated storage (Berjak *et al.*, 1989; Farrant *et al.*, 1989; Pammenter *et al.*, 1994; 1997; Motete *et al.*, 1997). However, recalcitrant seeds of temperate species can tolerate months of wet storage as they are shed either dormant or immature. The latter continue development and/or growth prior to the initiation of germination in a phenomenon Tompsett (1987) described as after-ripening.

The situation for seeds that initiate germination in hydrated storage is exemplified for *Camellia sinensis*, *Landolphia kirkii* and *Avicennia marina*. Embryonic axes from those seeds in hydrated storage reveal an ultrastructure commensurate with their active metabolic condition. Subcellular organisation increases in a manner similar to seeds set out to germinate immediately after they are shed (Berjak *et al.*, 1989).

ii. The requirement of additional water: The germinative events that occur in recalcitrant seeds in wet storage culminate in cell division and extensive vacuolation (Pammenter *et al.*, 1994; 1997; Motete *et al.*, 1997). However, unless more water is supplied thereafter, extensive degeneration rapidly follows with concomitant viability loss. Nonetheless, some species will produce extensive roots.

iii. Consequences of intracellular water-stress: The deteriorative processes in recalcitrant seeds during wet storage superficially appear to be similar to those in orthodox seeds during dry storage. They include, among others, biochemical changes (such as lipid peroxidation) and ultrastructural changes (such as damage to membranes).

From the events outlined above Pammenter and co-workers hypothesised that: (1) wet storage lifespan of recalcitrant seeds is inversely related to rate of germinative metabolism and (2) a progressively intense water-stress develops during hydrated storage. Thus, those authors concluded that reducing the rate of germinative metabolism would lead to: (1) an extended storage lifespan and (2) slowing down of the intensification of the water-stress (Pammenter *et al.*, 1997).

Those workers set out to test these hypotheses on the highly-recalcitrant seeds of *Avicennia marina*. To reduce the rate of germinative metabolism, seeds were coated with an alginate gel into which ABA had been incorporated. They found that coating seeds with alginate gel alone, as well with gel plus ABA, extended the storage lifespan of those seeds by a factor of three to four (Pammenter *et al.*, 1997). Germination rate, respiration, consumption of storage reserves and ultrastructural data indicated a reduction in the rate of germinative metabolism and may have contributed to, but could not fully explain the enhanced storage lifespan of coated seeds. However, no evidence of water-stress in bulk tissue could be detected during storage. On the contrary, osmotic and water potentials increased rather than decreased, although turgor did not change. This increase in osmotic and water potentials was attributed to the decline in soluble reserves.

Microorganisms, particularly fungi, whose inoculum is present in fresh seeds even when they have been newly hand-harvested, play a significant role in post-harvest deterioration of recalcitrant seeds (reviewed by Berjak, 1996). The same phenomenon is likely to be true for seed types classed as intermediate. As seeds naturally deteriorate, fungal proliferation and activity increase. It is thought that in wet-stored recalcitrant seeds, which have become debilitated, phytoalexin production and other defence mechanisms become impaired or non-functional.

Phytoalexins are small molecules, the synthesis of which is elicited by the presence of microorganisms or other factors in a wide variety of species (e.g. pea [Hadwiger and Webster, 1984], elm [Duchesne *et al*, 1990], pine [Gehlert *et al.*, 1990], citrus [Sulistyowati *et al.*, 1990], reviewed by Schäfer, 1994). In this regard, there is evidence to suggest that the methyltransferase activity, which converts a precursor molecule into the phytoalexin, (+) pisatin, in pea seedlings, is induced by microbial infection (Sweigard *et al.*, 1986). In addition, it is noteworthy that many pathogens are able to inactivate the host plant defence mechanisms, including the accumulation of

phytoalexins, and that phytoalexins are not normally present in any significant amounts in the absence of biotic or abiotic elicitors (Darvill and Albersheim, 1984).

A fungal succession occurs in hydrated recalcitrant seeds, with the most aggressive species present outcompeting the others (Mycock and Bejak, 1990). Besides being infected by fungi, recalcitrant seeds also generally harbour a range of bacteria although one species in particular may dominate.

In conclusion, it is noteworthy that the changes associated with damage and viability loss in ageing orthodox seeds and drying or wet storage in recalcitrant seeds cannot be observed directly in many cases (reviewed by Leprince and Golovina, 2002). Rather, they become apparent on extraction and *in vitro* assays. Hence, the damage may not become apparent until the system is hydrated. However, this situation may not accurately reflect that *in vivo*, especially when dealing with dehydrating desiccation-sensitive seeds.

1.8 Application of modern physical techniques to the study of the desiccation response

The development of physical techniques has brought substantial insights into the physical states of water and cellular components of seeds in recent years (reviewed by Leprince and Golovina, 2002). These techniques cover a large range of: (1) spectroscopic techniques such as (a) nuclear magnetic resonance (NMR), (b) electron paramagnetic resonance (EPR) also referred to as electron spin resonance (ESR) and (c) Fourier transform infrared (FTIR) spectroscopy as well as (2) differential scanning calorimetry (DSC).

The success of these techniques and spectroscopy, in particular, originates in their versatility and their ability to assess the state of seeds by non-invasive means. Here, these techniques are discussed briefly, although they were not used, to highlight the vast possibilities they open for future seed science research with respect to their responses to desiccation and storage.

NMR can be used to obtain information of biological interest such as the state of water, intracellular pH and membrane dynamics. Proton (^{1}H) NMR allows the non-destructive measurement of water content in biological systems with high precision. Two types of analytical NMR are commonly used in this respect: (1) continuous wave NMR (Pohle and Gregory, 1968) and (2) pulsed NMR (Martin *et al.*, 1980). Presently, the latter is generally adopted.

Such an approach is widely used for rapid non-invasive determination of water and oil content in air-dry seeds (Tiwari *et al.*, 1974; Gambhir and Agarwala, 1985; Brusewitz and Stone, 1987; Gambhir, 1992; Rubel; 1994; Warmsley, 1998). Drying or D_2O can be used to separate the NMR signal of free water from that of oil in hydrated seeds (Ratkovic *et al.*, 1982).

Only pulsed NMR can be used to characterise different water fractions in living tissues. The changes in water fractions can be followed during the dehydration and rehydration of anhydrobiotic systems. This tool gives insight into the role of different water fractions in biological systems (Seewaldt *et al.*, 1981; Ratkovic *et al.*, 1982; Aksyonov and Golovina, 1986a,b; Ishida *et al.*, 1987; 1988; Bacic *et al.*, 1992; Golovina and Aksyonov, 1993; Marconi *et al.*, 1993).

The behaviour of water in living systems can also be characterised by the water self-diffusion coefficient. This coefficient is measured by pulsed NMR technique (Fukushima and Roeder, 1981).

The NMR method proposed by Stejskal and Tanner (1965) can be used to study the *in situ* membrane permeability to water during drying. This approach has been applied to follow the changes in membrane properties in developing barley seeds (Ishida *et al.*, 1995) and to calculate the size of the oil bodies in rape seeds (Fleischer *et al.*, 1990; Fleischer and Werner, 1992).

NMR imaging (NMRI) is mainly based on the detection of water. Information on the spatial distribution of water and water properties can be obtained. Dynamic information can also be obtained. There are two experimental approaches in NMRI: (1) imaging large objects such as roots, stems or whole plants and (2) imaging small samples such as seeds and excised tissues with high resolution NMR microscopy (Ratcliffe, 1994; Ishida *et al.*, 2000).

The development of NMRI has led to a resolution that approaches the dimensions of single cells in plant tissues (Connelly *et al.*, 1987). The theoretical limit is considered as 10 X 10 X 10 μm (Ratcliffe, 1994). While NMRI is not yet able to compare with light microscopy in its resolution of cellular structures, it has the great advantage of being non-invasive. Thus, it can be used to monitor the functioning of plant tissues. Nitroxide radicals (Magin *et al.*, 1986; Swartz *et al.*, 1986) and paramagnetic ions (Ishida *et al.*, 2000) can be used as contrasting agents.

It is possible to map stationary, diffusing and flowing water in plant tissues (Ratcliffe, 1994). For instance, NMRI enables the water distribution inside seeds to be determined. The changes in water distribution during drying and rehydration have demonstrated the transfer routes for water (Ruan and Litchfield, 1992; Ruan *et al.*, 1992; Song *et al.*, 1992; Kovacs and Nemenyi, 1999).

The synthesis of storage substances and their hydrolysis during germination result in an apparent decrease or increase in brightness of the NMR image, respectively (Ishida *et al.*, 1990; 1995; McIntrye *et al.*, 1995), such that solubilised parts of the storage tissues can become visible. For example, the changes in image contrast during precocious germination of *Phaseolus vulgaris* after ethylene treatment have been attributed to the changes in the water status and redistribution from the cotyledon to the axis (Fountain *et al.*, 1998).

The spatial distribution of other compounds, mainly lipids and carbohydrates, that accumulate in storage tissues, can be mapped *in vivo* (Bottomley *et al.*, 1984). For instance, mung bean seeds, which have been germinating for one day, showed uniformly distributed oil. This phenomenon allowed the changes in the image with germination to be attributed to the bulk water fraction (Connelly *et al.*, 1987). Similarly, oil and sucrose have been mapped in fresh maize kernels (Koizumi *et al.*, 1995), barley seeds (Ishida *et al.*, 1990) and developing pea seeds (Tse *et al.*, 1996).

High-resolution NMR is used to detect ions and metabolites of low molecular weight, intracellular pH, subcellular compartmentation of compounds and flux through metabolic pathways (Ratcliffe, 1994; Schneider, 1997; Roberts, 2000). However, low concentrations of molecules of interest makes this approach rather insensitive.

^{1}H NMR is widely used to analyse tissue extracts for the presence of specific compounds such as, for example, betaine in wild-type and transformed *Arabidopsis thaliana* seeds (Alia *et al.*, 1998). ^{13}C NMR has been used to establish changes in soybean seeds during maturation and germination (Ishida *et al.*, 1987; 1988). The sensitivity of ^{13}C NMR can be enhanced (Ni and Eads, 1992; Heidenreich *et al.*, 1998). ^{13}C labelling gives opportunities for probing different metabolic pathways such as lipid synthesis in soybean ovules (Schaeffer *et al.*, 1975) and the metabolism of dormancy-breaking chemicals in red rice (Footitt *et al.*, 1995).

^{31}P NMR has many applications because of the physiological importance of the information that it provides. The measurement of cytoplasmic and vacuolar pH is one of the most important applications of *in vivo* ^{31}P NMR. Furthermore, a number of important phosphorylated metabolites can be resolved in ^{31}P spectra. For some of them such as polyphosphate, information on the subcellular distribution can also be obtained.

^{31}P NMR has been applied to study the pH of intracellular compartments in germinating seeds of *Phacelia tanacetifolia* (Espen *et al.*, 1995). Changes in the cytoplasmic pH and vacuolar inorganic phosphate concentration correlate with germination. ^{31}P can also be used to monitor phosphorus compounds and their changes during maturation and germination of seeds, both in extracts and *in vivo*. Phosphorus compounds can also be resolved *in vivo* (Ishida *et al.*, 1987; 1988) and in extracts (Ricardo and Santos, 1990). ^{31}P spectra can also be used for the identification of the appearance or disappearance of vacuoles in seeds during germination and maturation, respectively (Ishida *et al.*, 1990).

The ^{31}P NMR signal of phospholipids (PLs) depends on the orientation of the phosphate groups. Different PLs can be resolved (Smith, 1985). ^{31}P NMR is sensitive to the physical state of PLs.

There are very few examples of successful application of ^{31}P NMR in the field of desiccation-tolerance: for instance, Lee *et al.* (1986; 1989) studied the interaction of trehalose with the PL, dipalmitoylphosphatidylcholine (DPPC). On one hand, they showed that the head groups are in a rigid state above and below the phase transition for both dry DPPC and trehalose. On the other, Tsvetkova *et al.* (1998) used ^{31}P NMR in comparative study of the interaction of glucose, trehalose and hydroxyethyl starch with dry DPPC. Those authors related the differential effect of carbohydrates on the behaviour of head groups of PLs to the role of trehalose in membrane protection upon drying.

PLs arranged in lamellar bilayers or in an inverted hexagonal phase have different ^{31}P patterns (Cullis and Kruijff, 1979). These differences between lamellar bilayer and hexagonal phase arise from the fact that the lipids are restricted in motion to the plane of the membrane in the lamellar state. These differences in the ^{31}P pattern can be used to detect the presence of either phase.

Researchers have been interested in the membrane transition from the lamellar bilayer to the hexagonal phase upon drying for many years (e.g. Simon, 1974). Priestley and Kruijff (1982) applied ^{31}P NMR to several biological systems in an attempt to detect this transition. Pollen of *Typha latifolia* was the most suitable for analysis. However, ^{31}P NMR was not suitable for analysis at 0.052 g g^{-1} dm water content as only phosphorus low-weight molecules could be identified at water contents less than or equal to 0.088 g g^{-1} dm. In contrast, PLs organised in lamellar bilayers became evident at 0.109 g g^{-1} dm water content. As a result, no evidence was obtained for the presence of a hexagonal phase in the pollen on drying to 0.109 g g^{-1} dm water content.

Deuterium (^{2}H) is an ideal probe for membranes (Smith, 1985). This technique can be used to study membrane phase transitions, the influence of acyl chain saturation on membrane fluidity and changes in membrane fluidity.

Lee *et al.* (1986; 1989) applied ^{2}H NMR to study the effect of the interaction between trehalose and dry DPPC on the behaviour of acyl chains. ^{2}H spectra of dry DPPC showed that the disorder of lipid acyl chains is much greater in the case of interaction of DPPC with trehalose above phase transition, than in the hydrated state or in dry DPPC without trehalose. The type of liquid-crystalline phase observed in the dry mixture of trehalose and DPPC is believed to play the main role in maintaining membrane stability in dehydrating organisms.

^{13}C-labelled PLs can be used to study particular dynamics of membranes in the interfacial region. For example, Lee *et al.* (1989) used ^{13}C-labelled *sn*-2-carbonyl of DPPC to study the influence of the interaction of dry DPPC with trehalose on interfacial behaviour. Those authors reported observing no changes in ^{13}C NMR spectra during the phase transition of a dry mixture of DPPC/trehalose, whereas hydrated DPPC exhibited pronounced changes during the phase transition.

^{13}C-NMR is not a sensitive method and requires concentrations in the millimolar range. However, advantage can be taken of this low sensitivity by tracing metabolic changes through the detection of compounds that accumulate to high levels. These metabolites include compatible solutes that accumulate in cyanobacteria (Reed *et al.*, 1985), sugars and oil in seeds (Rutar, 1989; Ishida *et al.*, 1990; 1996; Koizumi *et al.*, 1995) and trehalose in fungal spores (Bécard *et al.*, 1991). The non-invasive character of NMR may allow the time-course of metabolic events to be followed and the

subcellular localisation of some metabolites such as in maturing or germinating seeds (Colnago and Seidl, 1983; Ishida *et al.*, 1990; 1996).

Alternatively, it is possible to label specific metabolites and monitor their fate through the cellular network of metabolic pathways *in vivo* or *in vitro* with crude extracts (Dieuaide-Noubhani *et al.*, 1995; Roberts, 2000; Roscher *et al.*, 2000). Similarly, ^{31}P- and ^{14}N-labelled compounds can be used to monitor the dynamics of phosphorylated metabolites and amino acids, respectively. The applicability of *in vivo* NMR to drying tissues remains to be ascertained.

It should be possible to pinpoint the metabolites, the concentrations of which are mostly sensitive to changes in water content during drying (Fan, 1996; Noteborn *et al.*, 2000). Approximately 0.5-1 g of fresh material is often required to take an NMR spectrum. This requirement could prove a limiting factor if the availability of the biological material is restricted.

In conclusion, NMR provides a rapid and non-invasive method for investigating the state of membranes in isolated cellular fractions and living tissues. It is the approach of choice in the study of membrane structure and dynamics. In addition, NMR spectroscopy appears to be the most appropriate technique to determine the effects of desiccation on the dynamics of metabolic pathways by analysing the flux of metabolites through the different pathways (Shachar-Hill and Pfeffer, 1996; Roberts, 2000). Several strategies can be adopted using ^{13}C-, ^{31}P-, ^{14}N-or ^{15}N-NMR, depending on the nature of the metabolite to be analysed. NMR studies may or may not be invasive.

The principle of the EPR method for the estimation of the relative amount of viable cells is based on the fact that membranes of viable cells are impermeable to some agents, whereas the membranes of damaged cells are not (Keith and Snipes, 1974). The EPR signal from the sample correlates with the amount of viable cells in a sample (Dobrucki *et al.*, 1990). It is possible to determine small amounts of viable cells in mostly dead tissue because of the high sensitivity of the method.

As an example, this approach has been successfully applied in the study of desiccation-tolerance acquisition of proembryonic cells in wheat kernels. Cells were dried slowly on the ear at an early stage when proembryos could not be detected morphologically in that investigation (Golovina *et al.*, 2001). Such an approach allowed the death of wheat endosperm cells during kernel development (Golovina *et*

al., 2000) and the progress of cell death after cold or imbibitional stress in neem seeds (Sacandé *et al.*, 2001) to be followed.

Changes in plasma membrane permeability can be estimated (Miller and Barran, 1977; Golovina *et al.*, 1998; Hoekstra *et al.*, 1999). The method is based on the presence of temporary defects in membranes

EPR can be used to determine cell volume changes under osmotic stress. The total volume is the product of the number and volume of viable cells. As a result, cell division and enlargement of cells during imbibition and germination (Golovina *et al.*, 2001) and osmotically induced changes in cell volume (Miller, 1978) can be detected.

Cytoplasmic viscosity can be studied (Keith and Snipes, 1974). The changes in cytoplasmic viscosity with drying of desiccation-tolerant and sensitive samples (Leprince *et al.*, 1999) and with the acquisition of desiccation-tolerance during seed development (Golovina *et al.*, 2001) have been established based on such an approach. EPR can also be used to characterise biological glasses (Buitink *et al.*, 2000b,c,d,e).

EPR is also used to study the physical properties of membranes (Berliner, 1976; Marsh, 1981; Morse, 1985). Information can be obtained from different depths in membranes from surface to core.

EPR is particularly suitable to characterise the glassy state. EPR has been used to study the motion of proteins in biological membranes (Marsh, 1981; Hemminga, 1983) and glasses (Roozen *et al.*, 1991). This method has also been successfully applied in the study of biological glasses in anhydrobiotic systems (Buitink *et al.*, 1998b; 1999; Buitink *et al.*, 2000b,c,d,e).

This approach has given insight into the differences between biological glasses and sugar or polymer glasses. For example, the occurrence of a second kinetic change in mobility at a definite temperature above the glass transition temperature was observed from EPR measurements (Buitink *et al.*, 2000e), which may have physiological relevance for survival in the dry state. EPR has been used to identify the glassy state in wheat seed (Dzuba *et al.*, 1993) and to characterise biological glasses of different water content (Buitink *et al.*, 2000a).

There are several biological applications in which EPR imaging (EPRI) has an advantage over NMRI: (1) the spatial distribution of O_2 and redox metabolism, (2) mapping viable and non-viable cells, (3) the diffusion of solutes and (4) mapping free

radicals at the site of radical production (Berliner and Fujii, 1986; Bacic *et al.*, 1989; Dobrucki *et al.*, 1990). In spite of the potential advantage of EPRI, there are only a few cases in which the method has been applied to desiccation-tolerant systems.

The pathways of bulk water penetration into wheat kernels during imbibition have been studied (Smirnov *et al.*, 1988; Golovina *et al.*, 1991). The image enabled contrast between embryo and storage tissue to be observed.

EPR has been used to determine the pH in vesicles and cells (Mehlhorn *et al.*, 1982). EPR can also be used to study the changes in pH in sample during drying (Khramtsov and Weiner, 1988).

With the introduction of FTIR spectrometers, *in vivo* studies became possible, which was not the case with grating infrared spectrometers. FTIR spectroscopy can be used for the analysis of certain compounds or to study the interaction between molecules. The technique is particularly useful in studies in dry organisms because of the absence of water. A considerable advantage of *in vivo* FTIR spectroscopy is that it permits the analysis of macromolecules in their natural environment as opposed to in a solvent.

An example of the *in vivo* analysis of certain compounds in seeds is the confirmation that the aleurone layer is enriched in proteins and the endosperm in starch (Leprince and Golovina, 2002). The change in molecular interactions or conformation is of interest in dehydrating organisms. It is possible to study, *in vivo*, membranes with dehydration (Cameron *et al.*, 1983; Crowe *et al.*, 1989a,b; Hoekstra *et al.*, 1992a). It is possible to determine the gel to liquid crystal transition temperature of these membranes.

In vivo FTIR spectroscopy has been successfully applied in the study of protein secondary structure during dehydration (Wolkers and Hoekstra, 1995; 1997; Golovina *et al*, 1997; Wolkers *et al.*, 1998a,c). The intermolecular extended β-sheet is indicative of the formation of large protein aggregates with drying in some model enzyme systems (Prestrelski *et al.*, 1993). These aggregates have also been found *in vivo* on heat denaturation. The stability of protein against heat denaturation can be followed (Wolkers and Hoekstra, 1997; Wolkers *et al.*, 1998c).

Recently, it was established that the glassy state can be studied *in vivo* (Wolkers *et al.*, 1998b; 1999). The interaction of sugars with proteins or polar head groups has been verified in dry model systems (Wolkers *et al.*, 1998d; Crowe *et al.*, 1996). FTIR

spectroscopy has considerable advantage in that molecules are studied in their native environment.

DSC is applied to the study of thermal events associated with lipid and water phase/state transitions. It is used for two main purposes in plant anhydrobiotes: (1) to determine the calorimetric properties of water present in the system and (2) to construct a phase/state diagram in which the glass (to liquid phase) transition temperature (T_g) and the ice formation/melting temperature are plotted as a function of water content (Vertucci, 1990; Leprince and Vertucci, 1995; Buitink *et al.*, 1996).

The heat released during the glass transition is sometimes below the sensitivity of the equipment. For example, T_g cannot be detected by DSC in seeds of rice and tobacco (Leprince and Golovina, 2002). In addition, the lipid melting transitions often mask the thermal events associated with water in oily seeds such as neem (*Azadirachta indica*) and bell pepper (*Impatiens*) (Buitink *et al.*, 1996; Sacandé *et al.*, 2000). However, the future of DSC in studying anhydrobiosis is questionable since no major difference in the calorimetric properties of water was found between desiccation-tolerant and -sensitive organisms (Sun *et al.*, 1994; Buitink *et al.*, 1996).

Two non-invasive techniques are worth mentioning owing to technical difficulties in studying ultrastructural characteristics of cells in the dry state and upon rehydration are: (1) atomic force microscopy (AFM) and low-temperature scanning electron microscopy (LTSEM). AFM is particularly suitable for imaging the surface topography of membranes. Furthermore, AFM can be used to obtain information on the mechanical properties of surfaces (Heinz and Hoh, 1999; Claessens *et al.*, 2000).

LTSEM overcomes problems linked to aqueous fixation. It allows a fast and direct observation of specimens. Application of LTSEM was found to be powerful for studying ultrastructural damage resulting from imbibitional injury in seeds (Leprince *et al.*, 1998; Nijsse *et al.*, 1998; Sacandé *et al.*, 2001) and cellular collapse in lichens (Scheidegger *et al.*, 1995). Non-invasive fixation (freeze-substitution) and a new non-aqueous fixative for immunocytochemistry, acrlein, are becoming available for transmission electron microscopy (TEM) studies (Grote *et al.*, 1999; Wesley-Smith, 2001), allowing observation without disturbing the sample water content.

1.9 The effects of drying rate on desiccation sensitivity – an overview

Desiccation-sensitive seed tissues are thought to be subjected to metabolic damage at intermediate water contents during dehydration. It has been argued that desiccation differentially affects enzyme activities and hence results in metabolic imbalance (Farrant *et al.*, 1985; Leprince *et al.*, 1992; 1993a; 1994; 1995b; 1998; 1999; 2000; Finch-Savage *et al.*, 1993; reviewed by Vertucci and Farrant, 1995; reviewed by Côme and Corbineau, 1996; Li and Sun, 1999; Pammenter *et al.*, 1999; Leprince *et al.*, 2000). It has been suggested that the consequent unbalanced respiration may result in more leakage of electrons than normal in the electron transport chain thus generating the accumulation of free radical species, which are associated with lipid peroxidation (Leprince *et al.*, 1990b; 1994; 1995b; Hendry *et al.*, 1992; reviewed by Hendry, 1993).

Many recalcitrant seeds are characteristically large. As a result, they dry slowly. However, even when recalcitrant seed are of similar size to comparable orthodox counterparts such as in *A. pseudoplatanus* (recalcitrant) and *A. platanoides* (orthodox), recalcitrant *Acer* takes 12 times as long to reach 20% water content under the same drying conditions (Greggains *et al.*, 2000a).

A number of studies have shown that rapid drying of recalcitrant seeds permits survival to lower water contents than does slow drying (Berjak *et al.*, 1984; Farrant *et al.*, 1985; 1989; Grabe, 1989; Pritchard, 1991; Pammenter *et al.*, 1991; 1998; 1999; Leprince *et al*, 2000; Liang and Sun, 2000; Walters *et al.*, 2001; Wesley-Smith *et al.*, 2001; Wu *et al.*, 2001). It has been suggested that rapid dehydration removes water sufficiently fast to reduce the accumulation of damage resulting from aqueous-based deleterious reactions (Berjak *et al.*, 1989; 1993; Pammenter *et al.*, 1991; Pritchard, 1991). In this regard, it is interesting to note that although rapid (especially very rapid [flash]) drying of excised embryonic axes decreased the minimum water content to which recalcitrant seeds could be dried without loss of viability under specific drying conditions, it was never less than the amount of non-freezable (matrix-bound) water (Berjak *et al.*, 1992; 1993; Pammenter *et al.*, 1991; Finch-Savage, 1992; reviewed by Vertucci and Farrant, 1995). In addition, the lifespan of rapidly-dried material is very short (Walters *et al.*, 2001)

In contrast to the studies mentioned above, some authors have found no effect of the drying rate on desiccation sensitivity (e. g. Tompsett, 1982; Finch-Savage, 1992;

desiccation. Berjak *et al.* (1989) suggested that this apparent contradiction may be explained in terms of the rates of dehydration relative to the rates of germination. It should be remembered that germination is accompanied by cell division and hence high metabolic activity and extensive vacuolation both of which render recalcitrant seeds more sensitive to desiccation.

In a review, Berjak *et al.* (1989) categorised recalcitrant seeds into four groups on the basis of their rate of germination: (1) very slow germinators, (2) relatively rapid (slow) germinators, (3) rapid germinators and (4) very rapid germinators. Those authors argued that the effect of dehydration rate on survival of recalcitrant seeds following drying varies among the four categories of recalcitrant seeds.

In very slow germinators, the period of axis differentiation is quite extended. Consequently, the subsequent germination process might be slow. The period of low desiccation sensitivity can be of the order of months, and, unless drying rates are of this order, no differential effect will be apparent. *Araucaria hunsteinii* and *Araucaria cunninghamii* (Tompsett, 1982), *Scadoxus membranaceus* (Farrant *et al.*, 1989) and *Quercus robur* (Finch-Savage, 1992) exhibit this type of behaviour.

Seeds of relatively rapid (slow) germinators are shed with relatively undifferentiated axes. Before cell division and subsequent growth can occur, axis differentiation must be completed. This phase, and subsequent germination can be relatively rapid. *Hevea brasiliens*, where germination of newly-shed seeds occurs within about 10 days, exemplify this type of behaviour. If the drying rates are not sufficiently dissimilar, then a differential effect is unlikely to be apparent.

In rapid germinators, the embryonic axis is fully differentiated on seed shedding. This is followed by a short period of organisation and differentiation at the cell level. A time scale of approximately 1-3 days is involved. During this period the tissue is at, or near, its relatively most desiccation-tolerant. This period is followed by cell division and extensive vacuolation during which desiccation sensitivity increases. Differential drying rates will have a marked effect. *A. marina* exhibits this behaviour.

Finally, very rapid germinators may germinate so rapidly that the relatively desiccation-tolerant state is obviated. In such cases, not even the most rapid drying rate is fast enough to prevent death of most of the seeds on dehydration. Drying rate will have a minimal discernible effect, at most (Berjak *et al.*, 1989).

1.10 The purpose and scope of the present study

The aim and objective of the present study was to test the hypothesis of Berjak and co-workers which states that rapid dehydration of desiccation-sensitive seeds removes water sufficiently fast to reduce the accumulation of metabolic damage (Berjak *et al.*, 1989; 1993; Pammenter *et al.*, 1991). Additionally, the hypothesis that hydrated storage subjects desiccation-sensitive seeds to mild, but increasingly severe, water-stress causing oxidative damage if additional water is not supplied (Pammenter *et al.*, 1994), was tested.

To achieve the objectives of the present study, a number of tests were performed. Firstly, excised embryonic axes of mature seeds of a number of species (*Trichilia dregeana* [forest mahogany], *Trichilia emetica* [Natal mahogany], germinating *Pisum sativum* [garden pea], *Quercus robur* [English oak], *Avicennia marina* [white mangrove] and *Strychnos madagascariensis* [black monkey orange]) were dried rapidly or slowly or stored wet. During dehydration and wet storage, water contents were determined periodically to establish the relationship between water content and viability, and, to ascertain if it was influenced by drying rate and hydrated storage. Seed vigour was assessed at regular intervals using tetrazolium and germination tests. Membrane integrity was estimated by electrolyte leakage. To establish if rate of dehydration and hydrated storage affect metabolism differentially, the activities of respiratory enzymes, phosphofructokinase (PFK) and malate dehydrogenase (MDH), and the concentration of the oxidised form of the coenzyme nicotinamide adenine dinucleotide (NAD) were measured periodically. A simple peroxide test was performed to determine if the level of peroxidation was a function of rate of drying and wet storage. To evaluate the effect of rate of drying and wet storage on free radical scavenging systems, the activities of enzymes, SOD, CAT and GR and levels of the antioxidant, ascorbate (vitamin C), were monitored at regular intervals. It was hoped that the results of the present study would show whether or not there exists a relationship between the rate of drying and survival, such that the faster the drying proceeds, the better the survival of desiccation-sensitive tissues, and some biochemical markers of viability loss.

1.11 Importance of the present study

Our present agricultural system is almost totally dependent upon the ability of orthodox seeds to tolerate desiccation. Although recalcitrant seeds constitute less than 10% of the seeds with known storage behaviour (Ellis, 1984; Hong *et al.*, 1998; Hay *et al.*, 2000; reviewed by Dickie and Pritchard, 2002) and comprise a very small fraction of all seeds, they include economically-important species such as rubber, cocoa and coconut, timber species (e.g. families Dipterocarpaceae and Araucariaceae and genera *Quercus* and *Hippocastanum*) and several tropical fruit species (for example avocado, durian, jackfruit, mango, neem and rambutan). Furthermore, seeds of up to seven out of ten tree species in tropical rainforests may be recalcitrant (Gunn, 1991).

Often, it is not yet possible to store recalcitrant seeds from harvest till the next sowing season (Roberts and Ellis, 1989). As such, difficulties are experienced with storage of those seeds, in the short-term, for agricultural purposes and in the long-term, for conservation of biodiversity in seedbanks.

The purpose of the present study was to shed more light on our understanding of desiccation-tolerance/sensitivity. Such knowledge may eventually allow us to store recalcitrant seeds for periods comparable to their orthodox counterparts. Of immediate promise in this regard, is the rapid dehydration of embryonic axes and their subsequent cryostorage (reviewed by Berjak *et al.*, 1996; 1999; Berjak and Pammenter, 1997a,b; 2001; 2004; Pammenter and Berjak, 1999).

In conclusion, over 35% of the world's land surface is considered semi-arid, experiencing precipitation that is inadequate for agricultural uses. In addition, Ramanathan (1988) has suggested that developing crops that are more tolerant of water deficits while maintaining productivity will be the critical requirement in the early part of this century. As a result, it is vital to understand this complex trait to develop strategies that can influence crop productivity and survival under these conditions of decreasing water availability.

Chapter 2

Aspects of water relations during desiccation and moist storage

2.1 Introduction

Water plays multiple roles in supporting life (reviewed by Vertucci and Farrant, 1995; Walters *et al.*, 2002). At the cellular level, it plays a structural role. It fills spaces and provides turgor. On the other hand, it provides hydrophilic and hydrophobic associations, at the molecular level. Thus, it controls intermolecular distances that determine conformation of proteins and polar lipids and the partitioning of molecules within organelles and limits reactivity among molecules such as metals. Water also plays a role in controlling metabolism, as it is a reactant and product of many reactions. Furthermore, as a dilutant, it affects the chemical potential of other molecules, potentially shifting the likelihood of reactions. In addition, water provides the fluid matrix that allows the diffusion of substances to reactive sites. Consequently, it may be expected that removal of water would result in a number of strains.

There are many ways to express water loss in cells (reviewed by Pammenter *et* al., 2002; Sun, 2002; Walters *et al.*, 2002). They include: (1) water content, both absolute and relative (e.g. Berjak *et al.*, 1992; Sun *et al.*, 1994; Farrant, 2000), (2) water potential and related functions such as water activity (Roberts and Ellis, 1989; Vertucci, 1990; Vertucci and Roos, 1990; Tompsett and Pritchard, 1993; Vertucci *et al.*, 1995; Farrant and Walters, 1998), (3) cell volume (Merryman, 1974; Steponkus, 1979; Murai and Yoshida, 1998a,b), (4) intracellular viscosity (Vertucci and Roos, 1990; Koster, 1991; Williams *et al.*, 1993; Leopold *et al.*, 1994; Buitink *et al.*, 1998a; Leprince and Hoekstra, 1998; Bryant *et al.*, 2001), (5) intermolecular proximity (Lis *et al.*, 1982; Steponkus *et al.*, 1995) and (6) structural water (Ladbrooke and Chapman, 1969; Vertucci and Leopold, 1984; 1987; Crowe *et al.*, 1990; Pammenter *et al.*, 1991).

Additionally, water content can be expressed on a fresh or dry mass basis. Whilst, moisture levels on a fresh mass basis may give an accurate picture of the concentration of water in seeds, water content on a dry mass is usually preferred when dealing with studies on the effects of water loss or uptake. This situation arises because water content on a dry mass basis is a linear expression of water content in tissues (Sun, 2002). For instance, a tissue with a water content of 0.20 g H_2O / g dry

matter (g g^{-1} dm) is exactly twice as hydrated as that with a water content of 0.10 g g^{-1} dm and four-fold as much as a sample with a water content of 0.05 g g^{-1} dm. In contrast, water content on a fresh mass basis is not a linear expression of water content because fresh mass appears in both the numerator (fresh mass – dry mass [mass of the water content]) and denominator (fresh mass) terms. Indeed, the change in water content on a fresh mass basis during drying or rehydration is related to the reciprocal of tissue fresh mass (Sun, 2002). Hence, water content on a fresh mass basis does not reflect the extent of dehydration or rehydration stress. For example, when a tissue with a water content of 80% fresh mass basis is dried to 70% and then 60% water contents fresh mass basis, the tissue actually loses 41.7% and 62.5% of the initial water quantity, respectively, not just 12.5% and 25% reduction as implied by the values of water content on a fresh mass basis. Indeed, the quantity of water lost during desiccation from 80 to 70% water contents fresh mass basis is twice as much as water loss from 70 to 60% water content fresh mass basis.

The loss of water from tissues depends on three factors: (1) the gradient in water potential between tissue and external air, which, in turn, is affected by: (a) the vapour pressure of the surrounding air, which, in turn, is linearly related to relative humidity, which is an exponential function of temperature and water potential (Wexler, 1997), (b) temperature and (c) the rate of air movement around the tissue (Pammenter *et al.*, 2002; Sun, 2002), (2) the hydraulic conductivity of the tissue, which is dependent on permeability of the tissue which, in turn, is affected by the chemical composition and the presence and nature of physical barriers such as the testa/pericarp of the outer layer of the tissue and (3) surface area which, in turn, will be influenced by: (a) size and shape of the tissue and (b) the amount of material to be dried (Pammenter *et al.*, 2002; Sun, 2002). Thus, the volume flow of water from the tissue to air can be described by:

$$V_w = AL_p (\psi_o - \psi_i)$$

where:

V_w = the volume flow of water per unit time (m^3 s^{-1}),

A = the surface area of the tissue (m^2),

L_p = hydraulic conductivity of the tissue and boundary layer (m s^{-1} Pa^{-1}),

ψ_o = water potential of external air (MPa) and

ψ_i = water potential of the tissue (MPa).

Whilst, the difference in water potential (ψ_o - ψ_i) is the measure of the driving force, hydraulic conductivity of the tissue and boundary layer (L_p) are a measure of diffusional resistance of the water transport pathway within the tissue.

Under constant temperature and relative humidity (RH), the water content (wc) of the tissue may decrease exponentially over time until ψ_i reaches ψ_o. In this case, the curve of water loss can be described by:

$$wc = \alpha \exp(-\beta t)$$

where:

α = initial water content (g g^{-1} dm) and

β = the rate constant of water loss.

This relationship was first used by Tompsett and Pritchard (1998) to compare the dehydration rates of *Aesculus hippcastanum* seeds. It has since been tried on the drying curves of *Theobroma cacao*, and they conform (Li and Sun, 1999; Liang and Sun, 2000). Typically, water content curves are biphasic. During the first drying phase, the loss of water may follow a simple exponential function. In contrast, water content does not decrease much during the second phase as tissue approaches equilibrium with the surrounding air. Because water loss during the first phase may be described by an exponential function, the rate constant of water loss (β) can be used as an expression of drying rate.

Although an exponential relationship has been found to fit some data sets (e.g. Liang and Sun, 2000), particularly for seeds or excised axes, in many cases initial drying is considerably faster than described by an exponential function (e.g. Pammenter *et al.*, 1998; reviewed by Pammenter *et al.*, 2002; 2003). However, the modified inverse function:

$$wc = a*b/(b+t)$$

where:

wc = water content (g g^{-1} dm),

a = initial water content (g g^{-1} dm),

b = time required to dry the tissue to half its original water content (h) and

t = time (h)

was found to fit data reasonably well in many cases (reviewed by Pammenter *et al.*, 2002; 2003). *a* and *b* are constants and *b* describes the curvature of the function.

If data are expressed as a relative water content (RWC), normalised to the initial water content, then a becomes unity and the function simplifies to:

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

This function has only one constant and so b constitutes an objective desriptor of drying rate. The value of b is determined as the inverse of the slope of a plot of 1/RWC vs t.

Conventionally, when working with vegetative tissues, RWC is normalised to the water content of fully hydrated tissue. However, when seeds or excised axes are placed in water to attain 'full hydration', they continue to absorb water and start germinating. In effect, the concept of 'full hydration' is meaningless for seed material and so RWC is calculated relative to the water content at shedding.

As a generalisation, if tissue is dried relatively slowly, the relationship between water content and drying time is exponential. However, for material dried more rapidly, the initial water loss is considerably faster than that predicted by the exponential relationship. It must be emphasised that the terms 'slow' and 'rapid' are relative. There is no drying rate common across species where drying changes from faster-than-exponential to exponential. Furthermore, a fast drying rate in one experiment could be equivalent of slow in another.

For example, during 'slow' drying of whole seeds of *Landolphia kirkii* (Pammenter *et al.*, 1991) and of *Camellia sinensis* (Berjak *et al.*, 1993), the water content of the axes within the seeds followed an exponential relationship with time, but during 'rapid' drying of excised axes did not (Pammenter *et al.*, 2002). Similarly, initial faster-than-exponential drying rates have been observed in rapidly dried excised axes of *Syzigium guiniense*, *Castanospermum australe*, *Trichilia dregeana*, *Artocarpus heterophyllus*, *Azadirachta indica* and radicle tips of axes of *Podocarpus henkelii* (Pammenter *et al.*, 2002). Conversely, axes of *Avicennia marina* and the entire axes of *P. henkelii*, which are relatively large, show exponential drying (Pammenter *et al.*, 2002).

However, when excised axes of *A. heterophyllus* (Pammenter *et al.*, 2002), *C. australe* (Pammenter *et al.*, 2002) and *T. dregeana* (Pammenter *et al.*, 1999; 2002) were dried slowly at 96% RH, drying was exponential. In contrast, when axes of these species were dried rapidly over silica gel, the initial drying was faster than predicted by an exponential rate (Pammenter *et al.*, 1999; 2002).

Similarly, when seeds of *Ekebergia capensis* with endocarp were dried slowly by burying in silica gel, drying was exponential. Conversely, when seeds of this species without endocarp were dried more rapidly, initial drying was faster than predicted by an exponential relationship (Pammenter *et al.*, 1998).

Pammenter *et al.* (1998) suggested that the drying kinetics of *E. capensis* indicated that uneven drying of the tissue might be occurring under rapidly dehydrating conditions. This observation was borne out by a later study of the ultrastructure of axes of *A. heterophyllus* during desiccation at two rates (Wesley-Smith *et al.*, 2001).

Wesley-Smith *et al.* (2001) found that a short exposure of axes to rapid drying resulted in preferential withdrawal of water from the outermost tissue layers and a corresponding contraction of these cells, while those in the core of the axis (stele) remain relatively unaffected. In contrast, slow dehydration caused more even distribution of water throughout the entire embryonic axes. This finding has implications for axes dried slowly: germinative cells from the core endured less dehydration, but for longer periods, than those dried rapidly.

The response of plant tissues to desiccation is significantly affected by dehydration conditions, such as dehydration rate and temperature. Plant tissues stay longer at intermediate water contents under slow drying conditions. In contrast, fast drying reduces damage accumulation of recalcitrant plant seeds (reviewed by Pammenter and Berjak, 1999).

The response of tissue to water-stress varies with drying rate. Therefore, the change in water potential ($d\psi/dt$) can be used to quantify the rate of application of the stress (Sun, 2002). Under the condition of constant temperature and RH, such plots ($d\psi/dt$) may be straight lines down to the fraction of apoplastic water. Water potential decreases faster and deviates away from the straight line when the apoplastic water is lost. The slope of the straight-line portion of each plot represents the degree of direct physical stress under different desiccation conditions. The relationship between $d\psi/dt$ and the rate constant of water loss (β) is linear if drying is exponential.

The 'critical water content' to which mature embryos can be dried under specific conditions without inducing desiccation damage has been suggested to be species-dependent (Levitt, 1980b). However, it is now known that the rate of drying influences the response. These 'critical water contents' correspond to the critical moisture levels for particular metabolic activities. Discrete changes in metabolic

activity with water content are hypothesized to be associated with discrete changes in the physical properties of water (e.g. Clegg, 1978; Rupley *et al.*, 1983; Bruni *et al.*, 1989; Leopold and Vertucci, 1989; Vertucci, 1989; 1990; 1992). This hypothesis is based on the observation that the characteristics of water change with the degree of hydration (reviewed by Vertucci and Farrant, 1995; Walters *et al.*, 2002).

At least five levels of hydration can be distinguished from calorimetric and motional properties (Clegg, 1978; Rupley *et al.*, 1983; Vertucci, 1990; Vertucci and Farrant, 1995; Walters *et al.*, 2002). Hydration level V water behaves as water would in a dilute solution and occurs at water contents and water potentials between 0.6 to 0.9 g H_2O g^{-1} dry matter (g g^{-1} dm) and 0 to –1.5 MPa, respectively. At level IV of hydration, water behaves as it would in a concentrated solution or syrup and is detected at water contents and water potentials between 0.45 and 0.7 g g^{-1} dm and –1.8 and –4MPa, respectively. Hydration level III water forms bridges over hydrophobic moieties on macromolecules and behaves as water would in rubber. It occurs at water contents and water potentials between 0.25 and 0.45 g g^{-1} dm and –5 and –12 MPa, respectively. Hydration level II water has characteristics of water in leathers and glasses and has strong interactions with polar surfaces of macromolecules and hydroxyl groups of solutes. It is detected at water contents and water potentials between 0.08 and 0.25 g g^{-1} dm and –15 and –190 MPa, respectively. Hydration level I water corresponds to the theoretical level at which water binds to macromolecules as a structural component (e.g. Briarty and Leopold, 1992). It occurs at water contents and water potentials of 0.08 g g^{-1} dm and –220 MPa, respectively or less.

It appears that the water at different hydration levels performs different functions in plant cells (reviewed by Walters *et al.*, 2002). For example, hydration level V water is required for turgor and supports growth (Vertucci, 1990). Hydration level IV water is required for photosynthesis and stress-related metabolism, whilst hydration level III water is required for respiration, and hydration level II is required to carry out catabolic reactions. Hydration level I water appears to be in stasis (Clegg, 1986; Roberts and Ellis, 1989). Its removal is lethal to intermediate seeds (Ellis *et al.*, 1990a,b; 1991a,b,c; Kovach and Bradford, 1992a,b), and may affect the long-term viability of some orthodox seeds and pollen (Vertucci and Roos, 1990; 1993; Hoekstra *et al.*, 1992a). It is noteworthy that the response of tissue depends not only on the hydration level, but also the time it spends in that range.

Viscosity is reduced at the transition from hydration level II to I (Vertucci and Roos, 1990; Buitink *et al.*, 1998b). Similarly, there is a discrete change in the heat capacity of water at this moisture level (Rupley *et* al., 1983; Vertucci, 1990; Buitink *et al.*, 1996). Additionally, characteristics of water sorption are poorly understood at this hydration level (Vertucci and Leopold, 1987; Vertucci and Roos, 1990; Vertucci *et al.*, 1994; Buitink *et al.*, 1998a,b; Eira *et al.*, 1999). With the exception of hydration level I, the relationships between physical properties of water and water potential appear to be similar among diverse cells (Vertucci and Leopold, 1987; Eira *et al.*, 1999). However, subtle differences may distinguish desiccation-tolerant from sensitive material (Koster, 1991; Berjak *et al.*, 1993; Farrant and Walters, 1998; Leprince *et al.*, 1999).

Comparisons of the properties of water in mature orthodox and recalcitrant seeds have revealed no major differences between the two seed types (Vertucci, 1990; Pammenter *et al.*, 1991; Berjak *et al.*, 1992; 1993). This phenomenon has led Pammenter *et al.* (1991) and Vertucci and Farrant (1995) to conclude that, contrary to the previously widely-held view that desiccation-tolerance was a result of the amount of structured water retained (Adams and Rinne, 1980; Berjak *et al.*, 1984; Vertucci and Leopold, 1987; Welbaum and Bradford, 1989; Grange and Finch-Savage, 1992), desiccation-tolerance involves the ability to lose a considerable proportion of structural water.

The aim of the phase of the work described in this chapter was to establish the drying or storage water content vs drying or storage time curves of embryonic axes of recalcitrant and germinating orthodox seeds during drying at different rates or wet storage, respectively.

2.2 Materials and methods

2.2.1 Plant materials

Seeds of *Trichilia dregeana* L. Sond. (Meliaceae) were collected in 1997, 1999 and 2001 from a number of trees in Durban, South Africa. Their counterparts of *Trichilia emetica* L. Sond. (Meliaceae) were collected from a number of trees in Emthunzini, South Africa in 1998. Seeds of *Pisum sativum* L. var Greenfeast (Fabaceae) were bought from Pannar Seeds, Greytown, South Africa. Propagules of *Avicennia marina* L. [Forssk.] Vierh. (Avicenniaceae) were collected from a number of trees in 1999 and 2001 in Beachwood Nature Reserve and Isipingo Beach, Durban, South Africa, respectively. Seeds of *Q. robur* L. (Fagaceae) were collected from two trees in Wellesbourne, UK in 1999. Finally, fruits of *Strychnos madagascariensis* L. (Loganiaceae) were collected in 1999 from a number of trees in Durban, South Africa.

All seeds were collected within a day of shedding. They were then brought to the laboratory, major débris removed and rinsed thoroughly. Following the removal of the aril and the testa, seeds of *T. dregeana* and *T. emetica* were surface-sterilised for 20 minutes in commercial bleach diluted to 1% sodium hypochlorite (NaOCl) and rinsed three times with distilled water. After coating with Benlate®, they were stored in loosely-closed plastic bags at approximately 15°C until required, but for not longer than two weeks to minimise seed deterioration in storage. Seeds of *P. sativum* were allowed to imbibe water for 6 h, surface-sterilised and set out to germinate in germination trays on moist paper towel at room temperature under constant light for 72 h after which they were used immediately. Following immersion in water at room temperature for 10 min to stimulate the shedding of the pericarp, propagules of *A. marina* were surface-sterilised in commercial bleach diluted to 1% NaOCl and rinsed three times with distilled water. After the removal of the testa, seeds of *Q. robur* were surface-sterilised in 6.4 g sodium dichloroisocyanurate (Fichlor [$C_3Cl_2N_3O_3Na$]) per 100 ml H_2O for 20 min and rinsed three times with distilled water. They were then stored in loosely-closed plastic bags at 1 ± 1°C until required. Following the removal of the testa, seeds of *S. madagascariensis* were surface-sterilised in commercial bleach diluted to 1% NaOCl and rinsed three times with distilled water. After coating with Benlate®, they were stored in loosely-closed plastic bags in an air-conditioned room maintained at approximately 15°C until required, but for not longer than two weeks.

2.2.2 Excision of embryonic axes

Embryonic axes were excised manually from seeds of all species. For *T. dregeana* and *T. emetica* seeds, a small block (*c.* 2 mm^3) of cotyledon was left attached to each axis. This practice is thought to avoid injury to embryonic axes. Only axes longer than 10 mm were used for subsequent experiments on *P. sativum* seeds. Petioles were left attached to embryonic axes of *Q. robur* seeds,. This procedure was performed to avoid damage to axes. During excision, axes were accumulated on moist filter paper in closed Petri dishes.

2.2.3 Surface-sterilisation protocols

Excised axes from seeds of tropical species (*S. madagascariensis*, *T. emetica*, *T. dregeana* and *A. marina*) and *P. sativum* were surface-sterilised in commercial bleach diluted to 1% NaOCl containing a drop of Tween-80 in a laminar flow cabinet for 15 min. In contrast, those from the temperate *Q. robur*, were surface-sterilised in a laminar flow cabinet in 3.2g Fichlor / 100ml H_2O containing a few drops of Nonidet for 6 min. They were then all rinsed three times with sterile distilled water.

2.2.4 Dehydration treatments

Embryonic axes of each species were partitioned into two samples. Each sample was aseptically placed in sterile containers over: (1) activated silica gel with a fan mounted (rapid drying [≈0% RH]), or (2) a saturated solution of sodium chloride (slow dehydration [75 ± 1% RH]). For *T. dregeana* seeds, saturated solutions of magnesium chloride, potassium carbonate or sodium bromide (33 ± 0.5%, 43% and 58 ± 1% RH, respectively) were also used. The containers were stored in an air-conditioned room maintained at approximately 15°C.

2.2.5 Wet storage of axes

Embryonic axes were aseptically placed in sterile containers over distilled water. The containers were then stored in an air-conditioned room maintained at approximately 15°C.

2.2.6 Water content determinations

Water contents were determined gravimetrically by drying axes in the oven at 80 °C for 48 hours. They were determined periodically during dehydration and wet storage. The reported water contents represent means ± SE for five individual axes expressed on a dry matter basis. Drying rates, determined as rate constants of water loss (βs) and time taken to dry the tissue to half its original water content (*b*s) were calculated as slopes of log-transformed water contents vs time curves and inverse of the slope of a plot of 1/RWC vs *t*, respectively (Sun, 2002 and Pammenter *et al.*, 2002, respectively).

2.3 Results

No apparent differences were seen in the drying curves of embryonic axes of *T. dregeana* harvested in 1997 and dried over saturated salt solutions of magnesium chloride (33 ± 0.5% RH), potassium carbonate (43% RH), sodium bromide (58 ± 1% RH) and sodium chloride (75 ± 0.5% RH) (Fig. 2.1). This observation indicates that transfer resistances (within the axes, axes surface and boundary layer) were controlling the drying rate.

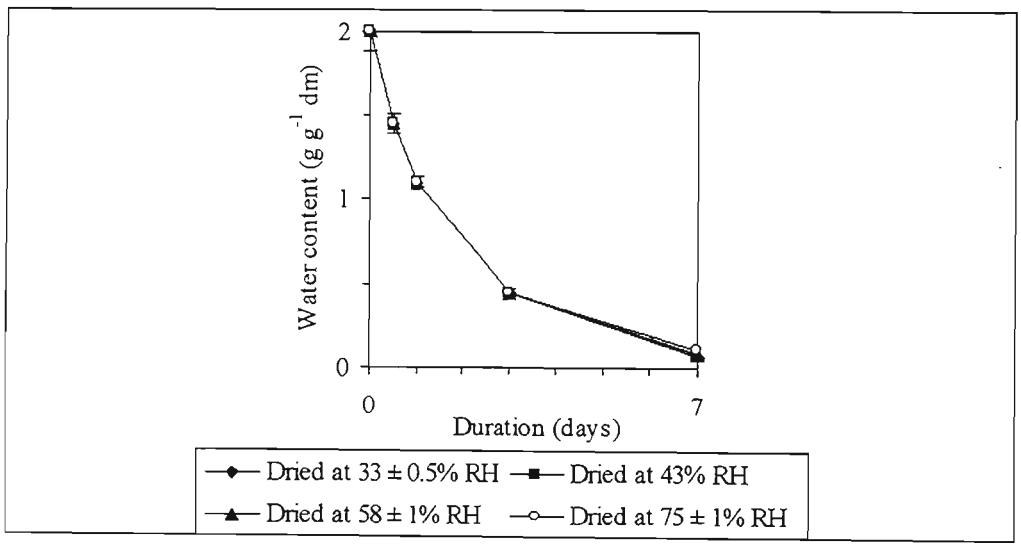

Figure 2.1 Water contents of embryonic axes of *Trichilia dregeana* harvested in 1997 during drying over various salt solutions. Data points represent means of five replicate readings. Error bars, in some cases hidden within the data symbols, show standard errors.

Embryonic axes of *P. sativum* seeds reached a water content of *c.* 2.0 g H_2O /g dry matter (g g^{-1} dm) after imbibition for 6 h. A water content of 4.5 g g^{-1} dm was attained after germination for 72 h subsequent to imbibition for 6 h of *P. sativum* axes (Figs 2.2 and 2.4A). Axes of *P. sativum* of various lengths showed different drying curves (Fig. 2.2).

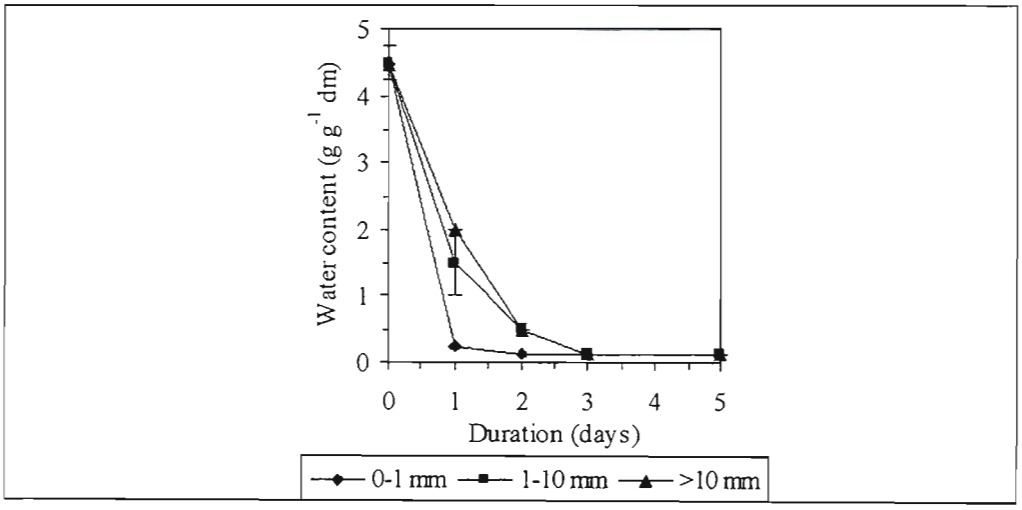

Figure 2.2 Water contents of germinating *P. sativum* axes of different lengths during drying over sodium chloride solution. Data points represent means of five replicate readings. Error bars, in some cases hidden within the data symbols, show standard errors.

No discernible differences were observed in the water contents of axes and three axial tissues (hypocotyls, distal portions of the hypocotyl containing root primordia and plumules) of *A. marina* harvested in 1999 before, during and after rapid or slow drying or wet storage (Fig. 2.3).

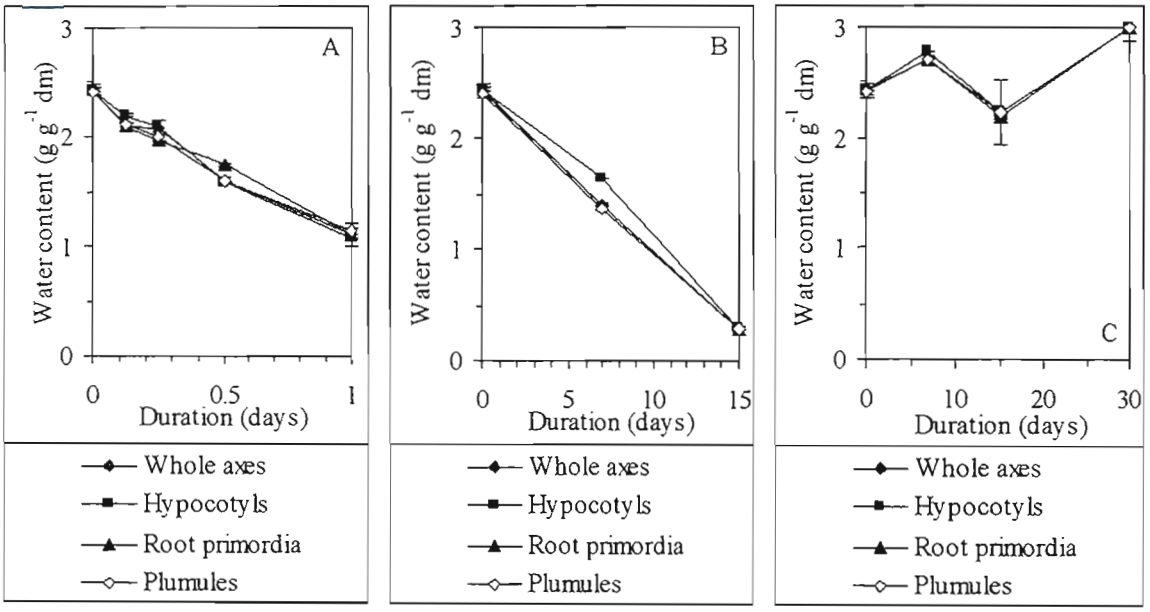

Figure 2.3 Water contents of axes and different axial tissues of *Avicennia marina* harvested in 1999 and dried rapidly (A) or slowly (B) or stored wet (C). Data points represent means of five replicate readings. Error bars, in some cases hidden within the data symbols, show standard errors.

Axes of *Q. robur* were shed at a water content of 1.6 g g^{-1} dm (Fig. 2.4B). *S. madagascariensis* axes were at a water content of 1.9 g g^{-1} dm immediately after shedding (Fig. 2.4C). Whilst axes of *T. emetica* were shed at a water content of 1.9 g g^{-1} dm (Fig. 2.4D), those of *T. dregeana* axes harvested in 1997, 1999 and 2001were at a water content of 2.1, 1.9 and 1.9 g g^{-1} dm immediately after shedding, respectively (Figs 2.1 and 2.4E). A water content of 2.4 and 2.7 g g^{-1} dm was attained by axes of *A. marina* harvested in 1999 and 2001 following immersion in water for 10 min, respectively (Figs 2.3 and 2.4F).

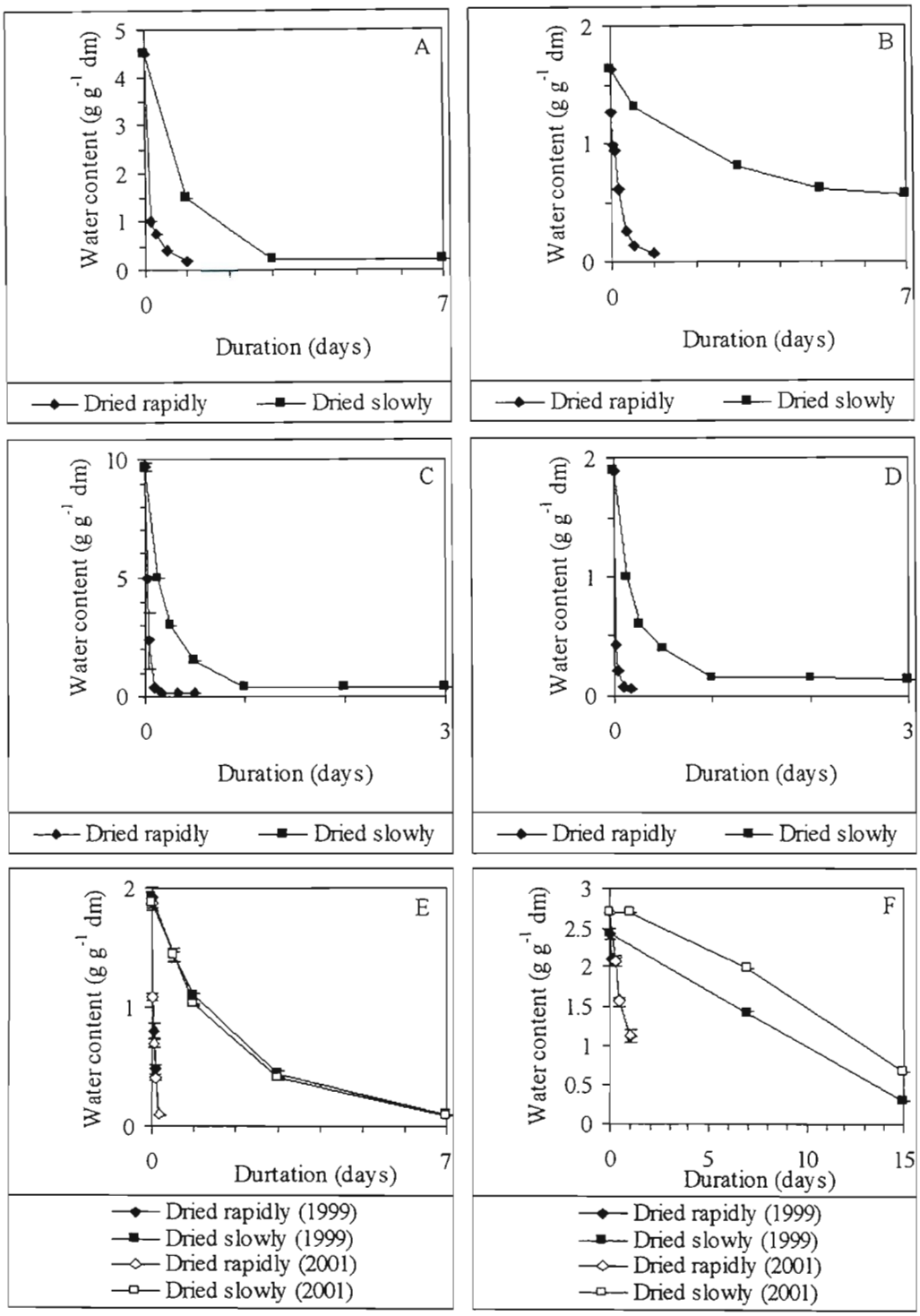

Figure 2.4 Water contents of axes of *Pisum sativum* (A), *Quercus robur* (B), *Strychnos madagascariensis* (C), *Trichilia emetica* (D), *Trichilia dregeana* (E) and *Avicennia marina* (F) harvested in 1999 (closed symbols) or 2001 (open symbols) where applicable during drying at different rates. Data points represent means of five replicate readings. Error bars, in some cases hidden within the data symbols, show standard errors.

Both exponential and modified inverse functions were fitted to the drying time courses, and the exponential drying rate constant (ß) and the inverse function constant (*b*) were calculated. The r^2 and p values for the fit to each function were also calculated.

Axes of *P. sativum* reached a water content of 0.19 g g^{-1} dm in 24 h during rapid drying (ß = 1.4, r^2 = 0.75 and p = 0.01; *b* = 1.05 h, r^2 = 0.99 and p < 0.01 [Fig. 2.4A]). In contrast, a final water content of 0.22 g g^{-1} dm was attained in 7 days upon slow dehydration (ß = 0.19, r^2 = 0.58 and p = 0.24; *b* = 8.25 h, r^2 = 0.83 and p = 0.09 [Fig. 2.4A]). Both desiccation treatments fitted an exponential and a modified inverse relationship between water content and time, although the correlation was not statistically significant for slow drying for both relationships. The fit was better for the modified inverse function for both regimes. Water content remained constant during wet storage of *P. sativum* axes (data not shown). The axes maintained a water content of *c.* 4.5 g g^{-1} dm over four weeks.

Q. robur axes reached a water content of 0.08 g g^{-1} dm in 24 h during rapid drying (ß = 1.4, r^2 = 0.91 and p < 0.01; *b* = 1.14 h, r^2 = 0.99 and p < 0.01 [Fig. 2.4B]). Conversely, a final water content of 0.57 g g^{-1} dm was attained in 7 days upon slow dehydration (ß = 0.07, r^2 = 0.96 and p = 0.02; *b* = 100 h, r^2 = 0.98 and p = 0.01 [Fig. 2.4B]). Both desiccation regimes followed an exponential and a modified inverse functions of water content and time. The fit was better for the modified inverse relationship for both treatments. Water content remained constant during hydrated storage of axes of *Q. robur* (data not shown). The axes maintained a water content of *c.* 1.5 g g^{-1} dm over four weeks.

Axes of *S. madagascariensis* reached a water content of 0.06 g g^{-1} dm in 12 h during rapid drying (ß = 3.5, r^2 = 0.89 and p = 0.001; *b* = 0.14 h, r^2 = 0.76 and p = 0.01 [Fig. 2.4C]). In contrast, a final water content of 0.1 g g^{-1} dm was attained in 5 days upon slow dehydration (ß = 0.24, r^2 = 0.55 and p = 0.02; *b* = 4.55 h, r^2 = 0.63 and p = 0.01 [Fig. 2.4C]). Both desiccation treatments fitted an exponential and a modified inverse relationship between water content and time. The fit was better for the exponential function on rapid drying, and the modified inverse function on slow dehydration. Water content remained constant during moist storage of *S. madagascariensis* axes (data not shown). The axes maintained a water content of *c.* 1.5 g g^{-1} dm over four weeks.

T. emetica axes reached a water content of 0.06 g g^{-1} dm in 12 h during rapid drying (ß = 8, r^2 = 0.89 and $p < 0.01$; *b* = 0.12 h, r^2 = 0.92 and p = 0.01 [Fig. 2.4D]). Conversely, a final water content of 0.1 g g^{-1} dm was attained in 5 days upon slow dehydration (ß = 0.18, r^2 = 0.55 and p = 0.02; *b* = 8.33 h, r^2 = 0.77 and $p < 0.01$ [Fig. 2.4D]). Both desiccation regimes followed an exponential and a modified inverse function of water content and time. The fit was better for the modified inverse relationship for both treatments. Water content remained constant during wet storage of axes of *T. emetica* (data not shown). The axes maintained a water content of *c.* 1.5 g g^{-1} over four weeks.

Axes of *T. dregeana* reached a water content of 0.1 g g^{-1} dm in 4 h during rapid drying (ß = 7.4, r^2 = 0.99 and $p < 0.01$; *b* = 0.22 h, r^2 = 0.89 and p = 0.02 [Fig. 2.4E]). In contrast, a final water content of 0.1 g g^{-1} dm was attained in 5 days upon slow dehydration (ß = 0.18, r^2 = 0.99 and $p < 0.01$; *b* = 10 h, r^2 = 0.95 and $p < 0.01$ [Fig. 2.4E]). Both desiccation treatments fitted an exponential and a modified inverse relationship between water content and time. The fit was marginally better for the exponential function for both regimes although the fit for the modified inverse function was also good. Water content remained constant during hydrated storage of *T. dregeana* axes (data not shown). The axes maintained a water content of *c.* 1.8 g g^{-1} dm over four weeks.

Finally, axes of *A. marina* reached a water content of 1.1 g g^{-1} dm in 12 h during rapid drying (ß = 0.3, r^2 = 0.99 and $p < 0.01$; *b* = 20 h, r^2 = 0.98 and $p < 0.01$ [Fig. 2.4F]). Conversely, a final water content of 0.29 g g^{-1} dm was attained in 15 days upon slow dehydration (ß = 0.06, r^2 = 0.95 and p = 0.15; *b* = 50 h, r^2 = 0.85 and p = 0.25 [Fig. 2.4F]). Both desiccation regimes followed an exponential and a modified inverse function of water content and time, although the correlation was not statistically significant for slow drying for both relationships. The fit was better for the exponential function for both treatments. Water content remained constant during moist storage in *A. marina* axes (data not shown). The axes maintained a water content of *c.* 2.5 g g^{-1} dm over four weeks.

It is noteworthy that the oily axes of *Trichilia* species dried considerably faster than axes containing predominantly carbohydrates (*Pisum sativum*, *Quercus robur*, *S. madagascariensis* and *Avicennia marina*) during rapid dehydration (Table 2.1). A comparison of the desiccation rates of axes of different species with their dry mass of

individual axes and total amount of material dried showed that the bigger the individual axes and/or the consequent greater the amount of the total material dried, the slower the drying rate under a specific set of drying conditions (Table 2.1).

Table 2.1 A comparison of the drying rates of axes of various species with their individual dry mass and total amount of material dried. Data points represent means of five replicate readings. Variations show standard errors. Ranking in brackets indicate the highest to the lowest values.

Species	Dry mass of individual axes (mg)	Total dry mass of the material dried (g)	Drying rate (β)		Drying rate (b)	
			Rapid	Slow	Rapid	Slow
P. sativum	3.7 ± 0.06 **(5)**	1.1 **(5)**	1.4 **(4)**	0.19 **(2)**	1.05 **(3)**	8.25 **(5)**
Q. robur	9.1 ± 1.4 **(2)**	1.4 **(3)**	1.4 **(4)**	0.07 **(5)**	1.14 **(2)**	100 **(1)**
S. madagascariensis	0.35 ± 0.01 **(6)**	0.1 **(6)**	3.5 **(3)**	0.24 **(1)**	0.14 **(5)**	4.55 **(6)**
A. marina	51.4 ± 2.2 **(1)**	5.1 **(1)**	0.3 **(6)**	0.06 **(6)**	20 **(1)**	50 **(2)**
T. emetica	4.8 ± 0.7 **(4)**	1.4 **(3)**	8 **(1)**	0.18 **(3)**	0.12 **(6)**	8.33 **(4)**
T. dregeana	6.4 ± 0.4 **(3)**	1.9 **(2)**	7.4 **(2)**	0.18 **(3)**	0.22 **(4)**	10.0 **(3)**

2.4 Discussion

According to Pammenter *et al.* (2002) and Sun (2002), the loss of water from tissues depends, among other factors, on the hydraulic conductivity of the tissue which is dependent on permeability of the tissue which, in turn, is affected by the chemical composition, and surface area to volume ratio which is influenced by the size and shape of the tissue and the amount of material to be dried. The results of the present study are in good agreement with the ideas advanced by those authors.

Generally, bigger axes dried more slowly than smaller ones (Table 2.1). In addition, the larger the quantity that was dried, the slower the drying rate attained. Indeed, it appears that the total amount of material dried was more influential on the drying rate than the size of the individual axis. Furthermore, oily seeds dried faster than their starchy counterparts except for *S. madagascariensis*.

The relationship between water content and time fitted both the modified inverse and exponenial functions for each one species. However, the fit was better with the

modified inverse relationship during drying of axes of *P. sativum*, *Q. robur* and *T. emetica*. In contrast, a better fit obtained with the exponential function upon dehydration of *T. dregeana* and *A. marina* axes. The plot of water content against time fitted an exponential and a modified inverse function better during rapid and slow desiccation of axes of *S. madagascariensis*, respectively.

In this regard, it is noteworthy that it was suggested that, as a generalisation, if tissue is dried relatively slowly, the relationship between water content and drying time is exponential (Pammenter *et al.*, 2002). In the present study, this statement may be true for *A. marina* axes, which dried at the slowest rate, but is not for axes of *T. dregeana*, which attained the second fastest dehydration rate (Table 2.1). It is proposed that the mass of the indidvidual axes and, perhaps more importantly, the total mass of the material dried are better predictors of the kinetics of desiccation of excised axes than drying rate *per se*.

It should be remembered that the drying conditions of temperature and RH were similar during each drying or storage treatment for all species studied. As a result, any differences in drying or storage responses among species would be expected to be a consequence of differences in the hydraulic characteristics and surface area of the various species. In this regard, it is noteworthy that the rate of water loss could be described by an exponential and modified inverse function for all species investigated. It should be remembered that the vapour pressure of a system, which is linearly related to RH has an exponential relationship with its temperature and water potential (Wexler, 1997). In addition, the gradient in water potential between the tissue and the surrounding air drives the process of water loss.

It is apparent that both the initial water contents and the drying curves vary with season for species that were studied for more than one season (*Trichilia dregeana* and *Avicennia marina*), although the differences were marginal for *T. dregeana*. Recalcitrant seeds show marked differences in post-harvest behaviour within species both intra- and inter-seasonally (reviewed by Berjak *et al.*, 1996; Berjak and Pammenter, 1997a,b; Pammenter and Berjak, 1999; Berjak and Pammenter, 2004). For instance, inter-seasonal differences in germination capacity of *Aesculus hippocastanum* seeds were ascribed to differences in mean temperature during seed filling (Tompsett and Pritchard, 1998). Similarly, interseasonal differences in post-harvest behaviour of seeds such as initial water content and drying curves in the present investigation may

be attributed to seasonal changes in environmental factors such as mean annual rainfall and temperature.

The decrease in water content over time during slow drying of axes of *Avicennia marina* appeared linear. It is suggested that this observation is a result of a very low drying rate (β [constant of water loss] = 0.06). In this regard, it should be noted that as β approaches zero, the more linear the plot of the water content against time, in which $wc = \alpha \exp(-\beta t)$, becomes. *A. marina* axes are very large. Their large individual masses would have resulted in a greater amount of material dried for an equivalent number of axes during each run of every treatment and reduced the surface area-volume ratios. It is suggested that these two factors led to very low drying rates during slow dehydration of *A. marina* axes.

Axes of all species studied maintained a constant water content during wet storage. This response may be expected given that the surrounding air during storage was fully humidified and that there was no direct contact between stored axes and water as they were kept at a distance from it. Thus, both water loss to the surrounding air and water uptake by the axes were obviated.

In conclusion, considerably different drying rates were reached by the various species studied despite identical drying conditions. Bigger axes generally dried discernibly more slowly. This phenomenon is a result of the larger axes having lower surface area to volume ratios, which influence the process of water loss. In addition, oily axes dried noticeably faster than axes containing mainly carbohydrates. It is suggested that this event is a consequence of the exclusion of water from the predominantly hydrophobic environment. Furthermore, it appears that the mass of the indidvidual axes and the total mass of the material dried may affect the kinetics of desiccation of excised axes directly rather than through the drying rate.

Chapter 3

Biochemical, biophysical and physiological assessment of seed viability

3.1 Introduction

The primary function of seeds is to produce seedlings. Seeds must germinate to fulfil this function. Seed quality can therefore be equated to ability to germinate (germinability or viability). However, germination may be considered as activation of the embryo (e.g. Côme and Corbineau, 1990) on one hand, or establishment of the seedling, on the other. In addition, there is a lack of consistent relationship between laboratory germination tests and field emergence (Perry, 1973). The concept of seed vigour was developed in an attempt to rectify this anomaly. Vigour is simply defined as a concept describing several seed performance characteristics and is not a single measurable property (Perry, 1981).

The major challenge of seed vigour testing has been to find quantifiable parameters, associated with seed deterioration, which detect differences in performance potential among high germinating seed lots. A vigour test should provide a reproducible result which accurately describes the potential for rapid and uniform emergence under field conditions and/or describes the storage potential of a seed lot (reviewed by Hampton and Tekrony, 1995). Whilst many different vigour testing methods have been proposed, only very few are in international usage (Hampton, 1992). The primary difference between the evaluation of viability and vigour is that certain seed conditions, which are not critical in assessing seed viability, can be important in a seed vigour assessment (Hampton and Tekrony, 1995). Methods for the determination of quality of seeds fall into four basic categories: (1) biochemical, (2) biophysical, (3) ultrastructural and (4) physiological.

The main biochemical approaches used for assessing seed quality have been: (1) the tetrazolium (TZ) (staining) and (2) the (electrolyte) conductivity (of leachate) tests (reviewed by Pritchard, 1996). The TZ test has been used to estimate seed vigour as well as seed viability. Close attention is focused on all parts of the individual seed, in particular the internal condition of the embryo. The TZ test provides a rapid evaluation of the vigour of viable seeds and provides timely guidance concerning the extent and

the nature of seed quality problems during harvesting, conditioning, storage and distribution (AOSA, 1983; Moore, 1985). It is based on the observation that a colourless solution of 2,3,5-triphenyltetrazolium chloride interacts with reduction processes of living cells and accepts hydrogen from dehydrogenases. It forms a red, stable and non-diffusible substance called triphenyl formazan after reduction. The topographical tetrazolium test is an extension of the TZ test as described in Chapter 6 of the International Rules for Seed Testing (ISTA, 1996).

Although undoubtedly of value, there are difficulties associated with the TZ test: (1) extensive experience is needed in evaluating the seed as viable by staining in relation to germination, as the TZ test often overestimates germinability (e.g. Ntuli *et al.*, 1997), (2) the inconsistent application of the evaluation procedure between laboratories (e.g. barley seed quality testing at seven ISTA stations was found to differ [Don *et al.*, 1990]), (3) certain chemical pre-treatments have resulted in false positive scores for viability (e.g. glyphosphate treatment of barley plants can kill the seed but their staining pattern appears 'normal' [Don *et al.*, 1990]) and (4) the method of applying the tetrazolium test may also influence the interpretation of the desiccation response in recalcitrant seeds (e.g. rapid imbibition of dry split seeds of *Zizania palustris* can account for the loss of viability [Kovach and Bradford, 1992a]). The third difficulty may be of particular concern to the use of this test in recalcitrant seed storage studies utilising chemical pre-treatment to inhibit fungal or insect attack. Thus, use should be made of other complementary biochemical staining tests such as the indigo carmine test, as the TZ test can be unreliable at times (Hendry and Grimme, 1992).

The conductivity test provides measurement of the electrolytic conductivity of leachate from plant tissues. It was first recognised for seeds of several crop species by Hibbard and Miller (1928) (Hampton and Tekrony, 1995). This test has been used to identify seed lots that have high laboratory germination, but poor field emergence. Such seed lots have high electrolyte leakage and are classified as showing low vigour, while those with low leakage are considered to show high vigour.

Although conductivity measurements are usually made on bulk seed samples, equipment is available to determine electrolyte leakage from single seeds (e. g. Steere *et al.*, 1981; Hepburn *et al.*, 1984; Ntuli *et al.*, 1997). The conductivity test has the tremendous advantages of simplicity, rapidity and meets most of the requirements for a good vigour test (Hampton and Coolbear, 1990). It is based on the observation that

the integrity of cell membranes is different among seed lots of different vigour and can be measured indirectly as electrolyte leakage (Powell, 1988). However, a study on aged orthodox maize seeds which showed an increase in electrolyte leakage, which almost exclusively came from the embryo, demonstrated that the electrolyte content of the pericarp may interfere with quality testing by conductivity measurements (Bruggink *et al.*, 1991). Therefore, care must be exercised when applying this test to species for the first time. In addition, more specific chemical testing of the leachate has been performed. For example, Fu *et al.* (1990) measured the amounts of soluble sugars.

The most reliable indicator of seed quality is the germination test (Pritchard, 1996) although there may be a lack of consistent relationship between laboratory germination tests and field emergence (Perry, 1973). However, this method and its evaluation can be critical when dealing with some dormant recalcitrant seeds such as *Zizania palustris* (Kovach and Bradford, 1992b) and horse chestnut (Pritchard and Tompsett, 1995).

Germination rate may provide the first indication of stress in seeds. For instance, mango seeds exhibit a reduced vigour index before there is any noticeable fall in germination percentage (Fu *et al.*, 1990). In contrast, seed vigour and germination percentage and/or rate may increase during the early stages of drying of recalcitrant seeds (e.g. in *Avicennia marina* [Berjak *et al.*, 1989], lychee and longan [Xia *et al.*, 1992] and horse chestnut [Pritchard and Tompsett, 1995]). However, there is usually a strong correlation between germination percentage and vigour in both orthodox (Ellis and Roberts, 1980) and (partially) desiccated recalcitrant (Pritchard *et al.*, 1995b) seeds.

Characterisation of the germination response may also be valuable in identifying optimal conditions for the storage of recalcitrant seeds. Recalcitrant seeds often progress from development to germination without the need for a quiescent phase. As a result, it has been hypothesised that the rate of germinative metabolism is linked to storage lifespan (Pammenter *et al.*, 1994; 1997; Motete *et al.*, 1997). Indeed, an association has been observed between the minimum temperature for germination and that suitable for short-term storage in the chilling-tolerant seeds of *Araucaria hunsteinii* (Pritchard *et al.*, 1995a). Considerable viability was also retained over a 3-year period when horse chestnut seeds were maintained at a temperature which was not conducive to dormancy breakage and germination (Pritchard, 1996).

Embryo culture techniques could be employed in the event that the germination response is known to be protracted and there is a need to assess growth potential, rather than biochemical and/or biophysical quality, in a relatively short time period. This approach may be particularly important for seeds with hard endocarps such as palms. Moreover, embryonic axis culture underpins attempts to develop a routine cryopreservation method for embryonic axes of recalcitrant seeds.

In conclusion, it is recommended that the biochemical and biophysical tests should not be used in isolation, but in association with others and/or the germination test, where possible. This recommendation arises from the uncertainties associated with some of them and the fact that most, if not all, of the biochemical and biophysical tests measure a single aspect of seed quality.

The objective of the work described in the present chapter was to assess the effects of drying rate and wet storage on the viability and vigour of axes of different recalcitrant species and germinating pea seeds.

3.2 Materials and methods

3.2.1 Seed material

Excised embryonic axes of *Trichilia dregeana*, *Pisum sativum*, *Avicennia marina*, *Quercus robur*, *Trichilia emetica* and *Strychnos madagascariensis*, which were obtained and treated as described in the previous chapter, were used for the tests outlined below.

3.3.2 Surface-sterilisation protocols

Excised axes from seeds of tropical species (*S. madagascriensis*, *T. emetica*, *T. dregeana* and *A. marina*) and from *P. sativum* were surface-sterilised in commercial bleach diluted to 1% sodium hpochlorite containing a drop of Tween-80 in a laminar flow cabinet for 15 min. Axes of *Q. robur* were surface-sterilised in 3.2g dichloroisocyanurate / 100ml H_2O containing a few drops of nonidet in a laminar flow cabinet for 6 min. They were then all rinsed three times with sterile distilled water.

3.3.3 Pre-moistening of dehydrated axes

To minimise the effects of imbibitional injury, dehydrated axes were moistened on damp filter paper in Petri dishes overnight at 20°C before being subjected to the tetrazolium, germination and conductivity tests. Stored axes were also pre-moistened to avoid introducing an extraneous factor.

3.2.4 Tetrazolium (TZ) tests

Apparent axis viability was determined by the tetrazolium test. Twenty pre-moistened axes were cut through longitudinally, soaked in 1% (w/v) 2,3,5-triphenyltetrazolium chloride solution for 24 h in the dark at 20°C, and scored using intensity and location of staining as criteria (International Seed Testing Association, 1999).

3.2.5 Germination tests

Axes of *T. dregeana*, *P. sativum*, *T. emetica* and *A. marina* were cultured in Petri dishes on half-strength Musharige and Skoog (Musharige and Skoog, 1962) medium supplemented with 0.3 g l^{-1} sucrose (photoperiod 16 h) under sterile conditions for a period of 20 days at room temperature. Those of *S. madagascariensis* were germinated in the dark and the medium for the germination of axes of *Q. robur* was further supplemented with 1 mg l^{-1} benzylaminopurine. In all cases, axes were scored as germinated when they showed greening, elongation, expansion, or a combination of these characteristics. They were all taken to be equivalent.

3.2.6 Electrical conductivity tests

Electrolyte leakage from five individual replicates of moistened axes of tropical seeds was measured using a multi-cell conductivity meter (CM100; Reid and Associates cc, Durban, SA) over 12 h. For *Q. robur*, a temperate species, leakage from ten replicates of individual moistened axes was measured using a compact conductivity meter (Cardy, C-172/173; Horiba Ltd, Kyoto, Japan) after leaching for 12 h. All measurements were made at 2 V whilst axes were immersed in 1 ml of distilled water except for the large *A. marina* axes where 3 ml of distilled water was used. Leakage was recorded as the highest reading over the measurement period. The results are

reported as means ± SE of five or ten individual axes. After the test, axes were dried and data expressed on a dry mass basis.

3.3 Results

3.3.1 TZ staining

Fresh embryonic axes of germinating *P. sativum* seeds showed 100% tetrazolium (TZ) staining (Figs 3.1A and B). It remained at 100% throughout rapid drying (Fig. 3.1A). However, a sharp decrease took place at *c.* 0.26 g g^{-1} dm during slow dehydration. Similarly, a gradual decline occurred after two weeks of wet storage (Fig. 3.2B).

Newly-shed *Q. robur* axes recorded 100% TZ staining (Figs 3.1C and D). Nonetheless, a marked reduction was seen at *c.* 0.26 and *c.* 0.8 g g^{-1} dm during rapid and slow drying, respectively (Fig. 3.1C). Total loss of viability was observed at *c.* 0.08 and *c.* 0.57 g g^{-1} dm upon rapid and slow dehydration, respectively. A gradual decrease took place immediately after wet storage (Fig. 3.1D) with loss of viability being complete after two weeks of wet storage.

TZ staining of fresh axes of *S. madagascariensis* was 100% (Figs 3.1E and F). Nonetheless, an abrupt decline occurred at *c.* 0.14 and *c.* 0.37 g g^{-1} dm during rapid and slow drying, respectively (Fig. 3.1E). Total loss of viability was seen at *c.* 0.37 g g^{-1} dm upon slow dehydration. Similarly, a marked reduction was observed after a week of wet storage (Fig. 3.1F) with loss of viability being complete after four weeks of hydrated storage.

Newly-shed *T. emetica* axes showed 100% tetrazolium staining (Figs 3.1G and H). However, a sharp decrease took place at *c.* 0.22 and *c.* 0.4 g g^{-1} dm during rapid and slow desiccation, respectively (Fig. 3.1G). Total loss of viability occurred at *c.* 0.15 g g^{-1} dm upon slow dehydration. Similarly, an abrupt decline in staining was seen after a week of wet storage (Fig. 3.1H). Loss of viability was complete after three weeks of hydrated storage.

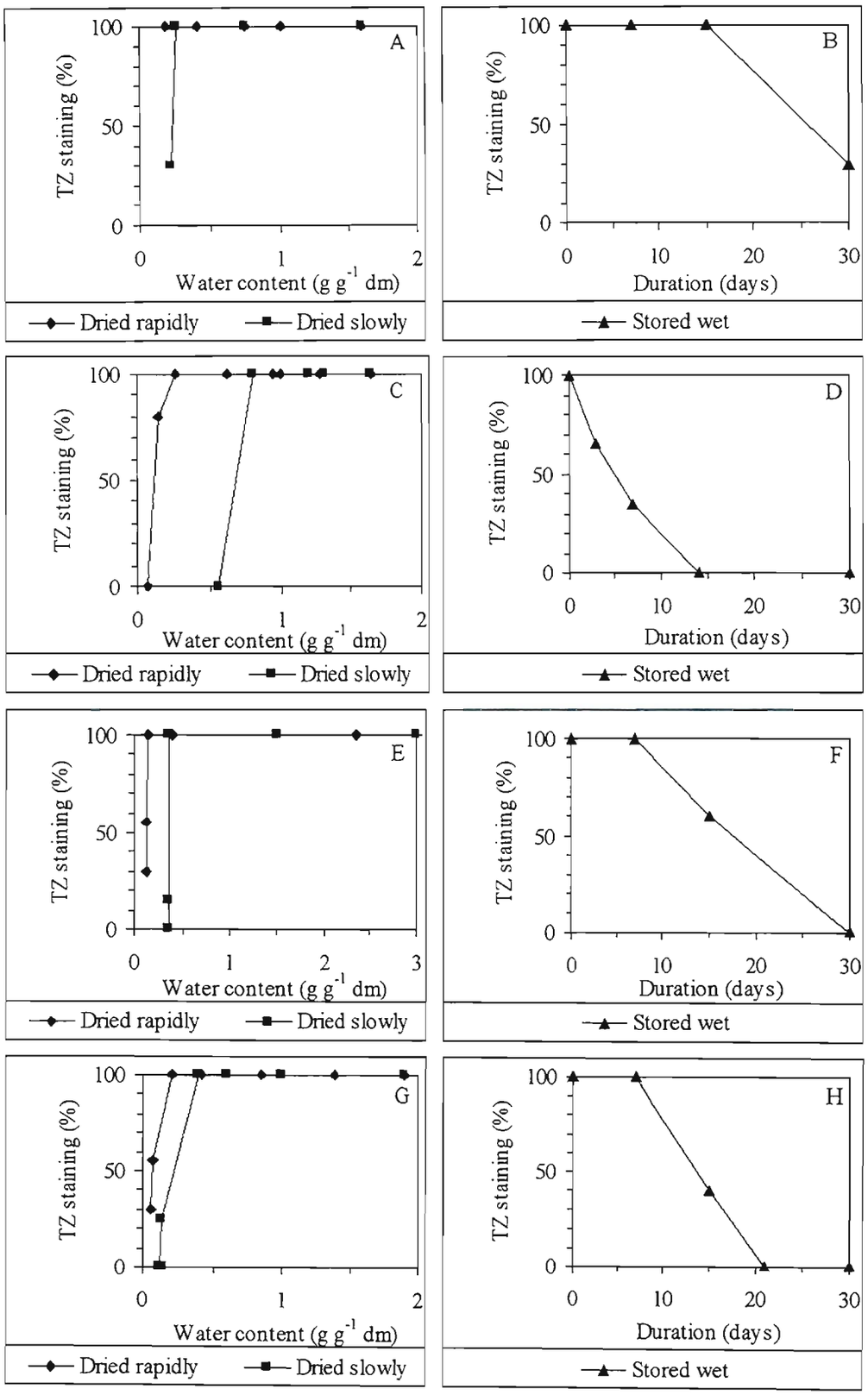

Figure 3.1 Level of tetrazolium (TZ) staining of axes of *P. sativum* (A-B), *Q. robur* (C-D), *S. madagascariensis* (E-F) and *T. emetica* (G-H) during drying at different rates or wet storage.

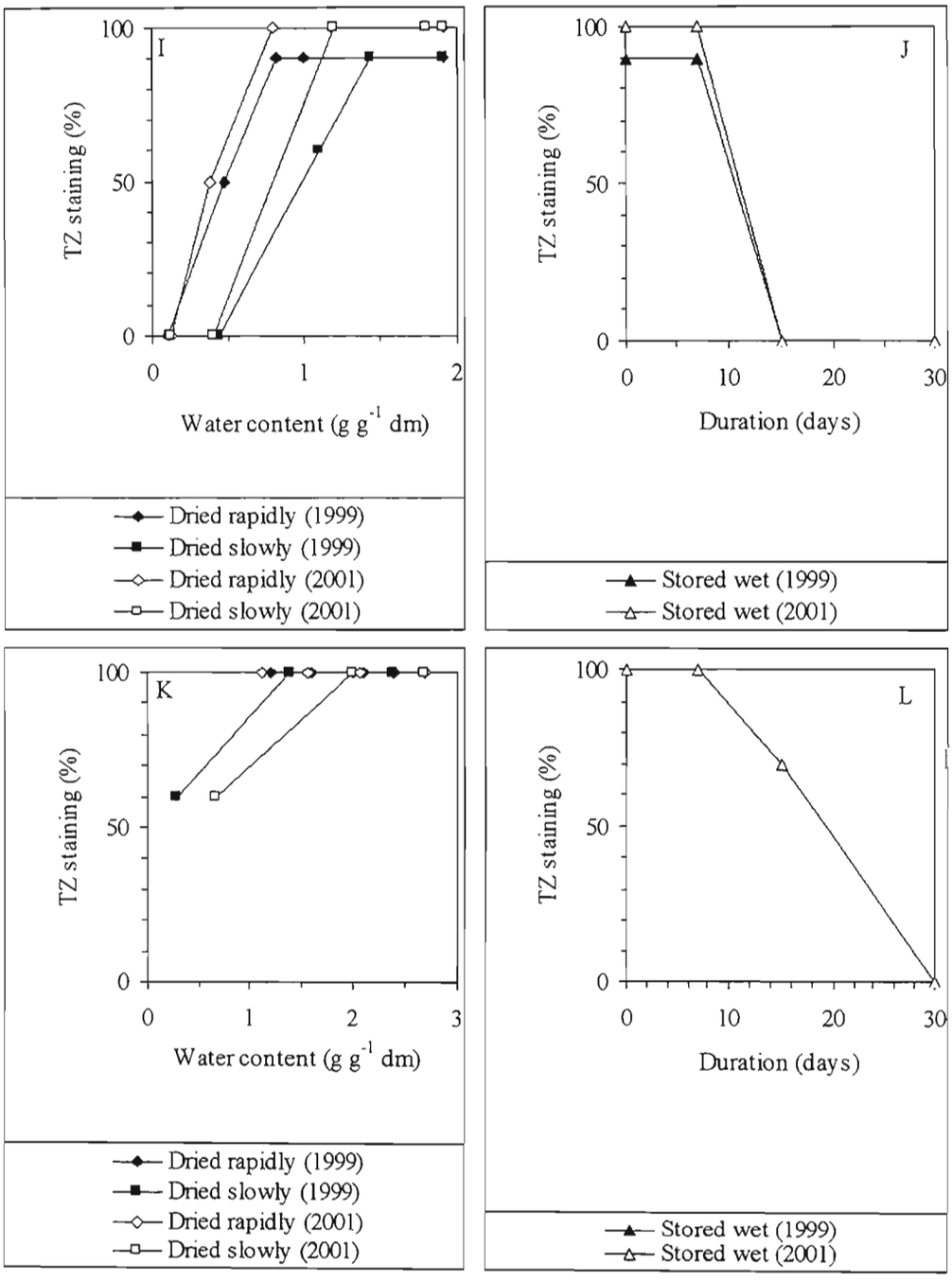

Figure 3.1 Level of tetrazolium (TZ) staining of axes of *T. dregeana* (I-J) and *A. marina* (K-L) harvested in 1999 (closed symbols) or 2001 (open symbols) during drying at different rates or wet storage. The storage curves of *A. marina* axes harvested in 1999 and 2001 were identical.

TZ staining of fresh axes of *T. dregeana* was 90% in axes from seeds collected in 1999 and 100% for those harvested in 2001 (Figs 3.1 and J). An abrupt decrease took place at *c.* 0.8 and *c.* 1.4 g g^{-1} dm during rapid and slow drying of axes from seeds collected in 1999, respectively (Fig. 3.1I). Similarly, a sharp decline took place at *c.* 0.8 and *c.* 1.4 g g^{-1} dm upon rapid and slow dehydration of axes from seeds collected in 2001, respectively. Total loss of viability occurred at *c.* 0.1 and *c.* 0.4 g g^{-1} dm during rapid and slow desiccation of axes from both harvests, respectively. Similarly, a marked decline was seen after a week of wet storage of axes from both harvests (Fig. 3.1J). Complete loss of viability was observed after two weeks of hydrated storage of axes from both harvests.

Fresh *A. marina* axes recorded 100% TZ staining (Figs 3.1K and L). It remained at 100% throughout rapid drying of axes from both harvests although the extent of drying was not marked – minimum value of 1.0 g g^{-1} dm (Fig. 3.1K). Nonetheless, a sharp decrease took place at *c.* 1.4 and *c.* 2.0 g g^{-1} during slow drying of axes collected from seeds harvested in 1999 and 2001, respectively. Similarly, a marked decline occurred after a week of wet storage of axes from both harvests (Fig. 3.1L). Total loss of viability was seen after four weeks of wet storage of axes from both harvests.

3.3.2 Germination

After various surface-sterilisation treatments, 100% germination of axes of *P. sativum* took place except after 2.5 min exposure to 0.1% mercuric chloride, which killed all the embryonic axes (Table 3.1). Nonetheless, axes showed different lengths, hence the choice of the surface-sterilisation treatment of 15 minutes exposure to 1% sodium hypochlorite in the present study for this species, which was inhibitory to growth but eliminated fungal proliferation.

The results of the germination test are generally in good agreement with those of the tetrazolium test. Fresh axes of *P.* sativum showed 100% germination (Figs 3.2A and B). Germination percentage remained at 100% throughout rapid dehydration (Fig. 3.2A). A sharp loss of germination took place at *c.* 0.26 g g^{-1} dm following slow drying. Germination percentage also decreased gradually after two weeks of hydrated storage (Fig. 3.8B).

Newly-shed *Q. robur* axes recorded 100% germination percentage (Figs 3.2C and D). However, a marked decline in germination totality occurred at *c.* 0.26 and *c.* 0.8 g g^{-1} dm during rapid and slow drying, respectively (Fig. 3.2C). Total loss of ability to germinate was seen at *c.* 0.08 and *c.* 0.57 g g^{-1} dm upon rapid and slow dehydration, respectively. Similarly, germination percentage decreased progressively during wet storage (Fig. 3.2D). Loss of the ability to germinate was complete after two weeks of hydrated storage.

Germination percentage of fresh axes of *S. madagascariensis* was 100% (Figs 3.2E and F) when kept in the dark. No germination was seen if axes were set out to germinate in the light. An abrupt reduction was observed at *c.* 0.37 and *c.* 0.6 g g^{-1} dm during rapid and slow drying, respectively (Fig. 3.2E). Total loss of ability to germinate took place at 0.14 and 0.37 g g^{-1} dm upon rapid and slow dehydration, respectively. Similarly, germination percentage decreased sharply after a week of wet storage (Fig. 3.2F). Loss of germination was complete after four weeks of hydrated storage.

Table 3.1 Germination of axes of *P. sativum* after different surface-sterilisation treatments. Lengths are shown as means of ten replicate readings. Variation indicates standard errors.

Treatment	Germination percentage (%)	Length of axes (mm)
None	100	13.6 ± 0.36
5 min 1% NaOCl	100	13.1 ± 0.41
10 min 1% NaOCl	100	12.3 ± 0.28
10 min 1% NaOCl + 1 drop Tween 80	100	11.6 ± 0.53
15 min 1% NaOCl	100	10.8 ± 0.21
20 min 1% NaOCl	100	10.4 ± 0.36
20 min 0.01% $HgCl_2$	100	8.6 ± 0.46
(+ 20min 1% NaOCl)		8.4 ± 0.56
2.5 min 0.1% $HgCl_2$	0	-

Newly-shed *T. emetica* axes showed 90% germination percentage (Figs 3.2G and H). A precipitous decrease in germination percentage took place at *c.* 0.42 and *c.* 0.6 g g^{-1} dm during rapid and slow drying, respectively (Fig. 3.2G). Total loss of ability to germinate occurred at *c.* 0.22 and *c.* 0.4 g g^{-1} dm upon rapid and slow dehydration, respectively. Similarly, germination declined markedly after a week of wet storage (Fig. 3.2H). Loss of germination was complete after four weeks of hydrated storage.

Germination percentage of fresh *T. dregeana* axes was 90% in axes from seeds collected in 1999 and 100% for those harvested in 2001 (Figs 3.2I and J). A sudden reduction was seen at *c.* 0.8 and *c.* 1.4 g g^{-1} dm during rapid and slow drying of axes collected in 1999, respectively (Fig. 3.2 I). Similarly, a sharp decrease was seen at *c.* 0.8 and *c.* 1.2 g g^{-1} dm during rapid and slow desiccation of axes collected in 2001, respectively. Total loss of germination was observed at *c.* 0.1 and *c.* 0.44 g g^{-1} dm upon rapid and slow drying of axes from both harvests, respectively. Germination percentage decreased precipitously after a week of wet storage (Fig. 3.2J). Loss of germination was complete after two weeks of hydrated storage of axes from both harvests.

Newly-shed *A. marina* axes recorded 100% germination percentage for both harvests (Figs 3.2K and L). A decline in germination percentage to 60% took place at *c.* 2.0 and 2.4 g g^{-1} dm during rapid and slow drying of axes from seeds harvested in 1999, respectively (Fig. 3.2K). Similarly, a reduction in germination percentage to 60% took place at *c.* 2.0 and 2.6 g g^{-1} dm during rapid and slow dehydration of axes from seeds harvested in 2001, respectively. Total loss of the ability to germinate occurred at *c.* 0.3 and *c.* 0.7 g g^{-1} dm upon slow desiccation of axes from seeds harvested in 1999 and 2001, respectively. Germination percentage decreased abruptly after a week of hydrated storage of axes from both harvests (Fig. 3.2L). Loss of germination was complete after two weeks of wet storage of axes from both harvests.

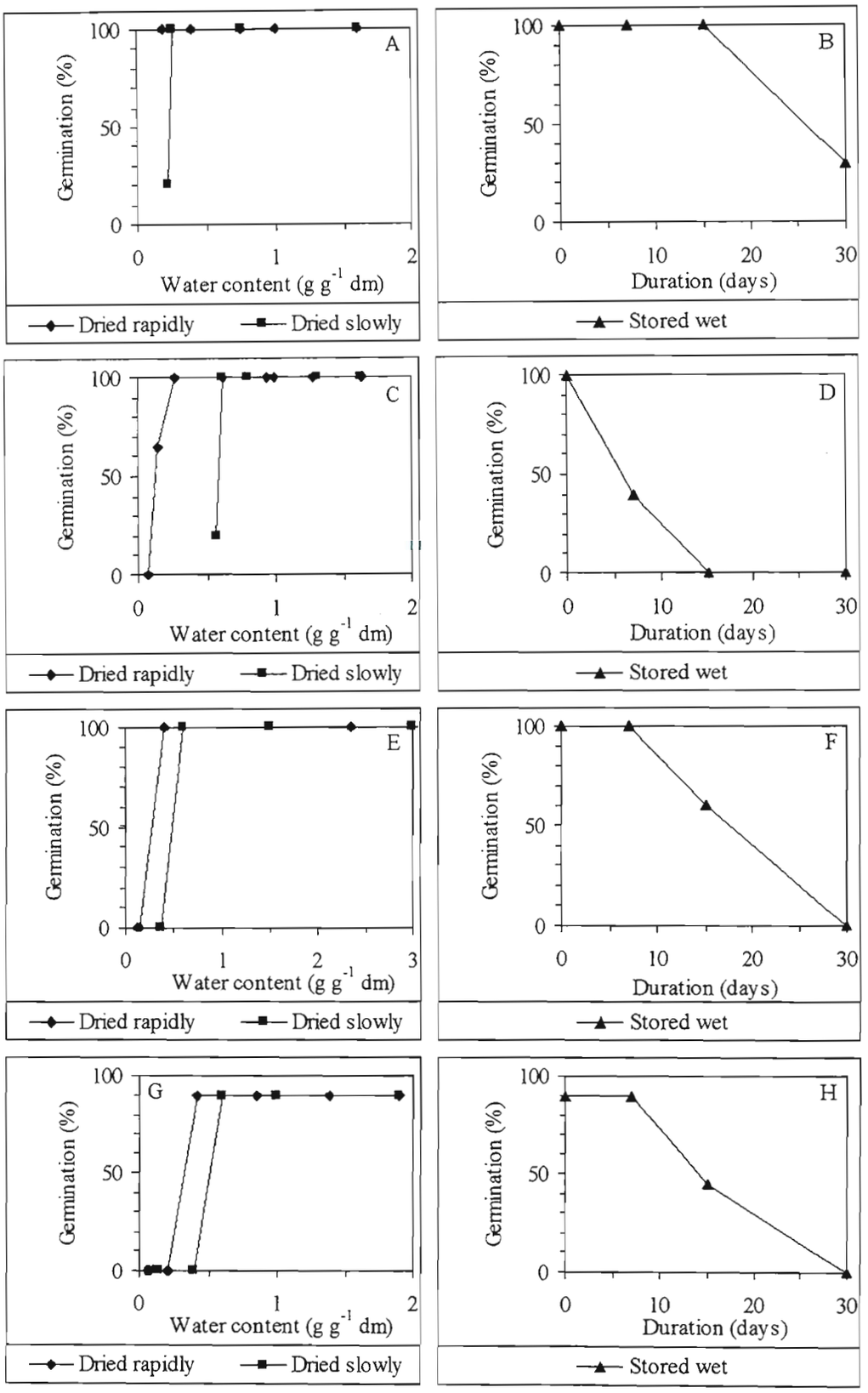

Figure 3.2 Germination of axes of *P. sativum* (A-B), *Q. robur* (C-D), *S. madagascariensis* (E-F) and *T. emetica* (G-H) during rapid or slow drying or wet storage.

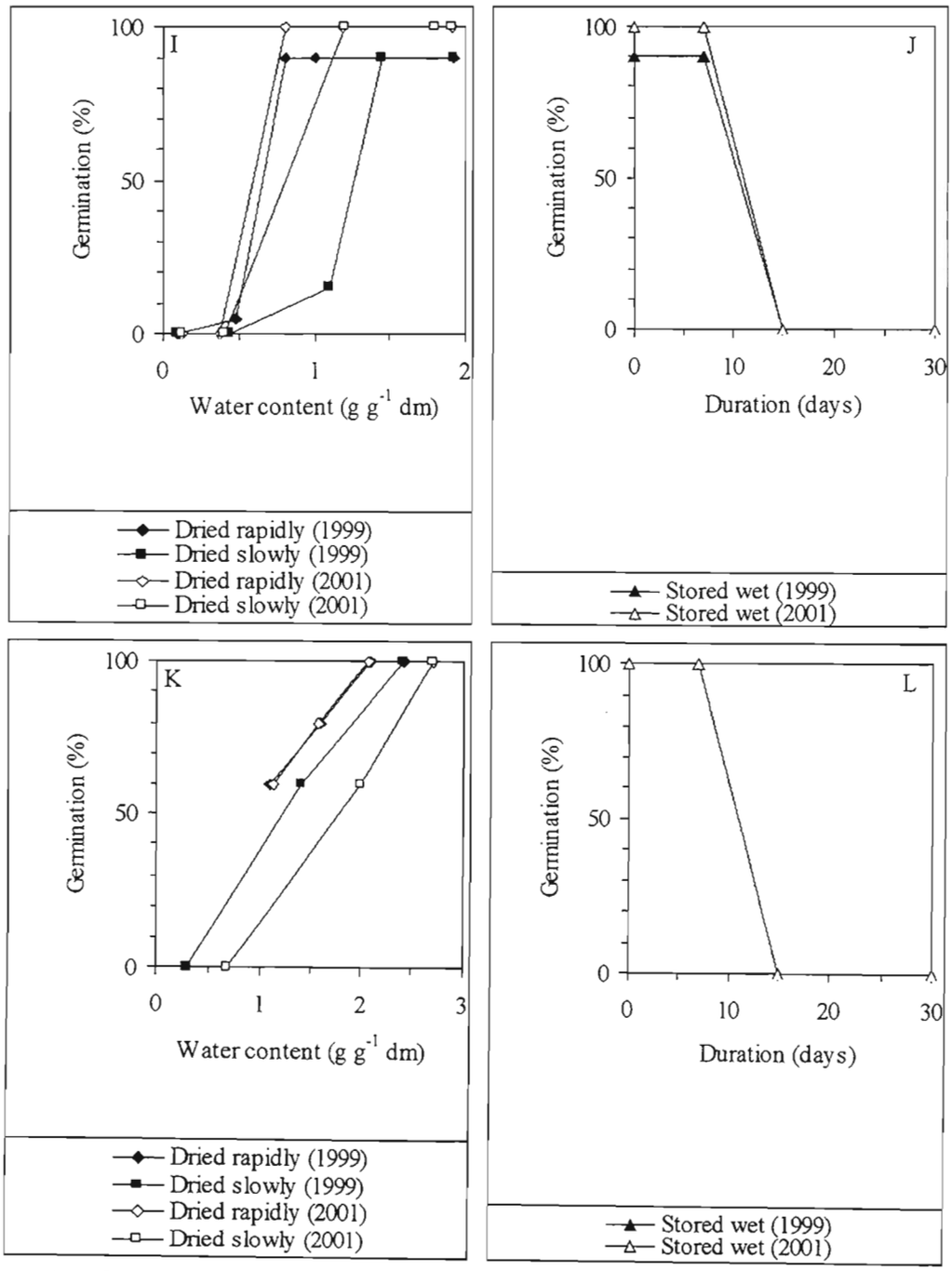

Figure 3.2 Germination of axes of *T. dregeana* (I-J) and *A. marina* (K-L) harvested in 1999 (closed symbols) or 2001 (open symbols) during rapid or slow drying or wet storage. The storage curves of *A. marina* axes harvested in 1999 and 2001 were identical.

Axes of the different species studied showed a wide range of 'critical water contents' (i. e. water contents at which viability decreased sharply) during drying (Table 3.2). Similarly, lifespans (prior to any loss of viability) of axes ranged from 0-15 days during wet storage. In addition, the 'critical water contents' varied greatly with the rate of drying within each species investigated such that they were lower upon rapid than slow dehydration. Furthermore, 'critical water contents' of axes of *A. marina*, *T. emetica* and *S. madagascariensis* were higher when assessed by *in vitro* culture in comparison to TZ staining. Moreover, the 'critical water content' was higher and storage lifespan was shorter than expected in *Q. robur* axes during slow drying and wet storage for both viability tests, respectively.

Table 3.2 'Critical water contents' and storage lifespans of axes of various species during drying at different rates or wet storage. Viability was assessed by *in vitro* culture and tetrazolium (TZ) staining. Where different from *in vitro* culture values, those of the TZ test are shown in brackets. – denotes no decrease in viability.

Species	**'Critical water content' (g g^{-1} dm)**		**Storage lifespan (days)**
	Rapid	Slow	
Pisum sativum	-	0.26	15
Quercus robur	0.27	0.8	0
Strychnos madagascariensis	0.37 (0.14)	0.6 (0.37)	7
Trichilia emetica	0.4 (0.22)	0.6 (0.42)	7
Trichilia dregeana	0.8	1.4	7
Avicennia marina	2.1 (-)	2.4 (1.4)	7

3.3.3 Electrolyte leakage

Leachate from fresh embryonic axes of *P. sativum* showed a mean electrolyte conductivity of *c.* 47 mS cm^{-1} g^{-1} dm after 12 h leakage. A gradual increase in electrolyte leakage took place upon drying and wet storage (Fig 3.3A and B). The highest conductivity readings of leachate of axes that were dried rapidly and slowly were *c.* 68.5 and *c.* 122 mS cm^{-1} g^{-1} dm, respectively. The material dried slowly recorded more leakage than that dried rapidly throughout the water content range monitored.

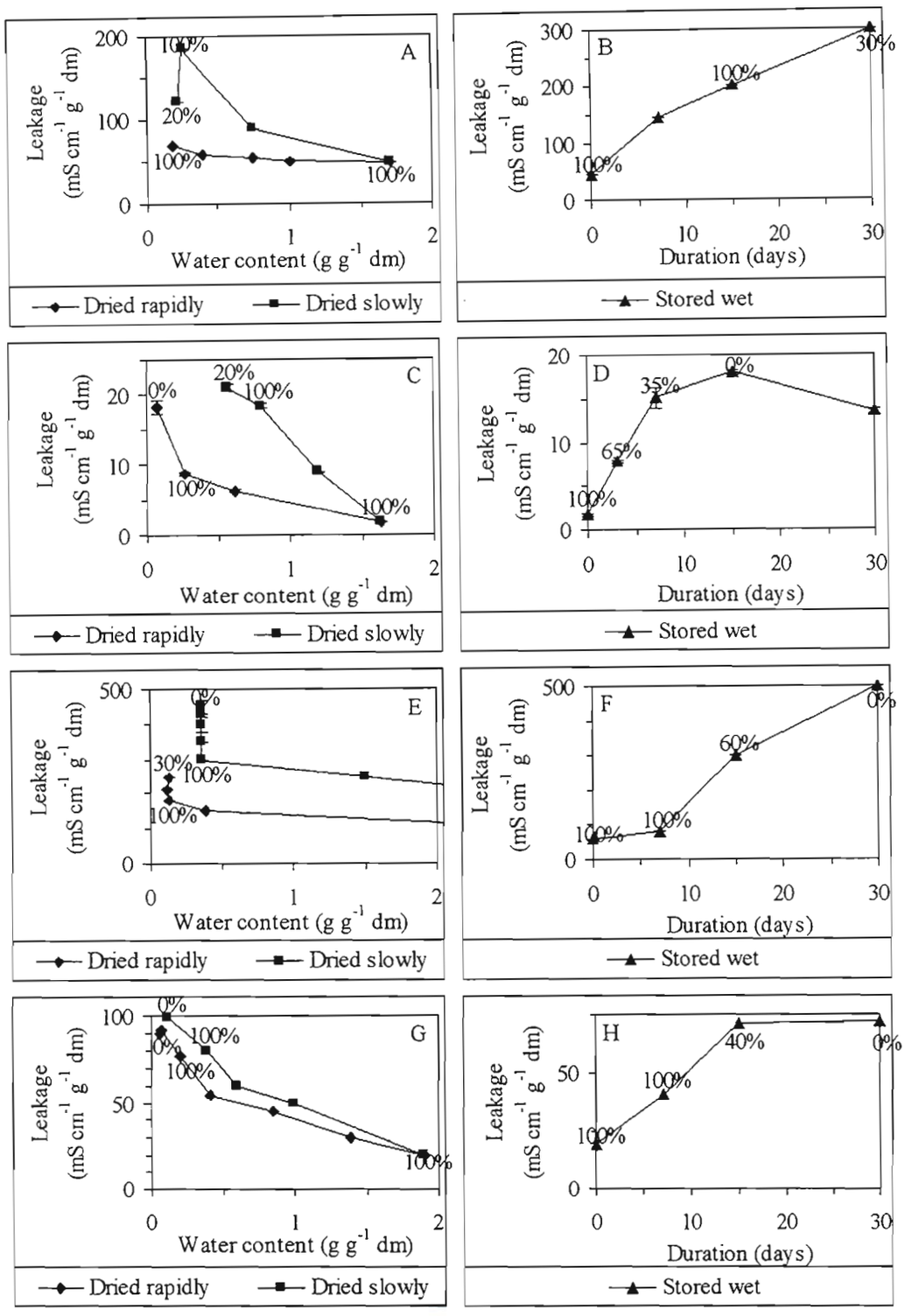

Figure 3.3 Pattern of electrolyte leakage of axes of *P. sativum* (A-B), *Q. robur* (C-D), *S. madagascariensis* (E-F) and *T. emetica* (G-H) during drying at different rates or wet storage. Data points represent means of ten replicate readings. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below or beside data symbols indicate germination. Data points without percentages are unchanged over the previous values.

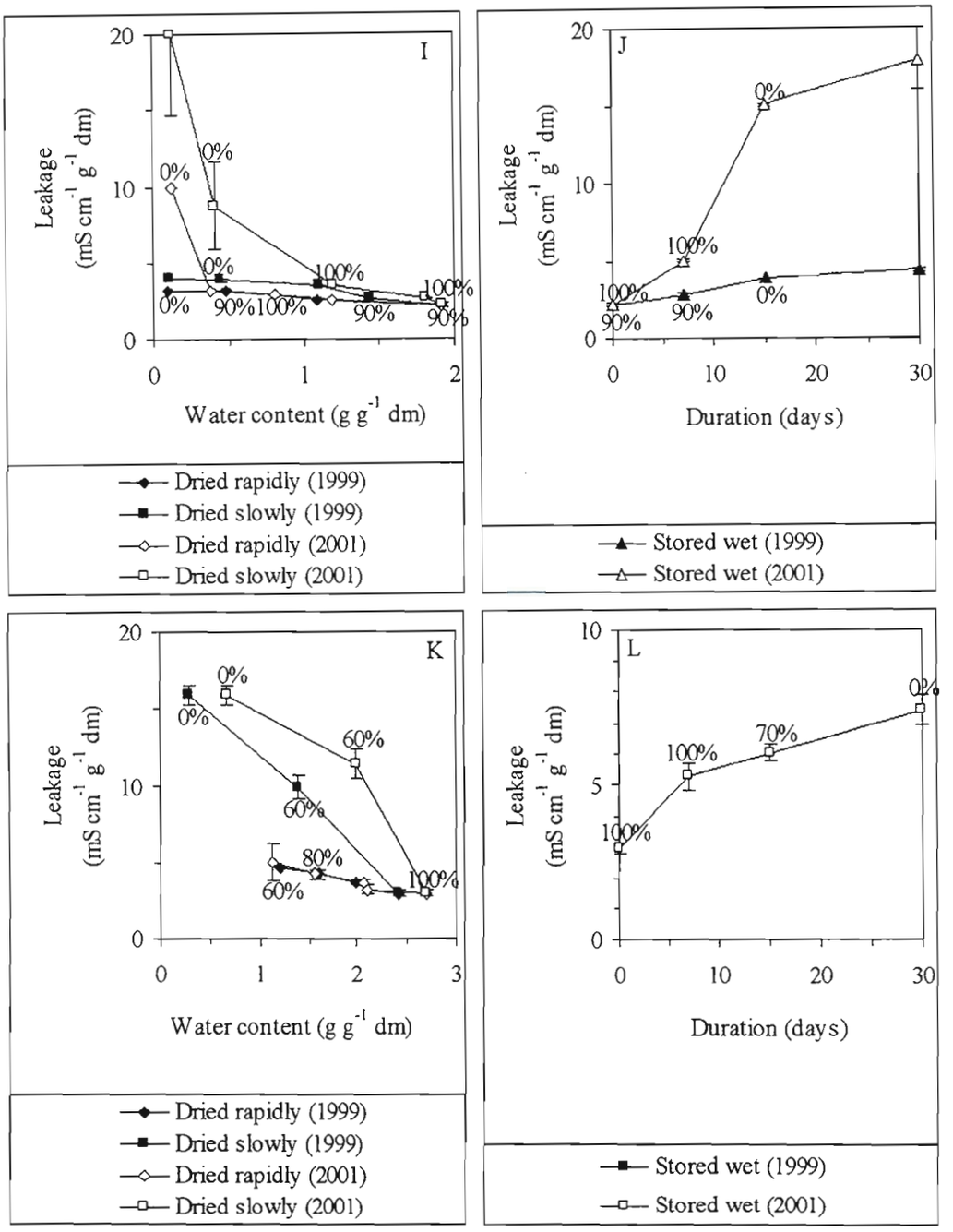

Figure 3.3 Pattern of electrolyte leakage of axes of *T. dregeana* (I-J) and *A. marina* (K-L) harvested in 1999 (closed symbols) or 2001 (open symbols) where applicable during drying at different rates or wet storage. Conductivity curves of *A. marina* axes harvested in 1999 and 2001 and stored wet overlapped.

A mean electrolyte leakage conductivity of *c.* 1.73 mS cm^{-1} g^{-1} dm was seen for newly-shed Q. *robur* axes after a 12 h measurement period. A progressive enhancement in electrolyte leakage occurred following dehydration and during hydrated storage (Fig 3.3C and D). The highest conductivity readings of leachate of axes that were dried rapidly and slowly were *c.* 18.3 and *c.* 21 mS cm^{-1} g^{-1} dm, respectively. The material dried slowly showed more leakage than that dried rapidly throughout the water content range monitored.

Leachate from fresh axes of *S. madagascariensis* demonstrated a mean electrolyte conductivity of *c.* 61 mS cm^{-1} g^{-1} dm after 12 h of leakage. A gradual elevation in electrolyte leakage was seen upon desiccation and moist storage (Fig 3.3E and F). The highest conductivity readings of leachate of axes that were dried rapidly and slowly were *c.* 250 and *c.* 450 mS cm^{-1} g^{-1}, respectively. The material dried slowly revealed slightly more leakage than that dried rapidly throughout the water content range monitored.

A mean electrolyte leakage conductivity of *c.* 20 mS cm^{-1} g^{-1} dm was recorded by newly-shed *T. emetica* axes after a 12 h measurement period. A progressive increase in electrolyte leakage was observed folowing drying and wet storage (Fig 3.3G and H). The highest conductivity readings of leachate of axes that were dried rapidly and slowly were *c.* 90.3 and *c.* 98.6 mS cm^{-1} g^{-1} dm, respectively. The material dried slowly recorded more leakage than that dried rapidly throughout the water content range monitored.

Leachate from fresh axes of *T. dregeana* revealed a mean electrolyte conductivity of *c.* 2.2 mS cm^{-1} g^{-1} dm over 12 h of leakage. A gradual enhancement in electrolyte leakage took place upon dehydration and hydrated storage (Fig 3.3I and J). The highest conductivity readings of leachate of axes that were dried rapidly and slowly were *c.* 3.2 and *c.* 3.8 mS cm^{-1} g^{-1} dm, respectively. The material dried slowly showed more leakage than that dried rapidly throughout the water content range monitored. In addition, material harvested in 2001 exhibited the classic pattern of a marked increase in electrolyte leakage at a particular water content, but this was not the case for the 1999 material.

A mean electrolyte leakage conductivity of *c.* 2.9 mS cm^{-1} g^{-1} dm was observed for fresh of *A. marina* axes after a 12 h measurement period. A progressive elevation in electrolyte leakage occurred following desiccation and moist storage (Fig 3.3K and L).

The highest conductivity readings of leachate of axes that were dried rapidly and slowly were *c.* 4.5 and *c.* 15.9 mS cm^{-1} g^{-1} dm, respectively. The material dried slowly demonstrated more leakage than that dried rapidly throughout the water content range monitored.

3.4 Discussion

Axes of *P. sativum* showed 100% survival after surface sterilization with 0.01% mercuric chloride for 20 minutes but none with 0.1% for 2.5 minutes. It appears that the higher (0.1%) concentration of mercuric chloride, which is in itself a relatively low concentration, is lethal to the rather robust *P. sativum* axes. It may be concluded that mercuric chloride is lethal not only to the contaminating microorganisms, but to the axes themselves. As a result, sodium hypochlorite was used as a surface-sterilisation agent for this species. Coincidentally, this sterilisation protocol is the method of choice for various species in the Plant Cell Biology Research Laboratory.

No germination was seen if axes of *S. madagascariensis* were set out to germinate in the light. Thus, it is suggested that darkness is a necessary condition for the *S. madagascariensis* axes to germinate *in vitro*.

Like in the previous studies (e.g. Ntuli *et al.*, 1997; reviewed by Pammenter *et al.*, 2002), the TZ test overestimated the viability during drying of axes of *A. marina*, *T. dregeana* and *S. madagascariensis* in comparison to the germination test. This discrepancy is usually attributed to the need for extensive experience of the evaluation of each species (reviewed by Pritchard, 1996). In contrast, the results of the TZ test of *P. sativum*, *Q. robur* and *T. dregeana* axes were in good agreement with those of *in vitro* culture.

Similarly, the findings of the germination and TZ tests of all species studied were in complete concurrence with regards to lifespans during wet storage. Hence, it appears that the TZ test may be a better indicator of viability during hydrated storage than it is of drying damage. It is suggested that the longer period of exposure and consequent longer measuring intervals during moist storage than dehydration may explain this anomaly. Nonetheless, lifespans during wet storage seem a poorer discriminator of the degree of desiccation sensitivity than 'critical water contents' during drying. Alternatively, this phenomenon may also be a result of longer measuring intervals. In

this regard, it is worth remembering that moist storage is equivalent to long-term and low-intensity water-stress.

In addition, it appears that the survival of axes of *Q. robur* during slow drying and moist storage was poorer than expected. It is proposed that this discrepancy is a result of the high temperature (20 °C) at which these treatments were carried out for this temperate species. However, temperature appeared not to be a major factor influencing longevity upon rapid dehydration presumably because of the limited time span of exposure to this procedure (24 h). Consequently, rapid desiccation is recommended as a method of choice to determine the 'critical water contents' accurately.

No loss of viability took place during rapid drying of axes of *P. sativum*. In contrast, viability loss occurred at low (≤ 0.5 g g^{-1} dm) water contents (*sensu* Vertucci, 1990) upon slow dehydration of *P. sativum* axes. As a result, it is suggested that metabolic disruption rather than desiccation damage (*sensu stricto*) underlied viability loss during slow desiccation of axes of *P. sativum* as removal of water did not kill the axes. It is proposed that metabolic damage may play a major role in loss of viability of *P. sativum* axes during wet storage.

Viability loss was seen mainly at intermediate (between 0.5 and 0.9 g g^{-1} dm) and low (≤ 0.5 g g^{-1} dm) water contents (*sensu* Vertucci, 1990) during slow and rapid drying of axes of *Q. robur*, *S. madagariensis* and *T. emetica*, respectively. Thus, it may be concluded that metabolic disruption is the major cause of loss of viability upon slow dehydration and desiccation damage (*sensu stricto*) brought about viability loss during rapid dehydration of axes of these species, respectively. It is suggested that metabolic damage may play a significant role in viability loss of *Q. robur*, *S. madagariensis* and *T. emetica* axes during wet storage.

Loss of viability was observed primarily at high (≥ 0.9 g g^{-1} dm) and intermediate (between 0.5 and 0.9 g g^{-1} dm) water contents (*sensu* Vertucci, 1990) during slow and rapid drying of axes of *T. dregeana*, respectively. Consequently, it may be concluded that physical and metabolic damage underlied viability loss upon slow and rapid dehydration of *T. dregeana* axes, respectively. It is proposed that metabolic damage may also play a major role in loss of viability of axes of *T. dregeana* during wet storage as there was no loss of water.

Viability loss occurred predominantly at high (≥ 0.9 g g^{-1} dm) water contents (*sensu* Vertucci, 1990) during drying of axes of *A. marina*. Hence, it appears that physical damage was a major cause of loss of viability upon dehydration of *A. marina* axes. It is suggested that metabolic damage also played a significant role in viability loss of axes of *A. marina* during wet storage.

In the present study, the relationship between electrolyte leakage and water content during drying and wet storage of axes of *P. sativum*, *Q. robur*, *S. madagascariensis*, *T. emetica*, *T. dregeana* and *A. marina* did not show the typical pattern in which leakage remains relatively constant to a 'critical water content', at which point a sharp increase is observed. Rather, there was a gradual increase in leakage as dehydration proceeded. Pammenter *et al.*, (1998) demonstrated a similar trend during desiccation of whole seeds of *Ekebergia capensis*. It is suggested that progressive deterioration of cellular membranes took place upon desiccation and hydrated storage of *P. sativum*, *Q. robur*, *S. madagascariensis*, *T. emetica*, *T. dregeana* and *A. marina* axes possibly as a result of oxidative attack.

However, less leakage occurred during rapid than slow drying of axes of all species investigated. It is proposed that less membrane damage was seen during rapid than slow dehydration because of the shorter period axes were subjected to stress during rapid in comparison to slow desiccation.

In contrast, axes of *T. dregeana* harvested in 2001 showed the classic pattern. This apparent discrepancy, as with initial water contents, drying curves and viability, is attributed to interseasonal variability in post-harvest behaviour of recalcitrant seeds (reviewed by Berjak and Pammenter, 1997a,b; 2001; 2004).

In conclusion, it seems that desiccation-sensitive seeds can be divided into three categories on the basis of the predominant mechanism of loss of viability during drying: (1) minimally desiccation-sensitive seeds such as *P. sativum*, *Q. robur*, *S. madagascariensis* and *T. emetica* which died largely as a result of desiccation damage *sensu stricto*, (2) moderately desiccation-sensitive seeds such as *T. dregeana* which lost viability predominantly due to metabolic damage and (3) highly desiccation-sensitive seeds such as *A. marina* which were mainly killed by physical damage. Irrespective of the mode of dying, the effect of the rate of drying on viability was always apparent.

In addition, excised embryonic axes do not seem to survive for appreciable periods in wet storage in comparison to whole seeds. For instance, considerable viability was retained over a 3-year period when horse chestnut seeds were maintained at a temperature which was not conducive to dormancy breakage and germination (Pritchard, 1996). Similarly, coating of *A. marina* propagules with alginate, which reduced the rate of germinative metabolism, extended the storage lifespan of those seeds by a factor of three to four (Pammenter *et al.*, 1997).

Furthermore, it appears that vigour, as assessed by the germination test and tetrazolium staining, may play a role in determining whether the responses of axes of desiccation-sensitive seeds to desiccation and wet storage show a typical pattern or not. For example, axes of *T. dregeana* harvested in 1999, which did not display the classical pattern, demonstrated lower vigour in terms of germination percentage and level of tetrazolium staining than those harvested in 2001, which exhibited the typical pattern in the present study. It is suggested that this phenomenon is a result of the ability of the more vigorous axes to better withstand water-stress.

Chapter 4

Some biochemical studies on respiratory metabolism

4.1 Introduction

Water has physical properties that make it an ideal biological solvent. Therefore, it is hardly surprising that it plays many roles in cellular metabolism. Because it is virtually incompressible, it fills cells and, thus, provides structure. The fluid environment it provides allows the diffusion of substrates to active sites of enzymes. Hydrophilic and hydrophobic interactions stabilise macromolecular conformations and allow for sequestering of cellular constituents. Water is a reactant or product in many important reactions. It also serves as a protectant of macromolecular structure by inhibiting deleterious reactions through preventing interaction between molecules (reviewed by Vertucci and Farrant, 1995; Walters *et al.*, 2002).

To appreciate the important role that water plays in cellular metabolism, it may be necessary to look at the damage that plant cells incur as a result of water removal. However, cells may be more susceptible to the rate of desiccation rather than loss of water *per se* and hence can survive to lower water contents with rapid dehydration (compare Probert and Brierley, 1989 and Kovach and Bradford, 1992a; Pammenter *et al.*, 1991; 1998; 1999; Pritchard, 1991; Finch-Savage, 1992; Berjak *et al.*, 1993; Walters *et al.*, 2001; Wesley-Smith *et al.*, 2001). It is thought that when cells are held at intermediate moisture levels during slow dehydration, they may suffer damage because they are subjected to the stress of unbalanced metabolism for longer periods than when dried rapidly (Pammenter *et al.*, 1991; 1998; 1999; Walters *et al.*, 2001). In effect, inadequate time is allowed for aqueous-based deleterious reactions to occur when cells are dried rapidly (Pammenter *et al.*, 1991; 1998; 1999; Walters *et al.*, 2001).

Because of the many roles it plays in metabolism, water controls the level of metabolism in plant cells (Clegg, 1978; Hegarty, 1978; Adams and Rinne, 1980; McIntyre, 1987; Leopold and Vertucci, 1989; reviewed by Walters *et al.*, 2002). It has been postulated that minimum critical moisture levels are required for different metabolic processes to take place. These processes include, among others, germination

(Hegarty, 1978; McIntyre, 1987; Palit, 1987), embryogenesis (Adams and Rinne, 1980; Finkelstein and Crouch, 1986; Rosenberg and Rinne, 1986; Fischer *et al.*, 1988; Xu *et al.*, 1990; Galau *et al.*, 1991; Morris *et al.*, 1991), growth (Adams and Rinne, 1980; Saab and Obendorf, 1989), cell division (Adams and Rinne, 1980; Myers *et al.*, 1992) and respiration (Leopold and Vertucci, 1989).

The discrete changes in metabolic activity with water content have been hypothesized to be associated with discrete changes in the physical properties of water (Clegg, 1978; Rupley *et al.*, 1983; Bruni *et al.*, 1989; Leopold and Vertucci, 1989; Vertucci, 1989; 1990; 1992). This hypothesis is based on the observation that the characteristics of water change with the degree of hydration (Vertucci and Roos, 1990). Thus, water becomes progressively capable of fulfilling the particular functions required for specific metabolic processes with increasing levels of hydration. On the other hand, removal of water from cells with the consequent loss of certain properties essential for particular metabolic activities, results in the loss of the capability for those activities.

For instance, it has been shown that phosphofructokinase (PFK) and malate dehydrogenase (MDH) were slightly and mildly affected by dehydration, respectively while the glucose-6-phosphate dehydrogenase (G6PDH) and NADH dehydrogenases of NADH-ubiquinone (coenzyme Q) reductase (complex I) and NADH-cytochrome *c* reductase (complex IV) were extremely sensitive to desiccation in germinating maize (Leprince *et al.*, 1993a; 1994). In contrast, Carpenter *et al.* (1987) observed that PFK was highly sensitive to *in vitro* desiccation. Furthermore, the activity of succinate dehydrogenase was elevated in the short-term by rapid or slow drying of propagules of *Avicennia marina* (Farrant *et al.*, 1985).

More recently, Bettey and Finch-Savage (1996) have shown an enhancement during germination in cabbage seeds in the activities of four enzymes which have a potentially regulatory role in glycolysis (PFK, pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase, pyruvate kinase and phosphoenol pyruvate carboxylase) and one enzyme which dominates the regulation of the oxidative pentose phosphate pathway, G6PDH. As expected, the ageing treatments caused a delayed increase in respiration on imbibition. However, priming the seeds after ageing brought forward the increase. It also brought the increase closer to the control levels.

In this regard, it is noteworthy that the cause of the differences in sensitivity to desiccation between enzymes is unknown. It has been suggested that desiccation-induced disruption of the electron transport chain of mitochondrial and microsomal membranes between the ubiquinone pool and cytochrome oxidase may result in more leakage of electrons from the electron transport chain than normal, thus generating the accumulation of free radical species, which are associated with lipid peroxidation (Leprince *et al.*, 1990b; 1994; 1995b; Hendry *et al.*, 1992; Hendry, 1993). However, it remains unclear whether oxidative damage is a cause or consequence of tissue death (Hendry *et al.*, 1992; Hendry, 1993; reviewed by Leprince and Golovina, 2002).

In addition to studies that have investigated the activities of respiratory enzymes, a number of investigations have examined the effects of drying and germination on respiration in terms of O_2 consumption and CO_2 production (Farrant *et al.*, 1985; Salmen Espindola *et al.*, 1994; Corbineau *et al.*, 1997; Leprince *et al.*, 1998; 2000; Walter *et al.*, 2001). In general, whilst dehydration diminishes the rate of respiration, germination elevates respiration.

Further evidence for the suppression of respiratory metabolism with desiccation comes from studies which investigated the effects of drying on respiratory substrates (monosaccharides [such as glucose and fructose], disaccharides [such as sucrose] and oligosaccharides [such as stachyose]). For example, there was a decline in the levels of these compounds with the onset of maturation drying in developing pea (Rogerson and Matthews, 1977) and cabbage (Leprince *et al.*, 1994) seeds. Moreover, germination was associated with an increase in the levels of respiratory substrates in maize seeds (Leprince *et al.*, 1992). In contrast, Ntuli *et al.* (1997) showed higher levels of these substances in control (hydrated) than their experimental (dried) counterparts in seeds of wild rice. Interestingly, *Zizania palustris* seeds dried at the optimal (25 °C) temperature showed a sugar profile more similar to the control than the experimental material.

In addition, dehydration induced a decrease in ATP and ADP levels and an increase in AMP content in *Araucaria angustifolia* embryos (Salmen Espindola *et al.*, 1994; Corbineau *et al.*, 1997). Consequently, there was a decline in the (adenylate) energy charge which was calculated as (ATP+0.5ADP)/(ATP+ADP+AMP) according to

Atkinson (1968). Moreover, there was a delayed reduction in the adenylate pool (ATP+ADP+AMP).

Furthermore, it was shown that desiccation of *A. angustifolia* embryos is associated with a rapid loss of the activity of 1-aminoacylpropane 1-carboxylic acid (ACC) oxidase which oxidises ACC to ethylene (Salmen-Espindola *et al.*, 1994; Corbineau *et al.*, 1997). This effect is more pronounced in the cotyledons than in the axes.

The objective of the work reported in the present chapter was to determine if drying rate and/or wet storage differentially affects respiratory metabolism of embryonic axes of desiccation-sensitive seeds.

4.2 Materials and methods

4.2.1 Seed material

Excised embryonic axes of *Pisum sativum*, *Quercus robur*, *Trichilia dregeana* and *Avicennia marina* were subjected to the slow or rapid drying or wet storage protocols outlined in Chapter 2.

4.2.2 Respiratory enzyme assays

Phosphofructokinase (PFK) activity was determined according to Leprince *et al.* (1993a). Axes (*c.* 5 mg dry matter [dm]) were homogenised to a fine powder under liquid nitrogen using a pestle and mortar. Soluble proteins were extracted from the frozen powder in 5 ml of 50 mM Tris-HCl (pH 7.6) in the presence of 0.1% polyvinylpyrolidone (PVP) (Hofmann and Kopperschläger, 1982) and the homogenate centrifuged at 8 000 *g* for 5 min. An aliquot of 2 ml of the supernatant was then transferred to 1 ml of a mixture of 0.2 mM ethylenediaminetetraacetic acid, 2 mM fructose-6-phosphate, 5 mM $MgCl_2$, 0.6 mM ATP, 0.33 U ml^{-1} aldolase, 10 U ml^{-1} triose phosphate isomerase, 1 U ml^{-1} glycerophosphate dehydrogenase and 0.2 mM NADH in Tris (pH 7.6) buffer. Activity was monitored by measuring the formation of fructose-1,6-bisphosphate, indicated by a coupled NADH-dependent reduction of dihydroxyacetone phosphate to glycerol-3-phosphate, as the change in absorbance at 340 nm over 3 min.

Malate dehydrogenase (MDH) activity was monitored using the procedure of Leprince *et al.* (1993a). Axes (*c.* 5 mg dm) were homogenised to a fine powder under liquid nitrogen using a mortar and pestle. Soluble proteins were extracted from frozen

powder in 5 ml of 50 mM potassium phosphate buffer (pH 7.4) in the presence of 0.1% PVP. The homogenate was then centrifuged at 8 000 *g* for 5 min. An aliquot of 0.1 ml of the supernatant was added to 2 ml of 0.1 M phosphate buffer (pH 7.55) and 0.1 ml of 2 mg/ml NADH. After 10 min, 0.1 ml of 0.5 M oxaloacetate in 0.1 M phosphate buffer (pH 7.0) was added. Activity was determined by observing the change in optical density of NADH at 340 nm over a 3 min period.

4.2.3 NAD assay

Following homogenisation to a fine powder under liquid nitrogen in the mortar with a pestle, axes (*c.* 5 mg dm) were further homogenised in 5ml of 0.2 M HCl, heated in a boiling water bath for 5 min, cooled in an ice bath and centrifuged at 14 000 *g* for 10 min (Zhao *et al.* 1987). An aliquot of 0.5 ml of the supernatant was then transferred to 1.0 M Bicine-NaOH buffer (pH 8.0) and neutralised with 0.2 M NaOH in the dark (Matsumura and Miyachi, 1980). Following the addition of 0.1 ml each of 40 mM EDTA, 4.2 mM 3-(4,5 dimethyl-thiazoyl-2)-2,5-diphenyltetrazolium bromide (MTT), 16.6 mM phenol ethosulfate and 5.0 M ethanol, 0.1 ml of 500 U ml^{-1} alcohol dehydrogenase was added after 5 min at 37 °C. The level of NAD was determined by measuring the rate of reduction of MTT as absorbance at 570 nm after 30 min.

For all three assays, the results are reported as percentage changes (between the present and previous values) of the initial values of means of three replicate extractions. After each assay, axes were dried and data expressed on a dry mass basis. Due to shortage of plant material, assays were only conducted after 7 days during wet storage of axes of *Avicennia marina*.

4.2.4 Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) test. Where significant effects were found to occur, the Tukey multiple range test was subsequently used to identify where they occurred.

4.3 Results

4.3.1 Respiratory enzyme activities

a. PFK

No statistically significant changes in the activity of PFK were seen during rapid and slow drying and during wet storage of axes of *P. sativum* ($F = 1.30$ and $p = 0.46$, $F = 30.80$ and $p = 0.13$ and $F = 0.90$ and $p = 0.51$, respectively [Fig 4.1A and B]).

The onset of loss of germination was preceded by a statistically significant *c.* 15% increase in the activity of PFK in axes of *Q. robur* during rapid drying at *c.* 0.26 g g^{-1} dm ($F = 5.60$ and $p = 0.01$ [Fig 4.1C]). In contrast, a marginally significant decrease was observed upon slow dehydration ($F = 0.80$ and $p = 0.6$). During wet storage, a highly significant *c.* 40% enhancement in PFK activity accompanied germination loss ($F = 48.9$ and $p < 0.01$ [Fig. 4.1D]).

A highly significant *c.* 70% decrease in the activity of PFK in axes of *T. dregeana* took place before the onset of loss of germination during rapid drying at *c.* 0.8 g g^{-1} dm ($F = 9.80$ and $p < 0.01$ [Fig 4.1E]). Similarly, a statistically significant *c.* 45% decline in PFK activity in *T. dregeana* axes occurred at *c.* 1.4 g g^{-1} dm prior to the onset of germination loss upon slow dehydration ($F = 13.80$ and $p = 0.01$). Loss of germination was associated with a statistically significant *c.* 20% increase in the activity of PFK during rapid desiccation. During wet storage, a statistically significant *c.* 60% reduction in PFK activity preceded the onset of germination loss ($F = 42.90$ and $p < 0.01$ [Fig. 4.1F]). There was a further highly significant *c.* 90% post-mortem decrease in PFK activity after total loss of germinability upon hydrated storage ($F = 42.90$ and $p < 0.01$).

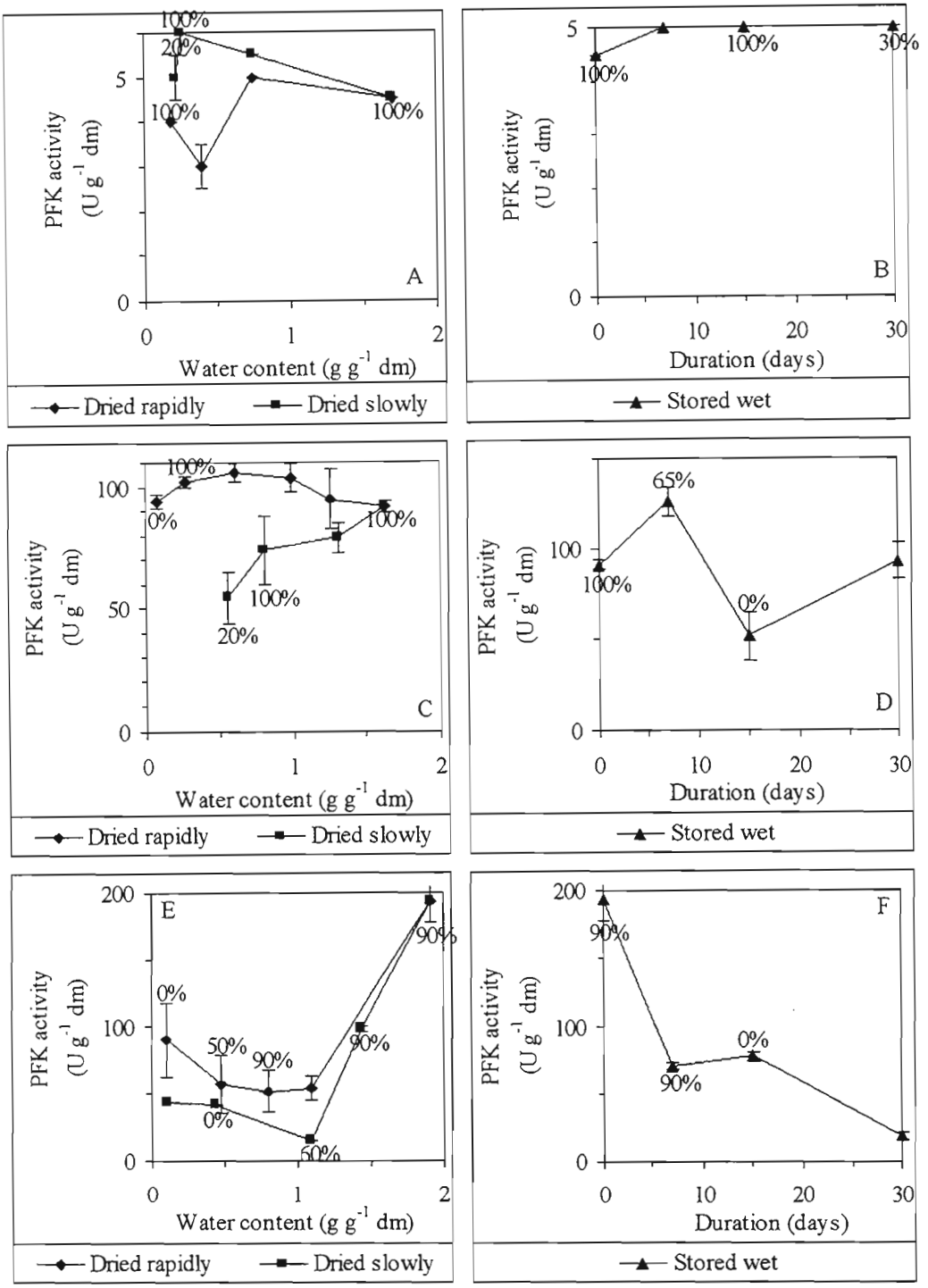

Figure 4.1 Activities of phosphofructokinase in axes of *P. sativum* (A-B), *Q. robur* (C-D) and *T. dregeana* (E-F) during drying at different rates or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below data symbols indicate germination. Data points without percentages are unchanged over the previous values. 1 U of PFK will convert 1 µmol of fructose-6-phosphate and ATP to fructose-1,6-bisphosphate and ADP per minute at pH 8.0 at 30 °C.

A statistically significant *c.* 70% decrease in the activity of PFK in whole axes of *A. marina* was seen during the initial 40% loss of germination at *c.* 1.1 g g^{-1} dm upon rapid drying (F = 1 585.70 and p = 0.02 [Fig 4.2A]). Similarly, a statistically significant *c.* 80% decline in PFK activity in whole axes was observed during the initial 40% loss of germination at *c. c.* 1.4 g g^{-1} dm upon slow dehydration (F = 113.32 and p = 0.01). During wet storage, there was no significant change in the activity of PFK in whole axes before the onset of loss of germination (F = 17.00 and p = 0.15 [Fig 4.2B]).

The activity of PFK in *A. marina* hypocotyls was highly significantly diminished by *c.* 83% during the initial 40% loss of germination upon rapid drying (F = 98.10 and p < 0.01 [Fig 4.2C]). Similarly, a marginally siginificant *c.* 30% decrease in PFK activity in hypocotyls was associated with 40% germination loss during slow dehydration (F = 16.70 and p = 0.06). Upon wet storage, a marginally significant *c.* 55% decrease in the activity of PFK in hypocotyls preceded the onset of loss of germination (F = 16.70 and p = 0.06 [Fig. 4.2D]).

There were no significant changes in the activity of PFK in root primordia of *A. marina* during rapid and slow drying (F = 1.7 and p = 0.35 and F = 1. 90 and p = 0.46, respectively [Fig 4.2E]). However, a marginally siginificant *c.* 73% decrease in PFK activity preceded loss of germination upon wet storage (F = 15.00 and p = 0.06 [Fig. 4.2F]).

No significant changes in the activity of PFK in *A. marina* plumules were associated with rapid and slow drying and wet storage (F = 0.20 and p = 0.13, F = 0.00 and p = 1.00 and F = 0.40 and p = 0.64, respectively (Fig 4.2G and H]).

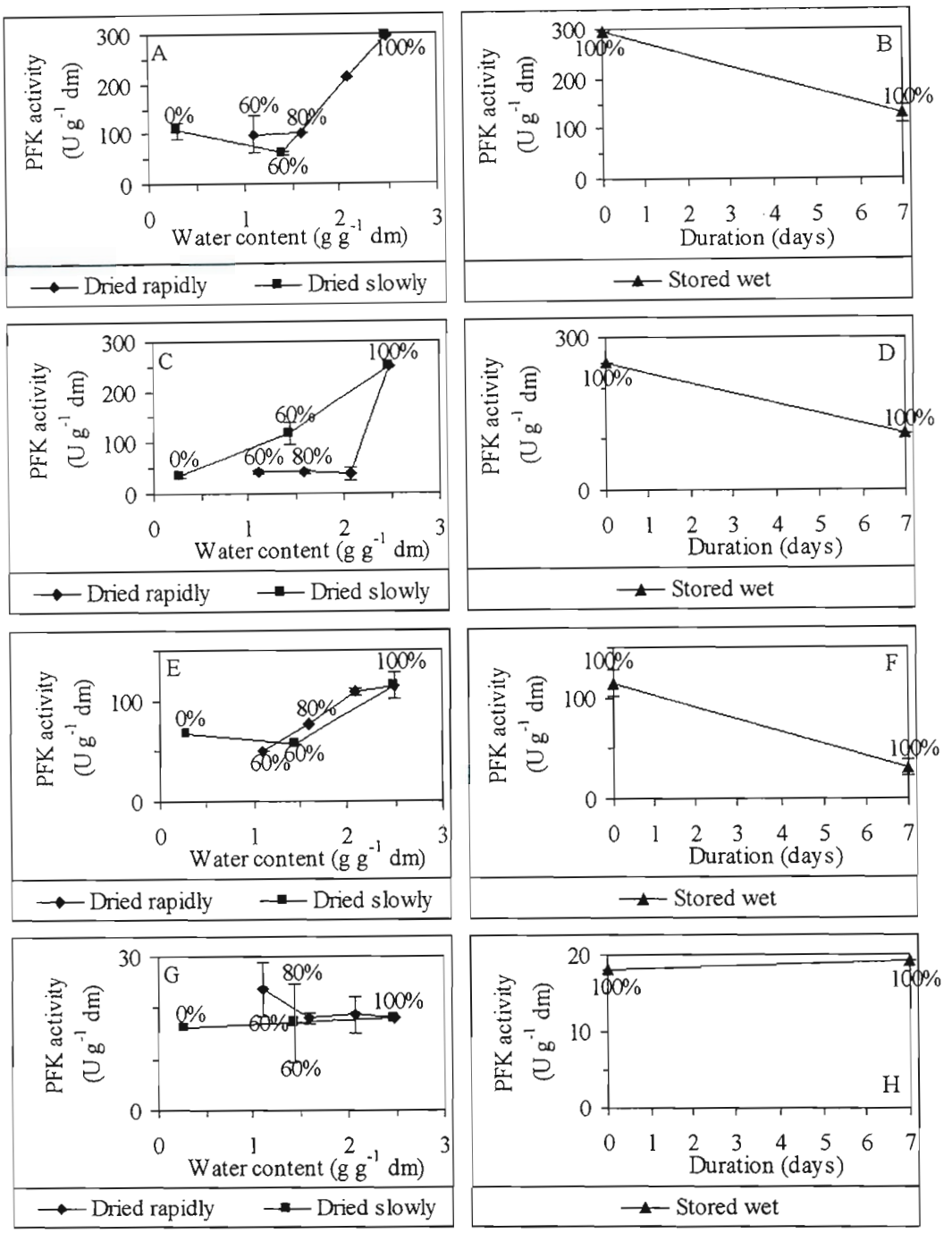

Figure 4.2 Activities of phosphofructokinase in whole axes (A-B) and different axial tissues (hypocotyls [C-D], root primordia [E-F] and plumules [G-H]) of *A. marina* during rapid or slow drying or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below data symbols indicate germination. Data points without percentages are unchanged over the previous values. 1 U of PFK will convert 1 μmol of fructose-6-phosphate and ATP to fructose-1,6-bisphosphate and ADP per minute at pH 8.0 at 30 °C.

b. MDH

Although changes in the activity of MDH in axes of *P. sativum* during rapid and slow drying and wet storage were considerable, they were not statistically significant ($F = 0.90$ and $p = 0.49$, $F = 1.50$ and $p = 0.33$ and $F = 1.70$ and $p = 0.26$ [Fig 4.3 A and B]).

A highly significant *c.* 50% increase in the activity of MDH in axes of *Q. robur* preceded the onset of loss of germination during slow dehydration ($F = 245.10$ and $p < 0.01$ [Fig. 4.3C]). Germination loss was associated with a statistically significant *c.* 80% enhancement of MDH activity upon rapid dehydration ($F = 106.00$ and $p = 0.01$). Similarly, a highly significant *c.* 120% elevation in the activity of MDH accompanied loss of germination during slow desiccation (and $F = 245.10$ and $p < 0.01$). There was a statistically significant c. 110% increase in MDH activity during germination loss upon wet storage ($F = 10.50$ and $p = 0.05$ [Fig. 4.3D]).

A highly significant *c.* 50% decrease in the activity of MDH in axes of *T. dregeana* was seen before the onset of loss of germination during slow drying ($F = 13.75$ and $p < 0.01$ [Fig. 4.3E]). Similarly, a statistically significant *c.* 60% decline in MDH activity was observed during germination loss upon rapid dehydration ($F = 22.80$ and $p < 0.01$). During wet storage, there were no significant changes in the activity of MDH ($F = 0.90$ and $p = 0.51$).

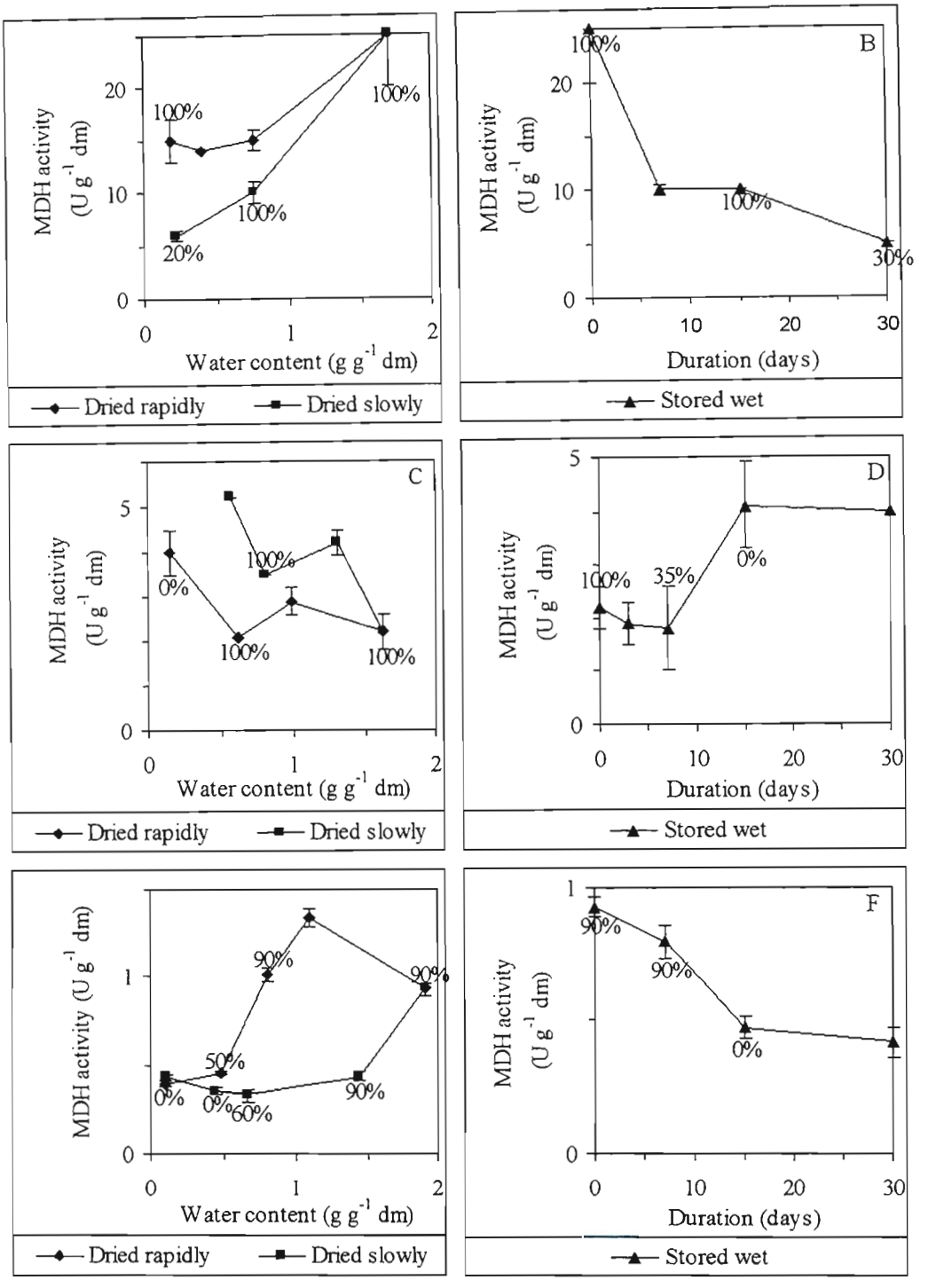

Figure 4.3 Activities of malate dehydrogenase in axes of *P. sativum* (A-B), *Q. robur* (C-D) and *T. dregeana* (E-F) during drying at different rates or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below data symbols indicate germination. Data points without percentages are unchanged over the previous values. 1 U of MDH will convert 1 µmol of oxaloacetate and NADH to malate and NAD per minute at pH 7.5 at 25 °C.

In whole axes of *A. marina*, a marginally significant *c.* 85% decrease in the activity of MDH was seen during the initial 40% loss of germination upon rapid drying ($F = 4.80$ and $p = 0.06$[Fig. 4.4A]). Similarly, a statistically significant *c.* 70% decline in MDH activity was observed during the initial 40% germination loss upon slow dehydration of whole axes ($F = 11.90$ and $p = 0.04$). During wet storage, a highly significant *c.* 85% reduction in the activity of MDH in axes preceded the onset of loss of germination ($F = 288.00$ and $p < 0.01$ [Fig. 4.4B]).

The activity of MDH in *A. marina* hypocotyls did not change significantly during rapid and slow drying and wet storage ($F = 2.20$ and $p = 0.23$, $F = 2.70$ and $p = 0.21$, and $F = 3.50$ and $p = 0.20$, respectively [Fig. 4.4C and D]). Similarly, there were no siginificant changes in MDH activity in root primordia of *A. marina* upon rapid and slow dehydration and hydrated storage ($F = 1.80$ and $p = 0.30$, $F = 0.50$ and $p = 0.65$ and, $F = 0.00$ and $p = 1.00$, respectively [Fig. 4.4E and F]). Also, the activity of MDH in *A. marina* plumules did not change significantly during rapid and slow desiccation and moist storage ($F = 0.01$ and $p = 1.00$, $F = 0.00$ and $p = 0.98$ and $F = 1.70$ and $p = 0.33$, respectively [Fig. 4.4G and H]).

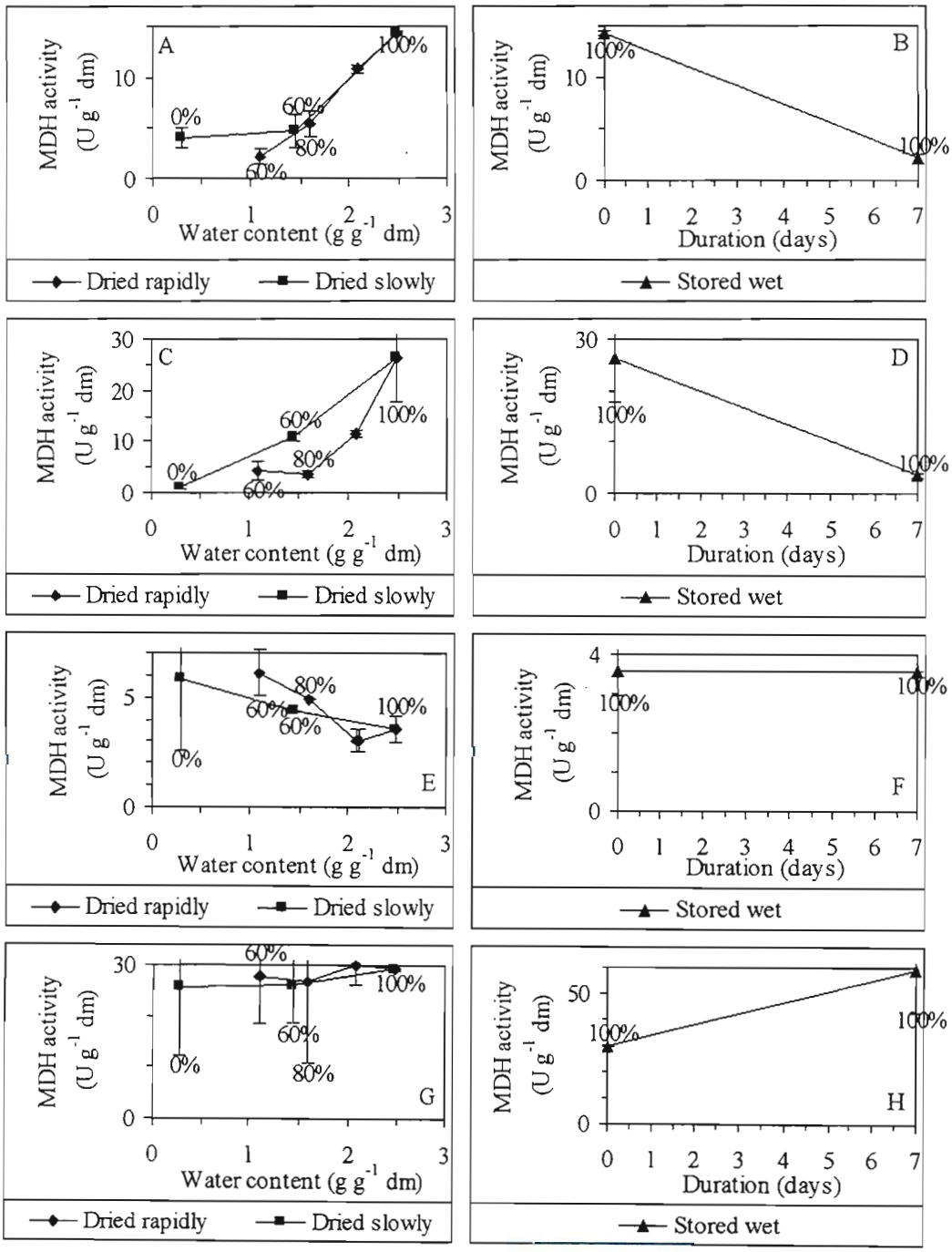

Figure 4.4 Activities of malate dehydrogenase in whole axes (A-B) and different axial tissues (hypocotyls [C-D], root primordia [E-F] and plumules [G-H]) of *A. marina* during rapid or slow drying or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below data symbols indicate germination. Data points without percentages are unchanged over the previous values. 1 U of MDH will convert 1 μmol of oxaloacetate and NADH to malate and NAD per minute at pH 7.5 at 25 °C.

4.3.2 NAD levels

A marginally siginificant *c.* 90% increase in the level of NAD occurred during rapid drying ($F = 4.50$ and $p = 0.09$ [Fig. 4.5A]). Germination loss was associated with a statistically significant *c.* 70% enhancement in NAD level as slow drying proceeded ($F = 3.50$ and $p = 0.01$). However, no siginicant change in the level of NAD accompanied wet storage ($F = 3.50$ and $p = 0.20$ [Fig. 4.5B]).

A marginally siginificant *c.* 50% decrease in the level of NAD was seen during rapid drying of axes of *Q. robur* ($F = 0.80$ and $p = 0.06$ [Fig. 4.5C]). Similarly, a statistically significant *c.* 60% decline in NAD level was observed in advance of the onset of loss of germination upon slow dehydration ($F = 5.64$ and $p = 0.01$). There was a marginally siginificant *c.* 86% reduction in the levels of NAD during germination loss upon wet storage ($F = 3.20$ and $p = 0.07$ [Fig. 4.5D]).

The levels of NAD in axes of *T. dregeana* did not change significantly during rapid and slow drying ($F = 2.60$ and $p = 0.17$ and $F = 0.90$ and $p = 0.5$, respectively [Fig. 4.5E]). Total loss of germinability was followed by a statistically significant *c.* 70% post mortem increase in NAD levels upon wet storage ($F = 23.40$ and $p < 0.01$[Fig. 4.5F]).

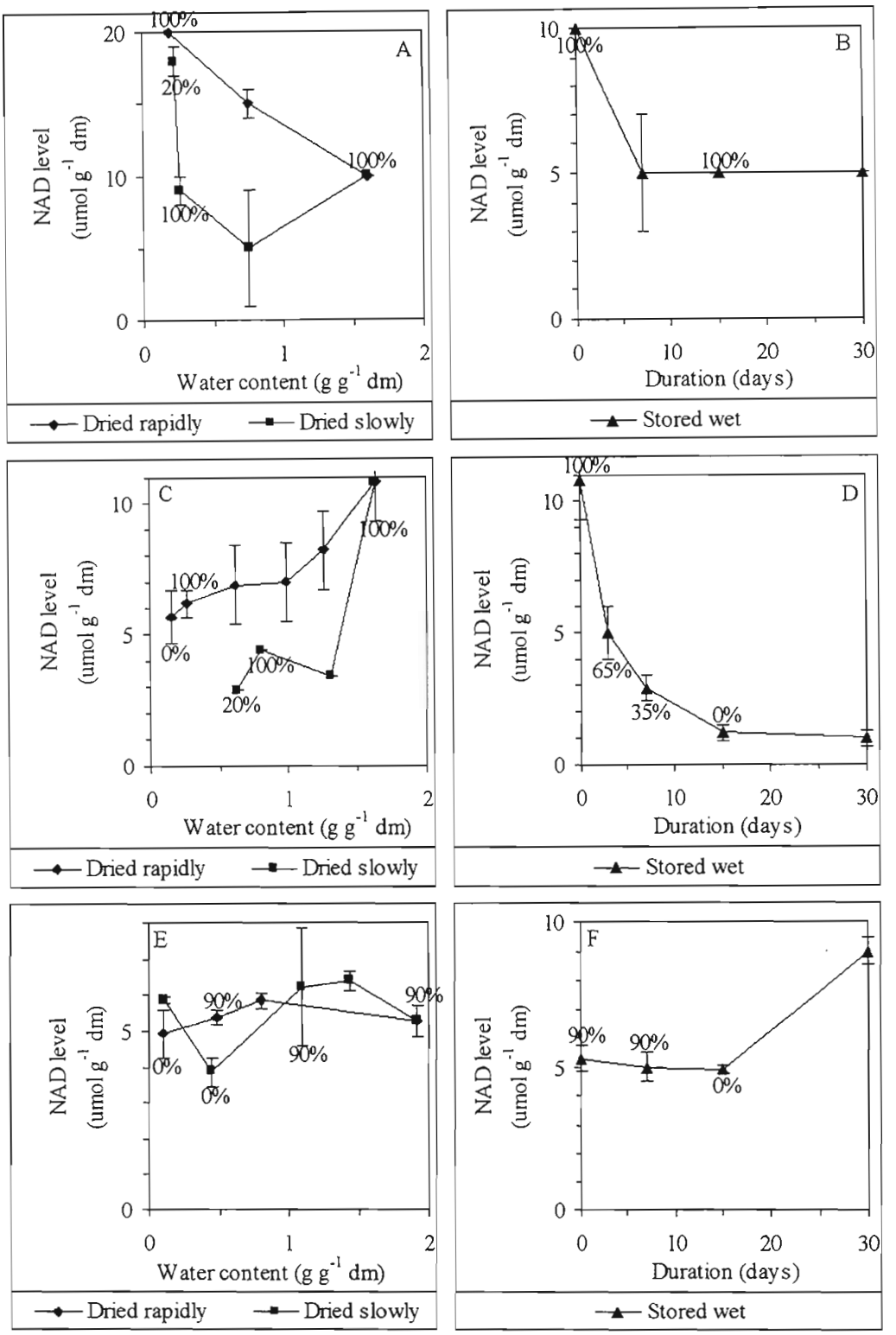

Figure 4.5 Levels of NAD in axes of *P. sativum* (A-B), *Q. robur* (C-D) and *T. dregeana* (E-F) during drying at different rates or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below data symbols indicate germination. Data points without percentages are unchanged over the previous values.

No siginificant changes in the levels of NAD were seen in whole axes of *A. marina* during rapid drying and wet storage ($F = 2.60$ and $p = 0.43$ and $F = 8.80$ and $p = 0.21$, respectively [Fig. 4.6A and B]). However, a statistically significant *c.* 80% decrease in NAD level in whole axes was observed during the initial 40% loss of germination upon slow dehydration ($F = 427.40$ and $p = 0.04$).

The levels of NAD in *A. marina* hypocotyls did not change significantly during rapid drying and wet storage ($F = 1.10$ and $p = 0.60$ and $F = 4.90$ and $p = 0.27$, respectively [Fig. 4.6C and D]). Nonetheless, a marginally significant *c.* 90% decrease in NAD level in hypocotoyls was associated with the initial 40% loss of germination upon slow deydration ($F = 157.50$ and $p = 0.06$).

There was no siginificant change in the level of NAD in root primordia of *A. marina* during rapid drying ($F = 7.10$ and $p = 0.13$ [Fig. 4.6E]). However, a highly significant *c.* 90% decrease in NAD level in root primordia accompanied the initial 40% loss of germination upon slow dehydration ($F = 791.50$ and $p < 0.01$). The onset of germination loss was preceded by a statistically significant *c.* 80% decline in the level of NAD in root primordia during wet storage ($F = 256.80$ and $p = 0.04$ [Fig. 4.6F]).

A marginally significant *c.* 70% decrease in the level of NAD in *A. marina* plumules took place during the initial 40% loss of germination upon rapid drying, respectively ($F = 14.60$ and $p = 0.07$ [Fig 4.6G]). Similarly, a statistically significant *c.* 90% decline in NAD level in plumules occurred during the initial 40% germination loss upon slow dehydration ($F = 2\ 792.00$ and $p = 0.01$). Nonetheless, no significant change in the levels of NAD in plumules was associated with wet storage ($F = 8.80$ and $p = 0.20$ [Fig. 4.6H]).

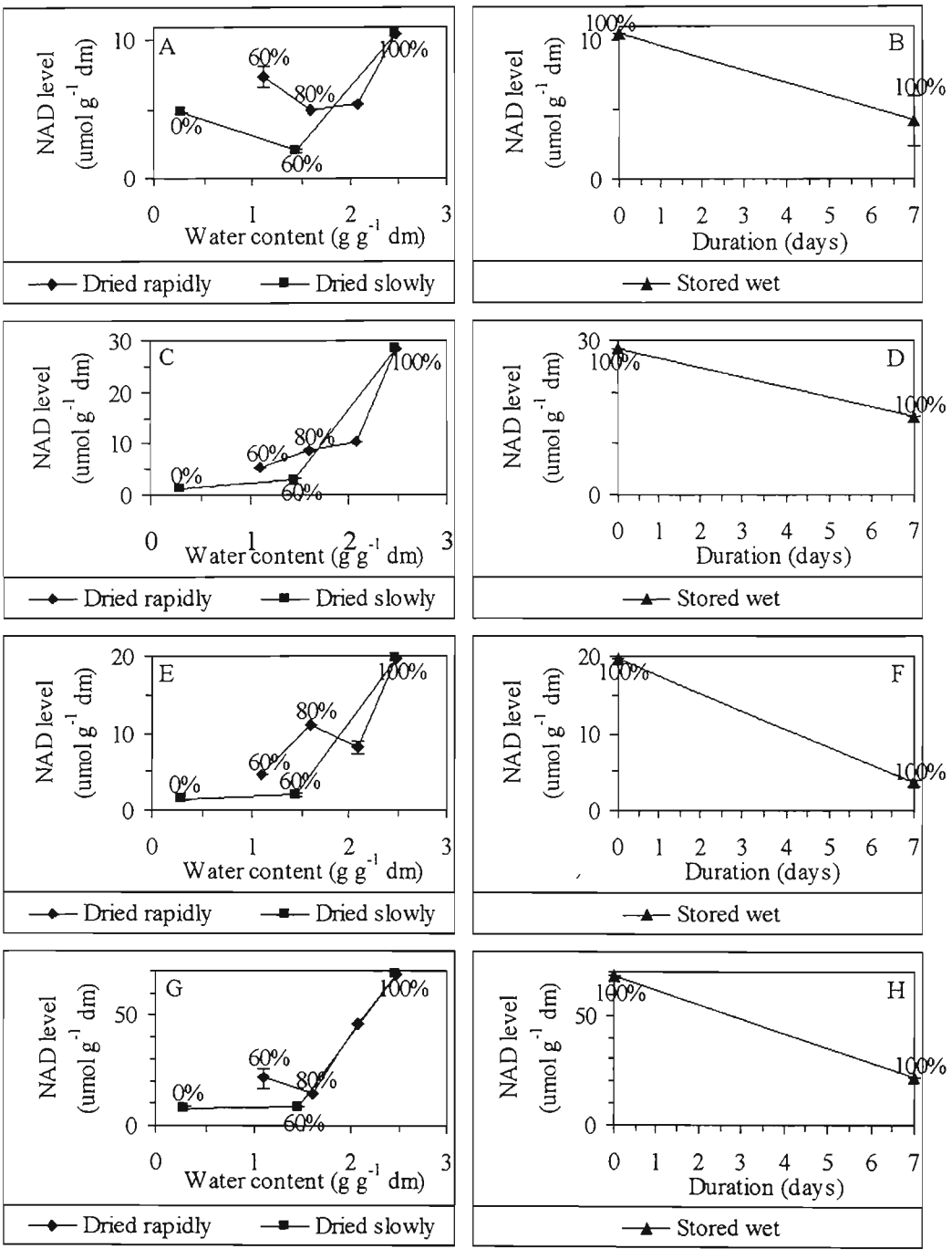

Figure 4.6 Levels of NAD in whole axes (A-B) and different axial tissues (hypocotyls [C-D], root primordia [E-F] and plumules [G-H]) of *A. marina* during rapid or slow drying or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below data symbols indicate germination. Data points without percentages are unchanged over the previous values.

4.4 Discussion

The activities of respiratory enzymes, PFK, MDH and NADH dehydrogenase, were reported to be adversely affected by drying (Leprince *et al.*, 1993a; reviewed by Côme and Corbineau, 1996). The results of the present study support those observations.

The activities of PFK in axes of *T. dregeana* and *A. marina* decreased during dehydration such that the activity of PFK was higher upon rapid than slow desiccation of *T. dregeana* axes. Similarly, PFK activity declined during wet storage of axes of *T. dregeana*. The activity of PFK in *Q. robur* axes also decreased during slow desiccation.

However, the activity of PFK in *A. marina* axes did not change significantly upon hydrated storage. The activity of PFK also remained constant during drying and hydrated storage of axes of *P. sativum*. Nonetheless, PFK activity actually increased upon rapid dehydration and wet storage of *Q. robur* axes. In this regard, it should be remembered that partially-germinated *P. sativum* and fresh *Q. robur* axes were found to be minimally desiccation-sensitive in the present investigation. In addition, PFK activity is only slightly adversely affected by drying (Leprince *et al.*, 1993a; reviewed by Côme and Corbineau, 1996).

A decline in activities of MDH in axes of *T. dregeana* and *A. marina* took place during dehydration and wet storage such that MDH activity was higher in *T. dregeana* axes upon rapid than slow desiccation. Nonetheless, the activity of MDH remained constant during drying and moist storage of axes of *P. sativum*. The activity of MDH in *Q. robur* axes was enhanced during dehydration and hydrated storage such that it was higher upon slow than rapid desiccation. The reason for this anomalous response is similar to the one advanced for the activity of PFK.

An elevation in the levels of NAD occurred in axes of *P. sativum* during drying. Similarly, there was an enhancement of NAD level in *T. dregeana* axes upon moist storage. It is suggested this response is a result of the impairment of the activity of the NADH dehydrogenases of NADH-ubiquinone (coenzyme Q) (complex I) and NADH-cytochrome c reductase (complex IV) of the electron transport chain, as shown in germinating maize (Leprince *et al.*, 1993a; 1994; 1995; 1998; 2000; reviewed by Côme and Corbineau, 1996).

In contrast, NAD levels were diminished during rapid and/or slow dehydration and/or wet storage of axes of *Q. robur* and *A. marina*. For *Q. robur* axes, their

response may be explained by a similar reason given for the activities of PFK and MDH. The reason for the increase in NADH dehydrogenase activity in *A. marina* axes is unknown.

The activities of PFK and MDH in axes of *A. marina* were adversely affected by desiccation and hydrated storage. However, the effect of the rate of drying was not apparent. It is possible that this discrepancy is a consequence of physical damage becoming more important than metabolic damage, as was shown by Greggains *et al.* (2001). The reason for the lack of the effect of drying rate on the response of the level of NAD in axes of *P. sativum* is unknown.

In addition, whole axes and different tissues of axes of *A. marina* showed dissimilar responses of the activities of PFK and MDH and level of NAD to desiccation and moist storage. These observations support those of Greggains *et al.* (2001) who demonstrated a similar phenomenon.

Desiccation-sensitive seeds ultimately die in wet storage; this has been suggested to be as a result of an initially mild but increasingly severe long-term water-stress (Pammenter *et al.*, 1994; 1997; Motete *et al.*, 1997). Nonetheless, it is often difficult to compare the responses of desiccation-sensitive tissues to hydrated storage with those to drying due to the vast difference in time scales (hours to days against weeks to months) and because water content remains constant during moist storage.

However, this problem may be overcome if the magnitude of the response to one or two of the treatments remain(s) constant whilst the other(s) change(s). For instance, the activity of PFK remained constant during wet storage of axes of *A. marina*. Nonetheless, it decreased upon rapid and slow drying. It may be concluded that *A. marina* axes incurred equivalent stress to PFK activity upon rapid and slow desiccation, but less during wet storage.

In conclusion, it appears that the responses of respiratory enzymes and, consequently, coenzymes to drying and hydrated storage vary with species and tissue. In this regard, it is noteworthy that, firstly, the degree of desiccation sensitivity of seeds plays a major role in determining the responses of their enzyme activities to water-stress.

On one hand, enzyme activities of minimally desiccation-sensitive seeds (such as *Q. robur*) may not be adversely affected but elevated. On the other hand, differential effects of desiccation and moist storage on enzyme activities of highly recalcitrant seed

types (such as *A. marina*) may not be apparent. In between, enzyme activities of moderately desiccation-sensitive (such as *T. dregeana*) seeds show a classic response to water-stress where they are adversely and are differentially affected by the rate of drying.

In addition, the sensitivity of the enzyme activities themselves to drying and wet storage is central to the responses of seeds for each species. For example, it appears that NADH dehydrogenase is more sensitive to water-stress than MDH, which, in turn, is more sensitive than PFK.

Chapter 5

The role of free radical processes in seed deterioration

5.1 Introduction

Free radicals are molecular species which contain unpaired electrons (reviewed by Benson, 1990). Consequently, they are some of the most chemically reactive structures known. Because of the need to pair its single electron, a free radical abstracts an electron from a neighbouring molecule. This event causes the formation of yet another free radical and a self-propagating chain reaction ensues.

In biological systems, free radical formation is involved in important processes of normal metabolism (reviewed by Benson, 1990; Hendry, 1993; Hendry and Crawford, 1994). They include, for instance, electron transport, substrate oxidation, lipid metabolism and pathological defence mechanisms. Under normal conditions, these processes are tightly controlled by metabolic coupling and cellular compartmentation. In addition, aerobic organisms have evolved extensive mechanisms to protect the cell against free radical injury and oxygen toxicity.

However, the tight control of the processes that involve free radical formation can, and does, become altered (e.g. by environmental stresses [Hendry, 1993; Hendry and Crawford, 1994], ageing and physical injury [Benson, 1990]). A situation arises in which metabolism enters a series of reactions which produce highly reactive, self-propagating free radical species. Perhaps, it is the biggest irony of life that oxygen, the primary agent of aerobic respiration, is often the primary promoter of the deleterious free radical reactions in biological systems.

The biochemical events, which lead to free radical production, are complex (reviewed by Benson, 1990). Furthermore, the high reactivity of the free radicals makes interpretation of their origin difficult. It is, therefore, not surprising that it is often difficult to determine if free radicals are a direct cause of injury or a consequence of preceding damage (reviewed by Benson, 1990; Hendry, 1993; Hendry and Crawford, 1994).

The free-radical theory of ageing originated in the medical sciences nearly half-a-century ago (Harman, 1956). It was soon introduced into seed biology when

Kaloyereas (1958) suggested that lipid oxidation might underlie loss of viability in seeds.

Since then, evidence to support the free-radical theory in seed ageing has been accumulating. The earliest study of Koostra and Harrington (1969) analysed phospholipid (PL) changes and raised the possibility that membrane peroxidative changes were associated with ageing. More recent studies have implicated lipid peroxidation involvement in membrane deterioration (e.g. Hailstones and Smith, 1988). These studies have shown a strong correlation between hydroperoxide (products of oxidation of unsaturated free fatty acids [FFAs] by free radicals) levels and viability loss. These findings are supported by a decline in levels of unsaturated FFAs during seed deterioration (Hailstones and Smith, 1988). Further evidence comes in the form of studies, which have analysed the headspace above seeds heated in a sealed container for aldehydes (thermal breakdown products of hydroperoxides [Frankel, 1982]). These studies have shown a link between increasing aldehyde evolution and viability loss (Hailstones and Smith, 1989; Ntuli *et al.*, 1997).

However, there are studies which contrast with the picture painted above. For instance, Fergusson *et al.* (1990) and Ntuli *et al.* (1997) did not find a decline in total and polar FFA levels in aged soybean axes and dried wild rice seeds during deterioration, respectively. Smith and Berjak (1995) attributed this contradiction to differences in methodology.

A number of factors determine the extent of peroxidation in ageing seeds. They include: (1) composition and saturation of FFAs (Priestley, 1986; Ponquett *et al.*, 1992), (2) level of hydration (Karel and Yong, 1981), (3) relative humidity (RH) and temperature (reviewed by Smith and Berjak, 1995; Leprince *et al.*, 1995b), (4) oxygen availability (Ohlrogge and Kernan, 1982; Leprince *et al.*, 1995) and (5) efficiency of antioxidative systems (Pokorný, 1987).

Membranes are one of the most important of the many targets of free radical attack (reviewed by Benson, 1990). Membrane lipids contain unsaturated bond systems. Thus, they are electron-rich. Consequently, they provide an ideal target for free radical attack. Free radical damage to membranes is thought to be primarily mediated through the process of lipid peroxidation, although associated proteins may also present targets (reviewed by Smith and Berjak, 1995).

Free radicals have been implicated in a number of processes in seed biology. These include: (1) ageing of orthodox seeds (reviewed by McKersie *et al.*, 1988; Tommasi *et al.*, 1999), (2) dehydration of recalcitrant seeds (Hendry *et al.*, 1992; Chaitanya and Naithani; 1994; Chandel *et al.*, 1995; Finch-Savage *et al.*, 1994a; 1996; Li and Sun; 1999; Tommasi *et al.*, 1999; Greggains *et al.*, 2001) and (3) deterioration of desiccation-sensitive seeds in wet storage (e.g. Greggains *et al.*, 2000b). Here, the involvement of free radicals during dehydration and hydrated storage of recalcitrant seeds is considered.

The free radicals and hydroperoxides produced during peroxidation are often not stable and can readily decompose to produce a whole range of breakdown products (Kappus, 1985; Sevanian and Hochstein, 1985; Chan, 1987). Apart from being cytotoxic, these breakdown products provide an indirect measurement of free radical-mediated damage (e.g. Hailstones and Smith, 1989).

Recent studies have provided evidence that recalcitrant seeds become exposed to activated forms of oxygen and lipid peroxidation, and accumulate stable free radicals under water-stress conditions (Finch-Savage *et al.*, 1994a). As an example, Hendry *et al.* (1992) have shown that loss of viability during drying in the desiccation-sensitive seeds of *Quercus robur* coincided with increased lipid peroxidation and free radical formation in the embryonic axes. More recently, Hendry and co-workers (Hendry *et al.*, 1994; Leprince *et al.*, 1994; 1995) and other authors (e.g. Wood *et al.*, 1995) have shown the occurrence of free radicals in a range of species subjected to dehydration.

The direct detection of free radicals requires sophisticated equipment (reviewed by Benson, 1990; Leprince and Golovina, 2002). The two commonly used methods (electron paramagnetic resonance [EPR] and electron nuclear double resonance [ENDOR]) exploit the fact that electrons possess both spin and charge and hence can behave like magnets (Williams and Wilson, 1975). If molecules containing unpaired electrons are placed in a magnetic field and electromagnetic radiation is applied, the electron can undergo a spin reversal ('electron spin resonance' [ESR]). The frequency at which spin reversal occurs depends on the free radical species and the applied magnetic field.

Because of the inhibitory cost and consequent lack of availability of EPR/ESSR and ENDOR equipment, a number of indirect methods have been developed to detect the products of free radical activity (reviewed by Wilson and McDonald, 1986). These

include monitoring changes in lipid bond saturation, lipid and PL content and measuring the release of FFAs, and the production of lipid peroxides and their breakdown products (e.g. volatiles such as hydrocarbons, aldehydes and alcohols).

Free radicals are naturally produced during plant metabolism, particularly in electron transport chains of chloroplasts and mitochondria during photosynthesis and respiration, respectively (reviewed by Halliwell, 1987; Benson, 1990; Puntarulo, 1991; Hendry, 1993; Hendry and Crawford, 1994). Consequently, plants are well endowed with both enzymatic and low-molecular-weight non-enzymatic free radical processing antioxidants (reviewed by Leprince *et al.*, 1993b; Côme and Corbineau, 1996).

An antioxidant is any compound capable of quenching free radicals without itself undergoing conversion to a destructive radical (reviewed by Rose and Bode, 1993; Nishikimi and Yagi, 1996; Noctor and Foyer, 1998). Free radical processing enzymes catalyse such reactions or are involved in the direct processing of free radicals. Of the numerous enzymes and metabolites potentially covered by these definitions, many remain uncharacterised (reviewed by Smirnoff and Cumbes, 1989; Halliwell *et al.*, 1995; Noctor and Foyer, 1998).

Non-enzymatic antioxidants include the fat-soluble α- and γ-tocopherol (TOC) isomers (vitamin E) and retinol (vitamin A) or β-carotene (provitamin A) and water-soluble ascorbic acid (AsA [vitamin C]) and glutathione. TOCs are chain-breaking antioxidants, thus block lipid peroxidation. Seeds are known to contain large concentrations of such antioxidant compounds varying in different tissues and seeds (Franzen and Haas, 1991).

Enzymic free radical processing enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and ascorbate and guaicol peroxidases (A/GPODs). SOD catalyses the dismutation of superoxide into hydrogen peroxide and molecular oxygen. CAT, GR and PODs are involved in the detoxification of hydrogen peroxide. Enzymatic systems are more likely to be involved in an early antioxidant response by neutralising potentially toxic activated oxygen formed during water deprivation (reviewed by Leprince *et al.*, 1993b).

Earlier studies have demonstrated the link between tolerance to oxidative stress, induced by water-stress during seed development, and the type and efficiency of free radical processing systems and antioxidants (reviewed by Leprince *et al.*, 1993b).

Those studies have shown that protective mechanisms against oxidative attack are predominantly enzymic. In addition, there was an increase in the activities of free radical processing enzymes with radicle emergence. However, there was severe impairment of the glutathione metabolism and activities of SOD and POD in geminating desiccation-intolerant maize radicles subjected to a desiccation treatment (Leprince *et al.*, 1990b). Similar events were observed in soyabean and wheat seeds (Puntarulo *et al.*, 1991 and Cakmak *et al.*, 1993, respectively). In contrast, the pattern of TOC metabolism did not show any convincing evidence that depletion in the supply of these antioxidants was the cause of loss of desiccation-tolerance in germinating maize radicles (Leprince *et al.*, 1990b).

More recently, it has been shown that accelerated ageing resulted in a decrease in activities of SOD, CAT and GR in sunflower seeds and seedlings (Bailly *et al.*, 1996; 1997; 1998; 2002). However, osmopriming with polyethylene glycol was associated with progressive increase in SOD, CAT and GR activities to levels similar to those found in unaged seeds and seedlings (Bailly *et al.*, 1997; 1998; 2000; 2002). Furthermore, the osmotreatment induced a second isoform of CAT.

Like germinating orthodox seeds, it appears that the patterns of physiological and biochemical response of the free radical processing systems in desiccation-sensitive seeds to oxidative stress differ among both tissues and species. For instance, the defence against oxidative attack on axial tissue of *Quercus robur* was largely dependent on antioxidants whereas it was predominantly enzymic in cotyledons (Hendry *et al.*, 1992). Moreover, the activities of SOD and GR in axes decreased during desiccation. In contrast, SOD and GR activity increased in the cotyledons upon drying. Furthermore, there was a decrease in the levels of α-tocopherol in axes during dehydration. Conversely, α-tocopherol levels increased in the cotyledon upon desiccation. However, the activity of SOD increased significantly during drying in *Shorea robusta* seeds (Chaitanya and Naithani, 1994). Furthermore, there was a rapid decrease in activities of SOD and peroxidases in *Theobroma cacoa* axes corresponding to loss of viability (Li and Sun; 1999). Additionally, an increase in both the amount of tocopherol and activity of SOD was observed in the plumules of axes of *Avicennia marina* following dehydration (Greggains *et al.*, 2001).

The aim of the work reported in the present chapter was to establish whether free radical-mediated damage was differentially affected by drying rate, or wet storage of embryonic axes from desiccation-sensitive seeds. Free radical damage was measured as level of hydroperoxides. In addition, the involvement of free radical processing enzymes and antioxidants during dehydration at different rates, or during wet storage, of embryonic axes from desiccation-sensitive seeds was ascertained.

5.2 Materials and methods

5.2.1 Seed material

Excised embryonic axes of *Pisum sativum*, *Quercus robur*, *Trichilia dregeana* and *Avicennia marina* were subjected to the slow or rapid drying or wet storage protocols outlined in Chapter 2.

5.2.2 Hydroperoxide determinations

Lipids were extracted from embryonic axes (*c.* 5 mg dm) that had been ground with liquid nitrogen in a mortar with a pestle, in 5 ml of dichloromethane/methanol (2:1 v/v) containing butylated hydroxytoluene (50 mg l^{-1}) according to Hailstones and Smith (1988) and modified as in Ntuli *et al.* (1997). Following centrifugation at 1 500 *g* for 5 min, 1 ml of 0.014 M ferrous chloride was added to 2 ml of the lipid extract in dichloromethane/methanol and shaken. Twenty µl of 30% potassium thiocyanate were then added. Hydroperoxide levels were estimated by the oxidation of Fe^{2+} as the absorbance recorded at 505 nm.

5.2.3 Antioxidant enzyme assays

Superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) were extracted in 10 ml of 50 mM potassium phosphate (pH 7.0), 0.25% (w/v) Triton X-100 and 1% (w/v) polyvinlypolypyrolidone, following homogenisation of axes (*c.* 5 mg dm) to a fine powder under liquid nitrogen in the mortar with a pestle, following the procedures of Mishra *et al.* (1993; 1995). The homogenate was then centrifuged at 8 000 *g* for 15 min. For SOD, the reaction was performed with an aliquot of 2 ml of the supernatant and 1 ml of 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA containing 18 µM cytochrome *c* and 0.1 mM xanthine and the reaction started with addition of 0.02 ml of 1 U ml^{-1} of xanthine oxidase (McCord and Fridovich, 1969;

Schoner and Krause, 1990). Activity was monitored by measuring the rate of reduction of cytochrome c as the change in absorbance at 550 nm.

CAT was assayed in an aliquot of 2 ml of the supernatant added to 1 ml of 50 mM potassium phosphate containing 11 mM hydrogen peroxide (pH 7.0). Activity was determined as the decomposition of hydrogen peroxide by the decrease in absorbance at 240 nm (Aebi, 1983).

GR was assayed in an aliquot of 2 ml of the supernatant added to 1 ml of 25 mM Tris-HCl containing 0.5 mM oxidised glutathione and 0.12 mM NADPH (pH 7.8) (Foyer and Halliwell, 1976). Activity was recorded by measuring the oxidation of NADPH as the decrease in absorbance at 340 nm.

5.2.4 Ascorbic acid (AsA) assay

AsA was extracted in 4 ml of 2.5 mM perchloric acid following homogenisation of axes (*c.* 5 mg dm) in liquid nitrogen in the mortar with a pestle according to Foyer *et al.* (1983). The homogenate was then centrifuged at 8 000 *g* for 5 min and neutralised to pH 5.6 with 1.25 M potassium carbonate. One ml of the extract was transferred to 1 ml of 0.1 M sodium phosphate buffer (pH 5.6) and reaction started with addition of 1 ml of 5 U ml^{-1} of ascorbate oxidase (Hewitt and Dickes, 1961). The level of AsA was estimated by measuring its oxidation as the decrease in absorbance at 265 nm.

For all five assays, the results are reported as percentage changes (between the present and previous values) of initial values of means of three replicate extractions. After each assay, axes were dried and data expressed on a dry mass basis. Due to shortage of plant material, assays were only conducted after 7 days during wet storage of axes of *Avicennia marina*.

5.2.5 Statistical analysis

Data were subjected to one-way ANOVA test. Where significant effects were found to occur, the Tukey multiple range test was subsequently used to identify where they occurred.

5.3 Results

5.3.1 Hydroperoxide levels

A marginally significant *c.* 70% decrease in the level of hydroperoxides occurred in axes of *P. sativum* during rapid drying ($F = 13.00$ and $p = 0.07$ [Fig. 5.1A]). However, no significant change in hydroperoxide level was associated with slow dehydration ($F = 0.00$ and $p = 0.96$). A *c.* 70% increase in the level of hydroperoxides was seen prior to the onset of the decrease in germination percentage during wet storage ($F = 13.00$ and $p = 0.07$ [Fig. 5.1B]).

Loss of germination was associated with a highly significant *c.* 190% increase in the level of hydroperoxide during rapid drying of axes of *Q. robur* ($F = 7.00$ and $p < 0.01$ [Fig. 5.1C]). Similarly, a highly significant *c.* 380% enhancement in hydroperoxide level was observed prior to the onset of germination loss upon slow dehydration ($F = 43.60$ and $p < 0.01$). A highly significant *c.* 190% decrease in the level of hydroperoxides was associated with loss of germination during slow desiccation ($F = 43.60$ and $p < 0.01$). Loss of germination percentage was accompanied by a highly significant *c.* 310% elevation of the hydroperoxide level upon wet storage ($F = 19.60$ and $p < 0.01$ [Fig. 5.1D]).

A marginally significant *c.* 40% decrease in the levels of hydroperoxide in axes of *T. dregeana* preceded the onset of loss of germination during rapid drying ($F = 3.00$ and $p = 0.09$ [Fig. 5.1E]). However, no significant change in hydroperoxide level preceded the onset of germination loss during slow dehydration ($F = 0.10$ and $p = 0.97$ [Fig. 5.1E]). During wet storage, a statistically significant *c.* 140% increase in hydroperoxide levels was seen before the onset of loss of germination ($F = 5.90$ and $p = 0.03$ [Fig. 5.1F]).

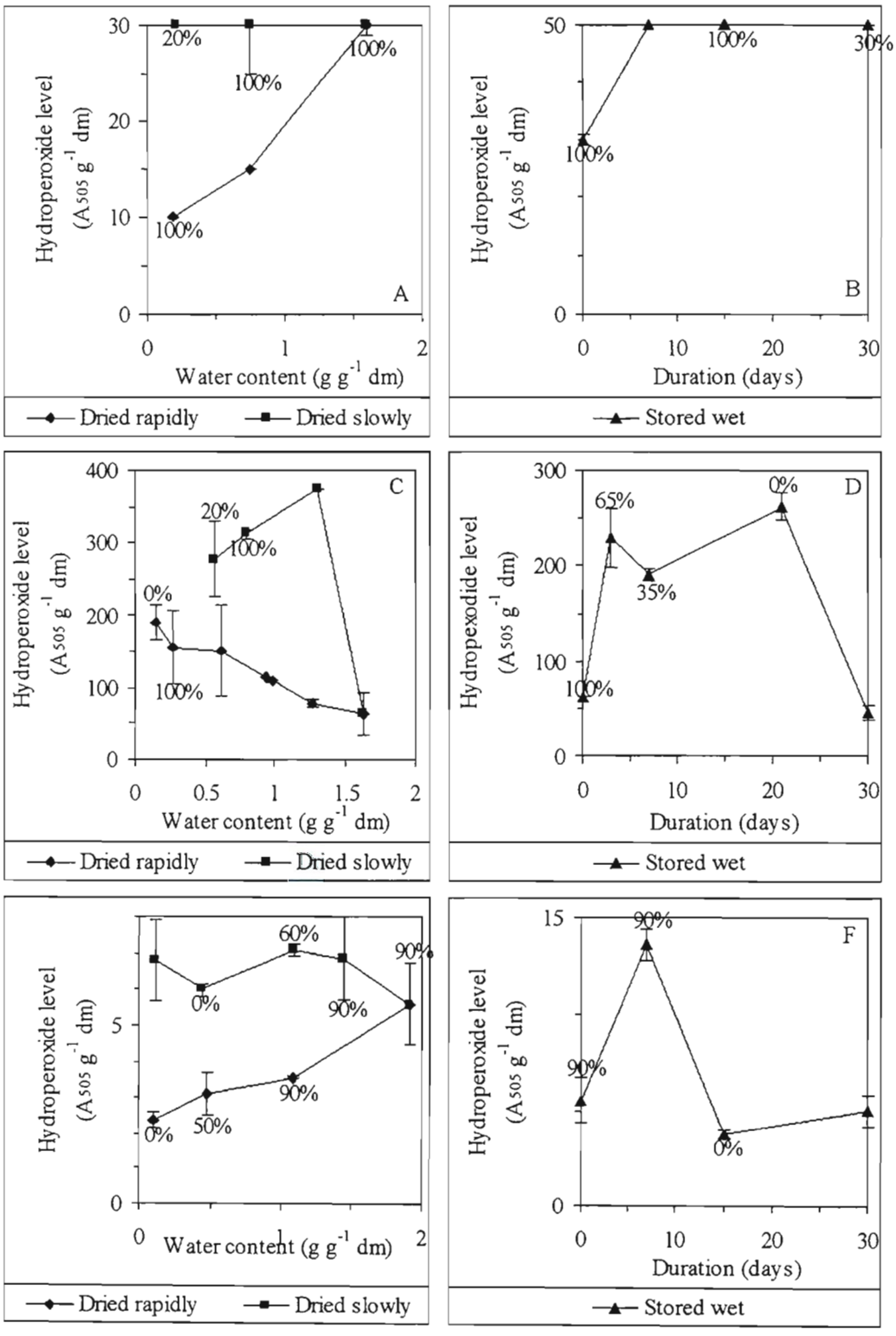

Figure 5.1 Levels of hydroperoxides in axes of *P. sativum* (A-B), *Q. robur* (C-D) and *T. dregeana* (E-F) during drying at different rates or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below data symbols indicate germination. Data points without percentages are unchanged over the previous values.

No significant changes in the level of hydroperoxides in whole axes of *A. marina* were associated with rapid and slow drying and wet storage ($F = 0.10$ and $p = 0.96$, $F = 0.30$ and $p = 0.80$ and $F = 33.06$ and $p = 0.11$, respectively [Fig. 5.2A and B]).

The level of hydroperoxides in *A. marina* hypocotyls increased highly significantly by *c.* 440% during the initial 40% loss of germination upon rapid drying ($F = 80.50$ and $p < 0.01$ [Fig. 5.2C]). Similarly, a highly significant *c.* 145% enhancement in hydroperoxide level in hypocotyls was associated with the initial 40% germination loss during slow dehydration ($F = 120.20$ and $p < 0.01$). However, no significant change in the level of hydroperoxides in hypocotyls accompanied wet storage ($F = 2.10$ and $p = 0.29$ [Fig. 5.2D]).

The levels of hydroperoxides in root primordia of *A. marina* did not change significantly during rapid and slow drying and wet storage ($F = 2.90$ and $p = 0.41$, $F = 0.40$ and $p = 0.76$ and $F = 1.00$ and $p = 0.50$, respectively [Fig. 5.2E and F]). Similarly, there were no siginificant changes in hydroperoxide levels in *A. marina* plumules upon rapid and slow dehydration and hydrated storage ($F = 5.80$ and $p = 0.30$, $F = 0.40$ and $p = 0.76$ and $F = 0.10$ and $p = 0.77$, respectively [Fig. 5.2G and H).

It is noteworthy that the level of hydroperoxides was considerably higher in the root primordia, and especially the plumules, than in the hypocotyls, so that the level in the whole axes reflected those in the plumules rather than in the hypocotyls which make up the bulk of the axes.

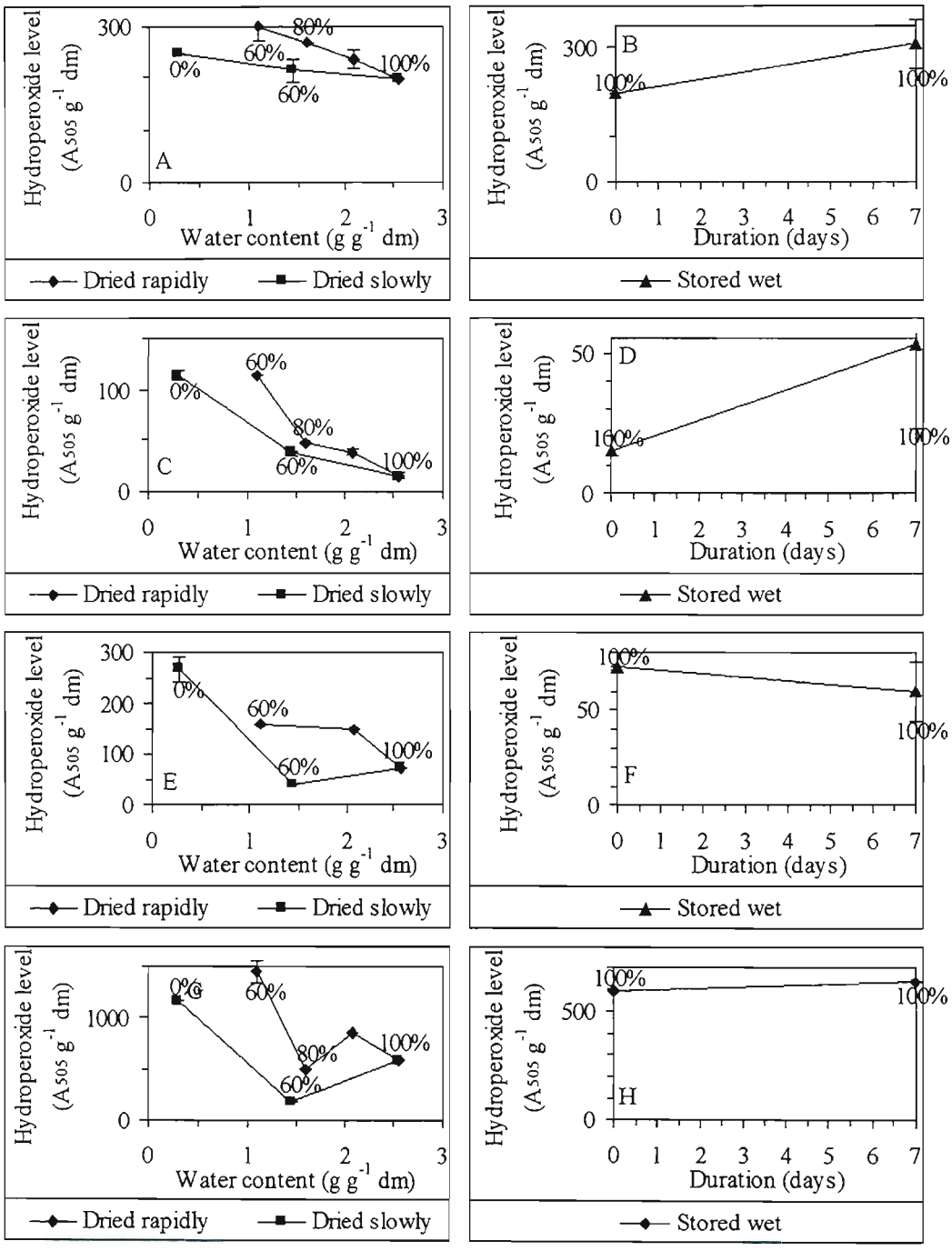

Figure 5.2 Levels of hydroperoxides in whole axes (A-B) and different axial tissues (hypocotyls [C-D], root primordia [E-F] and plumules [G-H]) of *A. marina* during rapid or slow drying or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below data symbols indicate germination. Data points without percentages are unchanged over the previous values.

5.3.2 Antioxidant enzymes activities

a. SOD

A marginally significant *c.* 50% transient decrease in the activity of SOD in axes of *P. sativum* took place during rapid drying ($F = 3.50$ and $p = 0.09$ [Fig. 5.3A]). A statistically significant *c.* 120% increase in SOD activity occurred before the onset of germination loss upon slow dehydration ($F = 10.50$ and $p = 0.01$). Similarly, there was a *c.* 140% enhancement in the activity of SOD prior to the onset of loss of germination during wet storage ($F = 20.40$ and $p = 0.02$ [Fig. 5.3B]).

No significant change in the activity of SOD in axes of *Q. robur* was seen during rapid drying and wet storage ($F = 0.90$ and $p = 0.58$ $F = 3.90$ and $p = 0.11$, respectively [Fig. 5.3C and D]). However, a statistically significant *c.* 30% decrease in SOD activity was observed before the onset of loss of germination upon slow dehydration ($F = 9.20$ and $p = 0.01$). The decline in germination percentage was accompanied by a further statistically significant *c.* 29% reduction in the activity of SOD during slow desiccation ($F = 9.20$ and $p = 0.01$).

Loss of germination was associated with a statistically significant *c.* 70% decrease in the activity of SOD during rapid drying of axes of *T. dregeana* ($F = 9.20$ and $p = 0.01$ [Fig. 5.3E]). Similarly, a highly significant *c.* 25% decline in SOD activity accompanied germination loss upon slow dehydration (and $F = 10.90$ and $p < 0.01$). In contrast, there was a statistically significant *c.* 110% increase in SOD activity prior to the onset of loss of germination during wet storage ($F = 10.70$ and $p = 0.01$ [Fig. 5.3F]) followed by a decline during germination loss.

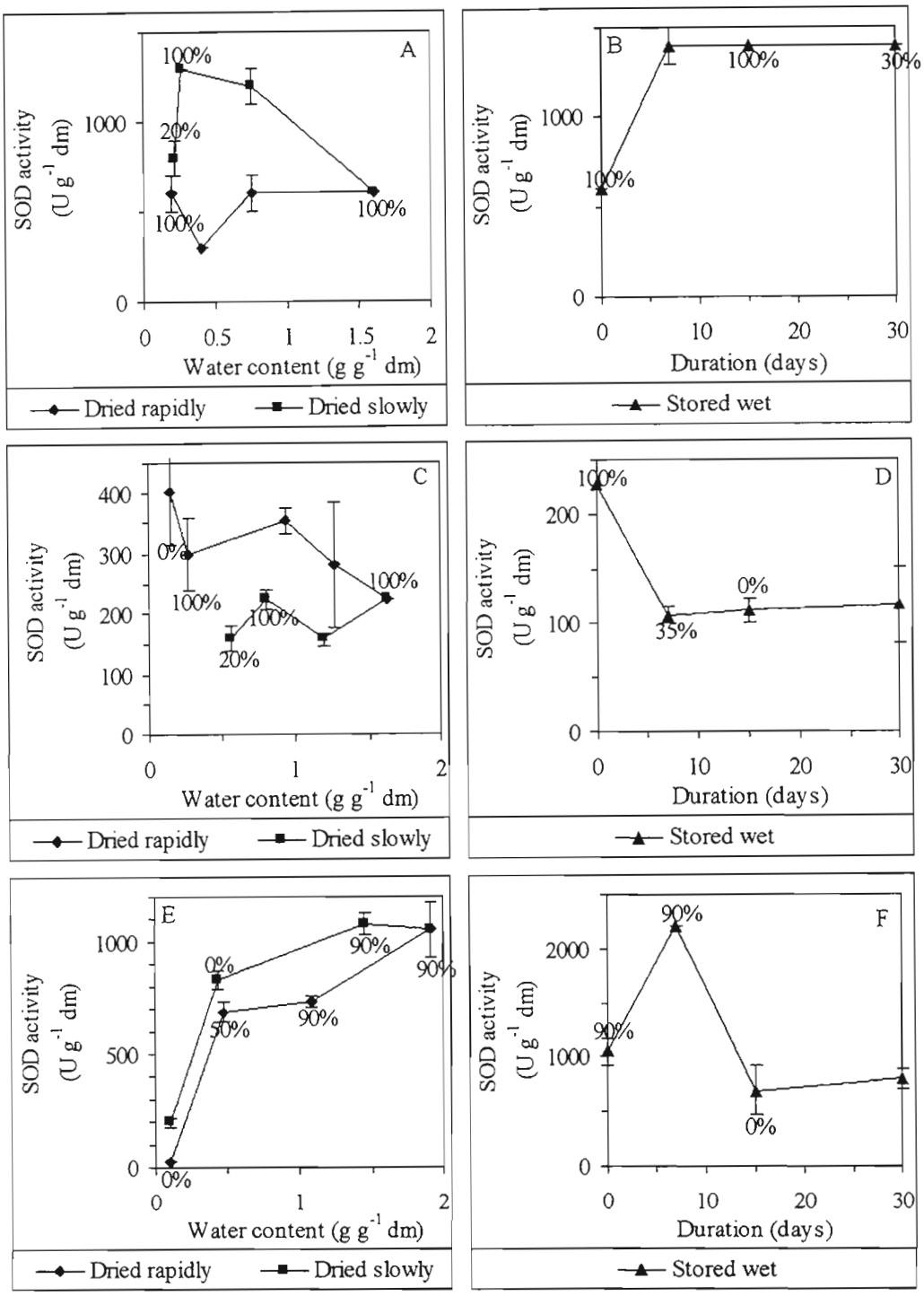

Figure 5.3 Activities of superoxide dismutase in axes of *P. sativum* (A-B), *Q. robur* (C-D) and *T. dregeana* (E-F) during drying at different rates or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below data symbols indicate germination. Data points without percentages are unchanged over the previous values. 1 unit (U) of SOD will inhibit the rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25 °C.

A statistically significant *c.* 540% increase in the activity of SOD in whole axes of *A. marina* took place during the initial 40% loss of germination upon rapid drying ($F = 1\ 789.80$ and $p = 0.02$ [Fig. 5.4A]). However, no significant change in SOD activity in whole axes occurred during slow dehydration ($F = 1.30$ and $p = 0.45$). There was a statistically significant *c.* 200% enhancement in the activity of SOD in axes prior to the onset of germination loss upon wet storage ($F = 54.70$ and $p = 0.02$ [Fig. 5.4B]).

No significant changes in the activity of SOD in *A. marina* hypocotyls were seen during rapid and slow drying and wet storage ($F = 5.50$ and $p = 0.30$, $F = 0.20$ and $p = 0.70$ and $F = 0.20$ and $p = 0.70$, respectively [Fig. 5.4C and D]). Similarly, there were no significant changes in SOD activity in root primordia of *A. marina* upon rapid and slow dehydration and hydrated storage ($F = 0.50$ and $p = 0.75$, $F = 1.30$ and $p = 0.46$ and $F = 1.30$ and $p = 0.46$, respectively [Fig. 5.4E and F]). It is noteworthy that the activity of SOD in *A. marina* plumules was very high compared with other axial tissues. Nonetheles, no significant changes in SOD activity were observed during rapid and slow desiccation and moist storage ($F = 1.60$ and $p = 0.41$, $F = 4.50$ and $p = 0.32$ and $F = 6.30$ and $p = 0.24$, respectively [Fig. 7.2G and H]).

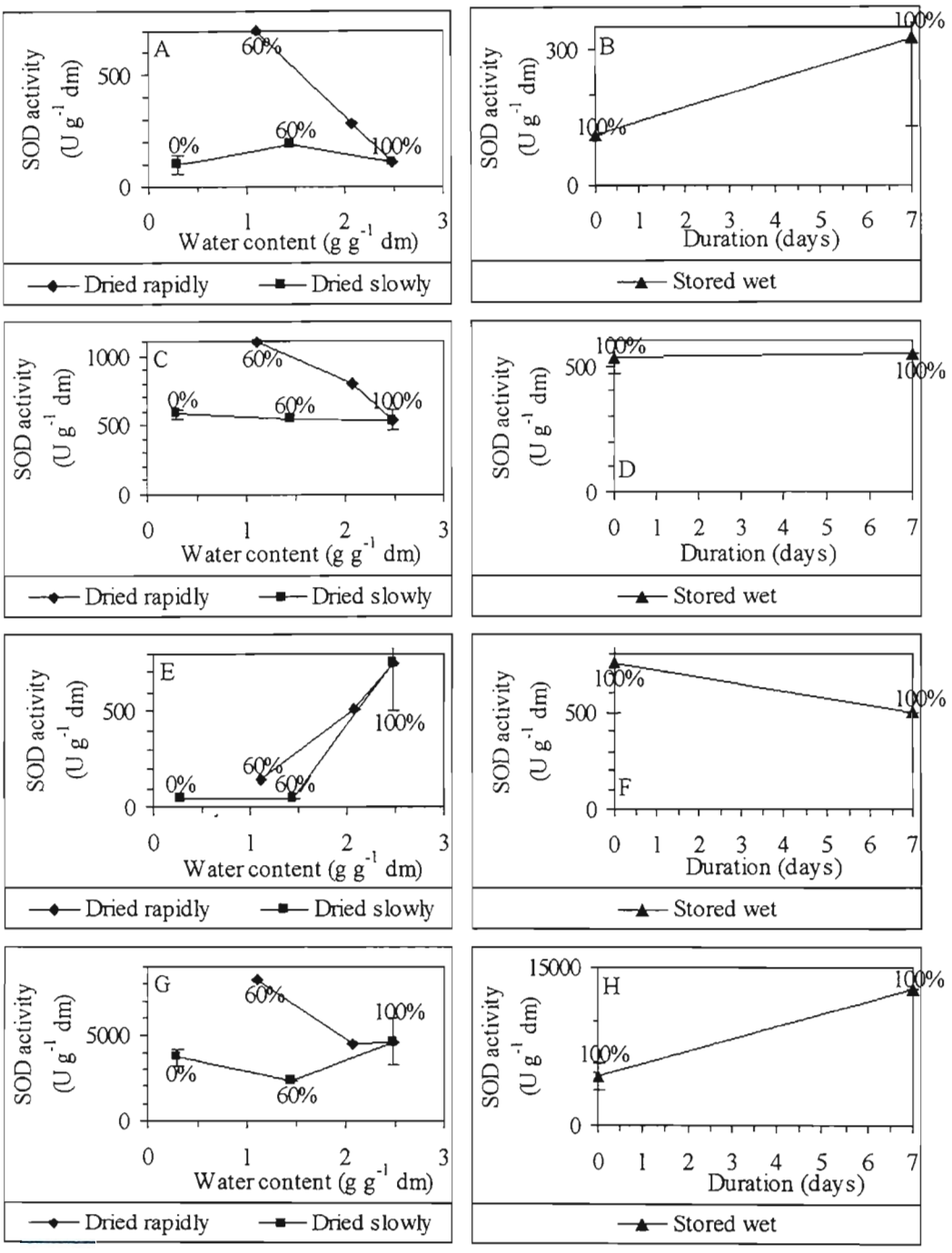

Figure 5.4 Activities of superoxide dismutase in whole axes (A-B) and different axial tissues (hypocotyls [C-D], root primordia [E-F] and plumules [G-H]) of *A. marina* during rapid or slow drying or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below data symbols indicate germination. 1 unit (U) of SOD will inhibit the rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25 °C.

b. CAT

There were no significant changes in the activity of CAT during rapid and slow drying and wet storage of axes of *P. sativum* ($F = 0.90$ and $p = 0.53$, $F = 4.70$ and $p = 0.12$ and $F = 0.20$ and $p = 0.69$, respectively [Fig. 5.5A and B]).

The activity of CAT in axes of *Q. robur* did not change significantly during rapid drying ($F = 2.00$ and $p = 0.15$ [Fig. 5.5C]). A marginally significant *c.* 80% decrease in CAT activity in *Q. robur* axes preceded the onset of loss of germination upon slow drying ($F = 3.20$ and $p = 0.07$). Similarly, a statistically significant *c.* 75% decline in the activity of CAT was associated with loss of germination during wet storage ($F = 6.90$ and $p = 0.03$ [Fig. 5.5D]).

A statistically significant *c.* 20% decrease in the activity of CAT in axes of *T. dregeana* accompanied loss of germination during rapid drying ($F = 4.30$ and $p = 0.03$ [Fig. 5.5E]). Similarly, a statistically significant *c.* 45% decline in CAT activity was associated with germination loss upon slow dehydration ($F = 10.10$ and $p = 0.01$). However, no significant change in the activity of CAT took place during wet storage ($F = 3.60$ and $p = 0.16$ [Fig. 5.5F]).

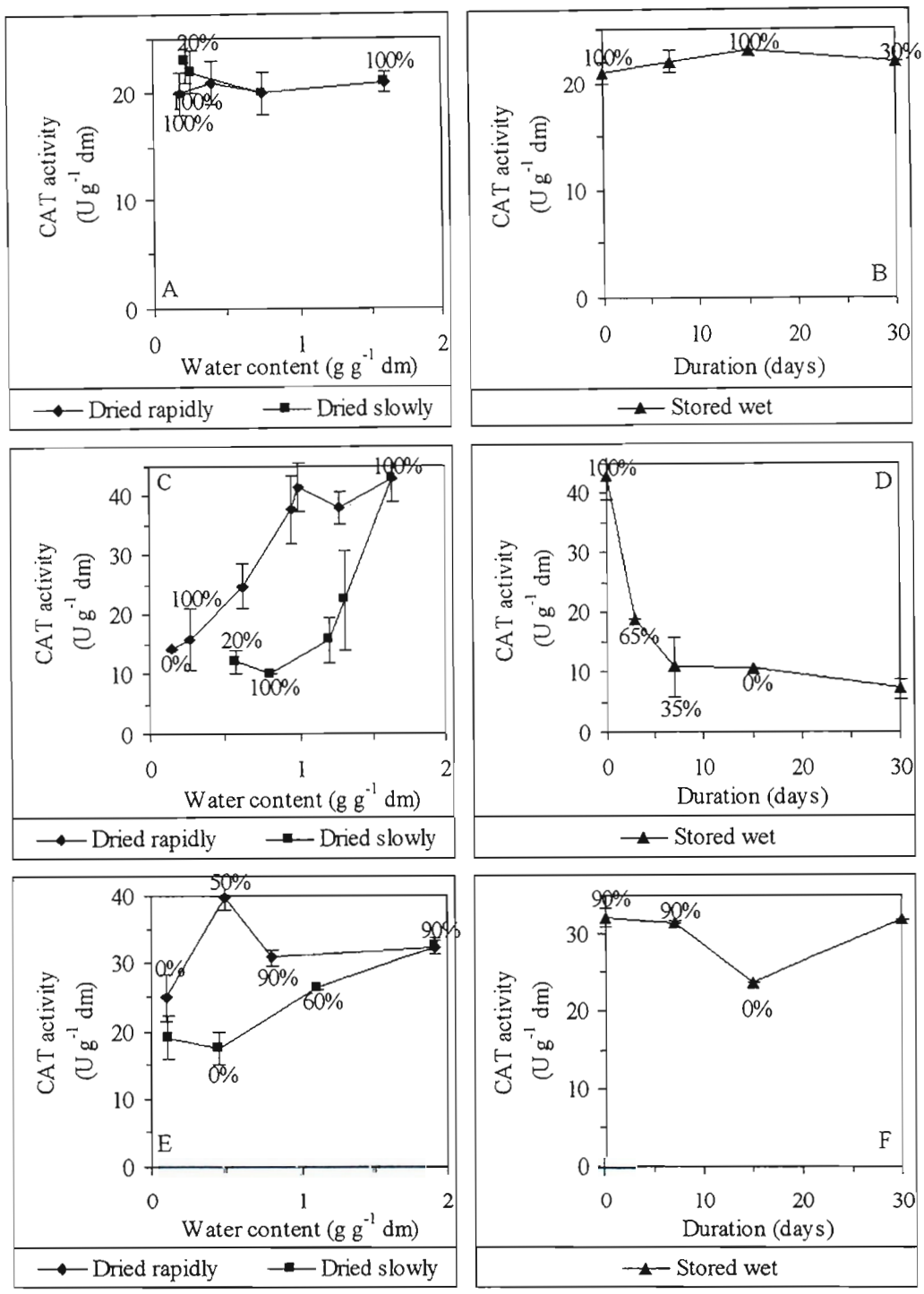

Figure 5.5 Activities of catalase in axes of *P. sativum* (A-B), *Q. robur* (C-D) and *T. dregeana* (E-F) during drying at different rates or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below data symbols indicate germination. Data points without percentages are unchanged over the previous values. 1 unit (U) of CAT will decompose 1 μmol of hydrogen peroxide per min at pH 7.0 at 25 °C.

No significant changes in the activity of CAT in whole axes of *A. marina* took place during rapid drying and wet storage ($F = 3.60$ and $p = 0.16$ and $F = 0.00$ and $p = 0.91$, respectively [Fig. 5.6A and B]). However, a marginally significant *c.* 40% elevation in CAT activity in axes was associated with loss of germination percentage beyond the initial 40% during slow dehydration ($F = 12.60$ and $p = 0.07$).

The activity of CAT in *A. marina* hypocotyls did not change significantly during rapid and slow drying and wet storage ($F = 2.90$ and $p = 0.21$, $F = 1.20$ and $p = 0.45$ and $F = 0.00$ and $p = 0.91$, respectively [Fig. 5.6C and D]).

Simlarly, no significant changes in the activity of CAT in root primordia of *A. marina* took place during rapid and slow drying ($F = 3.20$ and $p = 0.15$ and $F = 0.40$ and $p = 0.72$, respectively [Fig. 5.6E]). However, a statistically significant *c.* 360% increase in CAT activity preceded the onset of loss of germination upon wet storage ($F = 37.00$ and $p = 0.03$ [Fig. 5.6F]).

The activity of CAT in *A. marina* plumules also did not change significantly during rapid and slow drying and wet storage ($F = 1.40$ and $p = 0.40$, $F = 0.36$ and $p = 0.72$ and $F = 6.00$ and $p = 0.14$, respectively [Fig. 5.6G and H]).

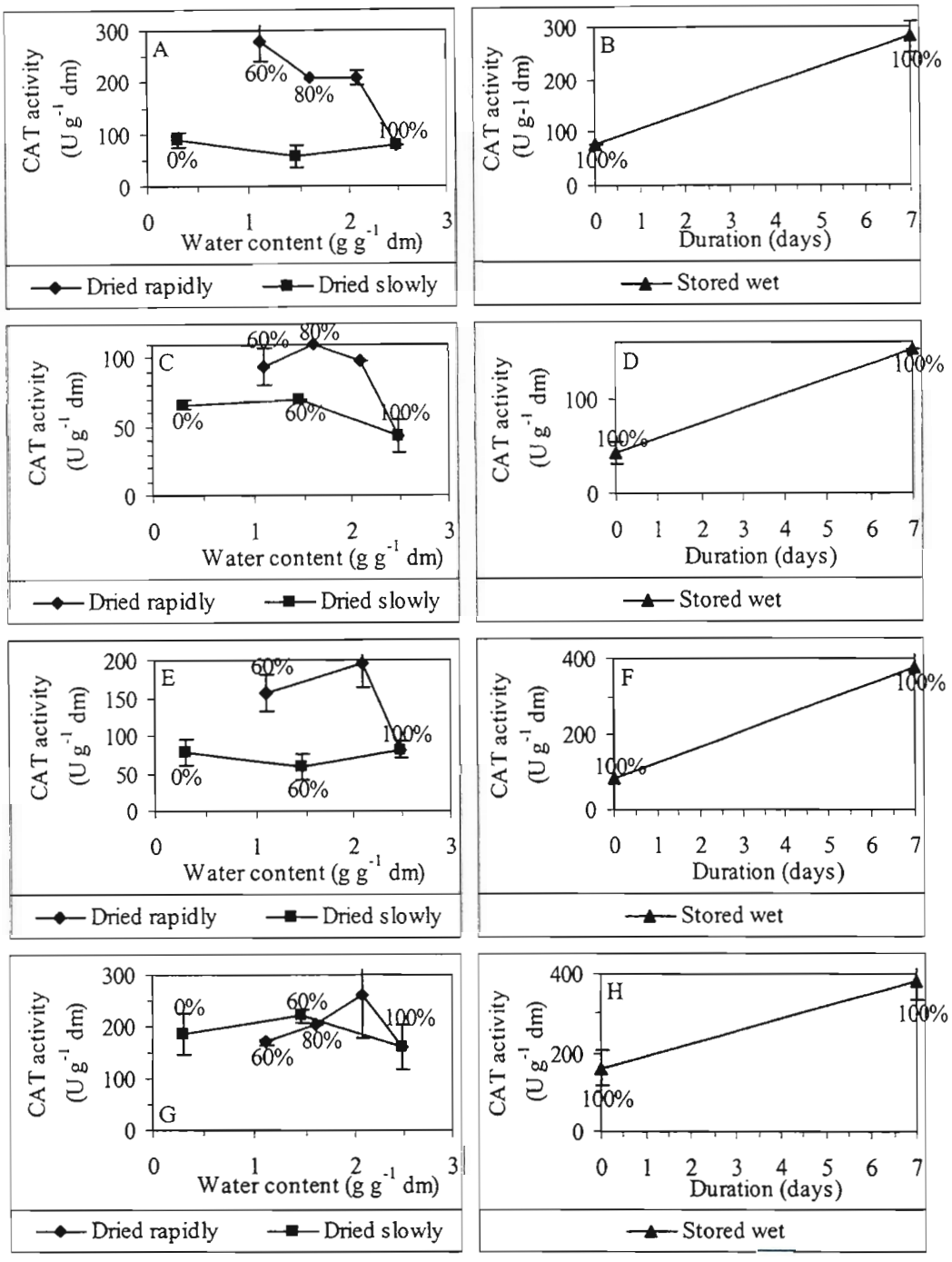

Figure 5.6 Activities of catalase in whole axes (A-B) and different axial tissues (hypocotyls [C-D], root primordia [E-F] and plumules [G-H]) of *A. marina* during rapid or slow drying or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below data symbols indicate germination. Data points without percentages are unchanged over the previous values. 1 unit (U) of CAT will decompose 1 μmol of hydrogen peroxide per min at pH 7.0 at 25 °C.

c. GR

The activity of GR statistically significantly increased by *c.* 240% during rapid drying of axes of *P. sativum* ($F = 10.50$ and $p = 0.01$ [Fig. 5.7A]). Similarly, GR activity was enhanced by a marginally significant *c.* 40% prior to the onset of germination loss upon slow dehydration ($F = 3.50$ and $p = 0.09$). During wet storage, the onset of loss of germination was preceded by a statistically siginificant *c.* 220% elevation in GR activity ($F = 20.40$ and $p = 0.02$ [Fig. 5.7B]).

The activity of GR in axes of *Q. robur* did not change significantly during rapid drying ($F = 0.80$ and $p = 0.60$ [Fig. 5.7C]). However, there was a statistically significant *c.* 70% decrease in GR activity before the onset of loss of germination upon slow dehydration ($F = 5.70$ and $p = 0.02$). The onset of germination loss was associated with a statistically significant *c.* 75% decline in the activity of GR during wet storage ($F = 5.50$ and $p = 0.03$ [Fig. 5.7D]).

No significant changes in the activity of GR accompanied rapid and slow drying of axes of *T. dregeana* ($F = 1.60$ and $p = 0.33$ and $F = 0.70$ and $p = 0.63$, respectively [Fig. 5.7E]). The onset of loss of germination was preceded by a marginally significant *c.* 50% decrease in GR activity during wet storage ($F = 6.00$ and $p = 0.06$ [Fig. 5.7F]) and a post mortem increase.

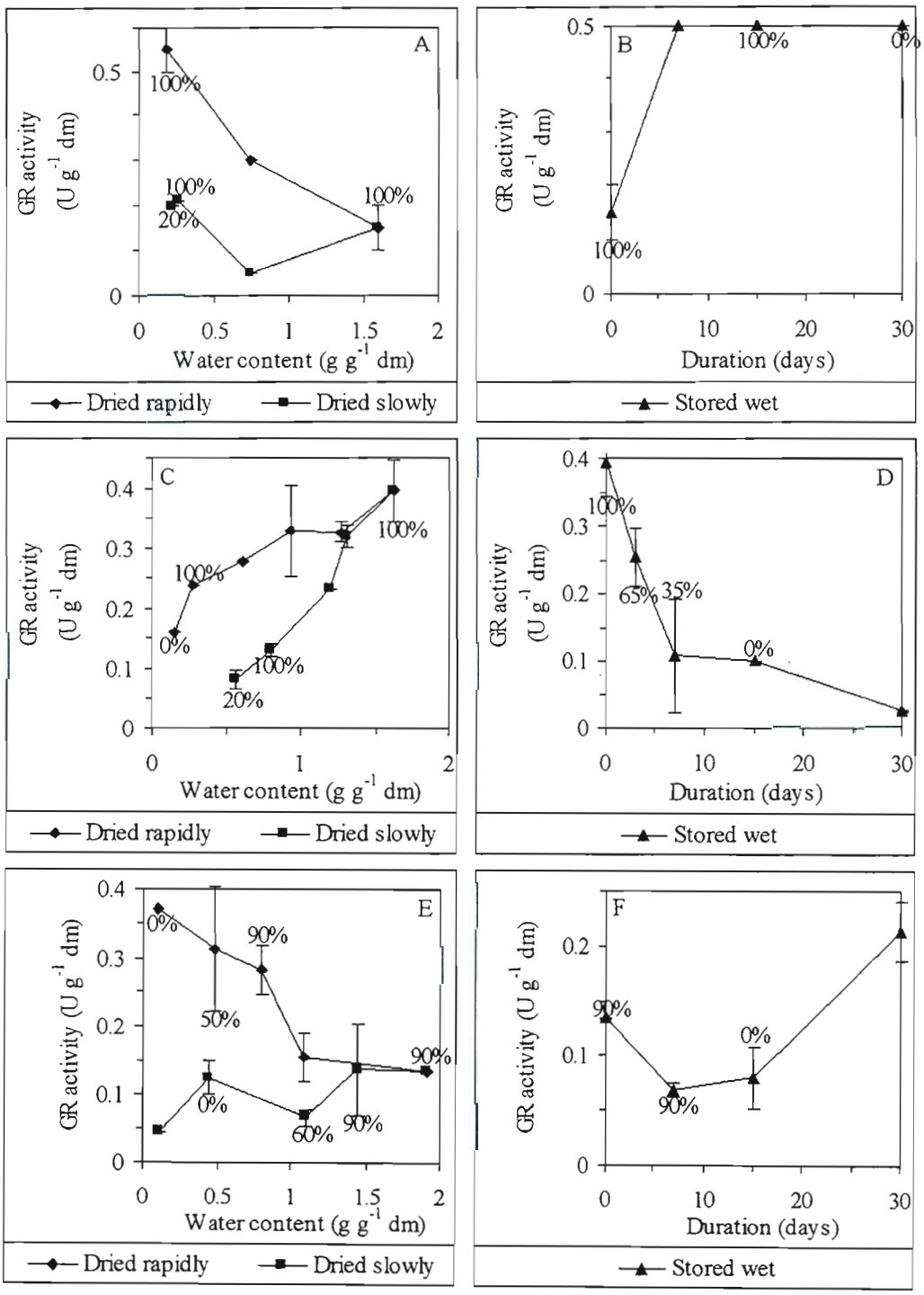

Figure 5.7 Activities of glutathione reductase in axes of *P. sativum* (A-B), *Q. robur* (C-D) and *T. dregeana* (E-F) during drying at different rates or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below data symbols indicate germination. Data points without percentages are unchanged over the previous values. 1 unit (U) of GR will reduce 1 μmol of oxidised glutathione per min at pH 7.6 at 25 °C.

The activity of GR in whole axes of *A. marina* decreased highly significantly by *c.* 90% during the initial 40% loss of germination upon rapid drying ($F = 33.74$ and $p < 0.01$[Fig. 5.8A]). Similarly, GR activity in axes was diminished by a highly significant *c.* 90% during the initial 40% germination loss upon slow dehydration ($F = 61.20$ and $p < 0.01$). During wet storage, the onset of loss of germination was preceded by a statistically significant *c.* 95% reduction in the activity of GR in axes ($F = 63.10$ and $p = 0.02$ [Fig. 5.8B]).

No significant changes in the activity of GR in *A. marina* hypocotyls were seen during rapid drying and wet storage ($F = 3.80$ and $p = 0.11$ and $F = 0.90$ and $p = 0.45$, respectively [Fig. 5.8C and D]). Loss of germination beyond the initial 40% was associated with a statistically significant *c.* 40% enhancement in GR activity in hypocotyls during slow dehydration ($F = 27.10$ and $p = 0.01$).

The activity of GR in root primordia of *A. marina* did not change significantly during rapid and slow drying and wet storage ($F = 2.60$ and $p = 0.23$, $F = 2.50$ and $p = 0.23$ and $F = 0.50$ and $p = 0.57$, respectively [Fig. 5.8E and F]).

No significant changes in the activity of GR in *A. marina* plumules were seen during rapid drying and wet storage ($F = 0.90$ and $p = 0.56$ and $F = 0.20$ and $p = 0.70$, respectively [Fig. 5.8G and H]). A statistically significant *c.* 90% decrease in GR activity in *A. marina* plumules was observed during the initial 40% loss of germination upon slow drying ($F = 25.70$ and $p = 0.01$).

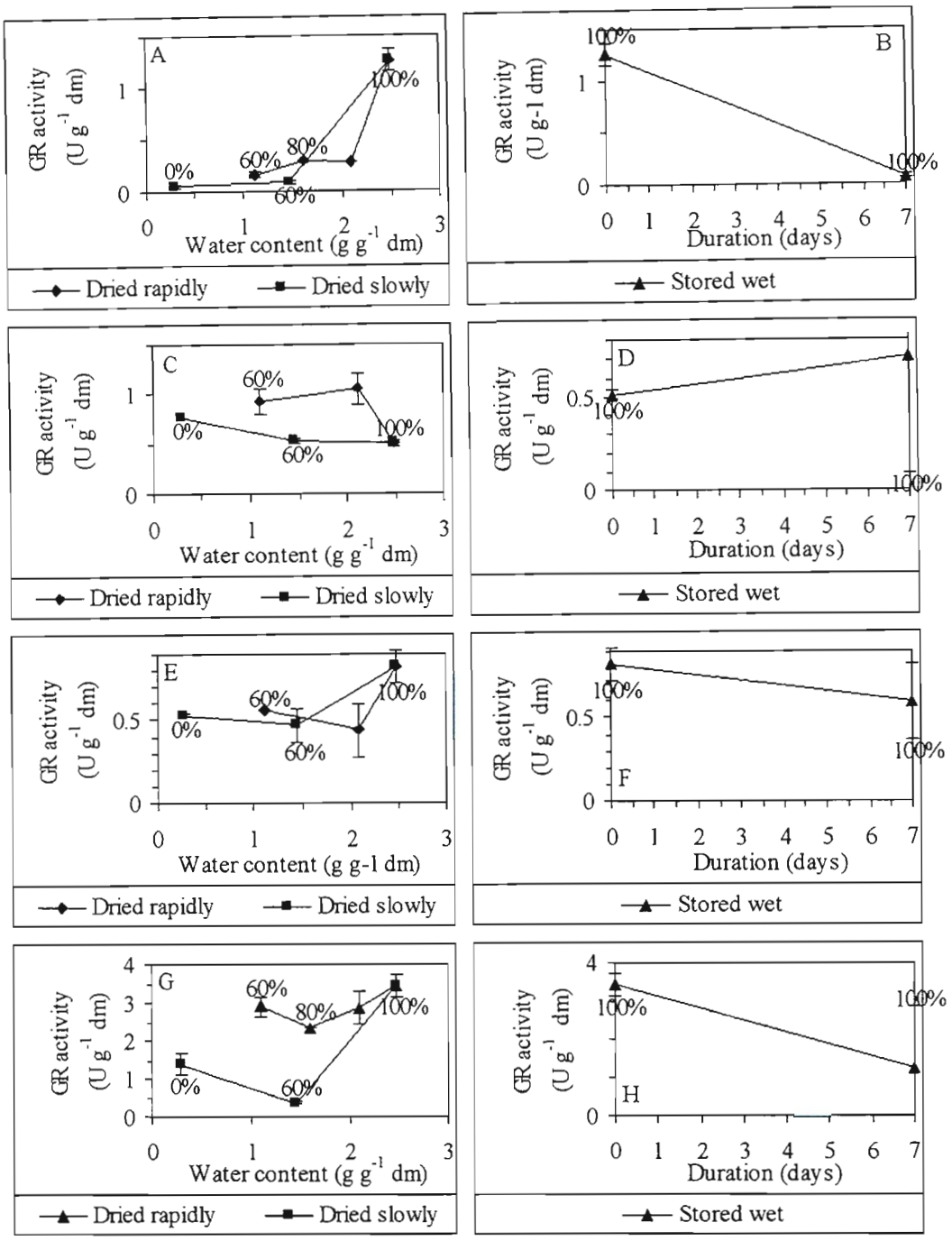

Figure 5.8 Activities of glutathione reductase in whole axes(A-B) and different axial tissues (hypocotyls [C-D], root primordia [E-F] and plumules [G-H]) of *A. marina* during rapid or slow drying or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below data symbols indicate germination. Data points without percentages are unchanged over the previous values. 1 unit (U) of GR will reduce 1 µmol of oxidised glutathione per min at pH 7.6 at 25 °C.

5.3.3 AsA levels

A marginally significant *c.* 50% decrease in the level of AsA took place during rapid drying of axes of *P. sativum* ($F = 3.50$ and $p = 0.09$ [Fig. 5.9A]) followed by an increase. Similarly, a marginally significant *c.* 28% decline in AsA level occurred pror to the onset of germination loss upon slow dehydration ($F = 3.52$ and $p = 0.09$) with a subsequent enhancement. The level of AsA did not change significantly during the experimental period upon wet storage ($F = 0.20$ and $p = 0.69$ [Fig 5.9B]).

Although the level of AsA during rapid and slow drying and wet storage of *Q. robur* axes were highly variable, they did not change significantly ($F= 1.30$ and $p = 0.32$, $F = 2.50$ and $p = 0.11$ and $F = 0.60$ and $p = 0.65$, respectively [Fig. 5.9C and D]).

A marginally significant *c.* 30% decrease in the level of AsA in axes of *T. dregeana* took place before the onset of the loss of germination during rapid drying, ($F = 2. 90$ and $p = 0.09$ [Fig. 5.9E]). However, no significant changes in AsA levels occurred upon slow dehydration and wet storage ($F = 2.20$ and $p = 0.21$ and $F = 2.40$ and $p = 0.25$, respectively [Fig 5.9E and F]).

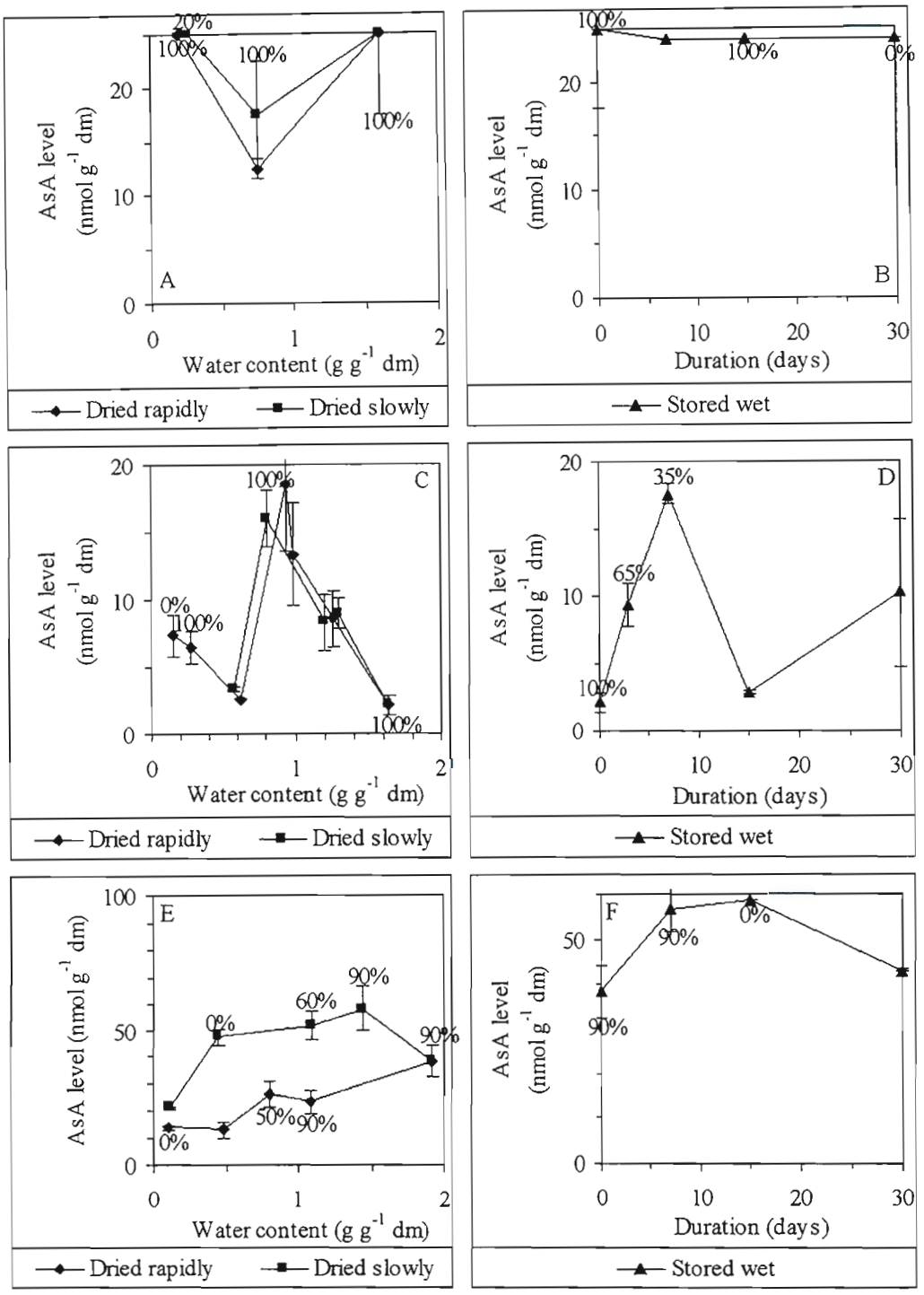

Figure 5.9 Levels of ascorbic acid in axes of *P. sativum* (A-B), *Q. robur* (C-D) and *T. dregeana* (E-F) during drying at different rates or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above or below or beside data symbols indicate germination. Data points without percentages are unchanged over the previous values.

No significant change in the level of AsA in whole axes of *A. marina* took place during rapid drying and wet storage ($F = 1.40$ and $p = 0.46$ and $F = 30.10$ and $p = 0.12$, respectively [Fig. 5.10A and B]). However, a statistically significant *c.* 80% decrease in AsA level in whole axes occurred during the initial 40% loss of germination upon slow dehydration ($F = 29.00$ and $p = 0.03$).

The level of AsA in *A. marina* hypocotyls did not change significantly during rapid and slow drying and wet storage ($F = 0.20$ and $p = 0.92$, $F = 0.70$ and $p = 0.59$ and $F = 4.30$ and $p = 0.29$, respectively [Fig. 5.10C and D]). Similarly, there were no significant changes in AsA level in root primordia of *A. marina* upon rapid and slow dehydration and hydrated storage ($F = 0.40$ and $p = 0.83$, $F = 1.90$ and $p = 0.35$ and $F = 4.30$ and $p = 0.29$, respectively [Fig. 5.10E and F]).

A marginally significant *c.* 10% decrease in the level of AsA was seen during the initial 40% loss of germination in *A. marina* plumules upon rapid drying ($F = 9.30$ and $p = 0.10$ [Fig. 5.10G]). Nonetheless, no significant changes in AsA levels in plumules were observed during slow dehydration and wet storage ($F = 0.40$ and $p = 0.72$ and $F = 19.80$ and $p = 0.14$, respectively [Fig. 5.10G and H]).

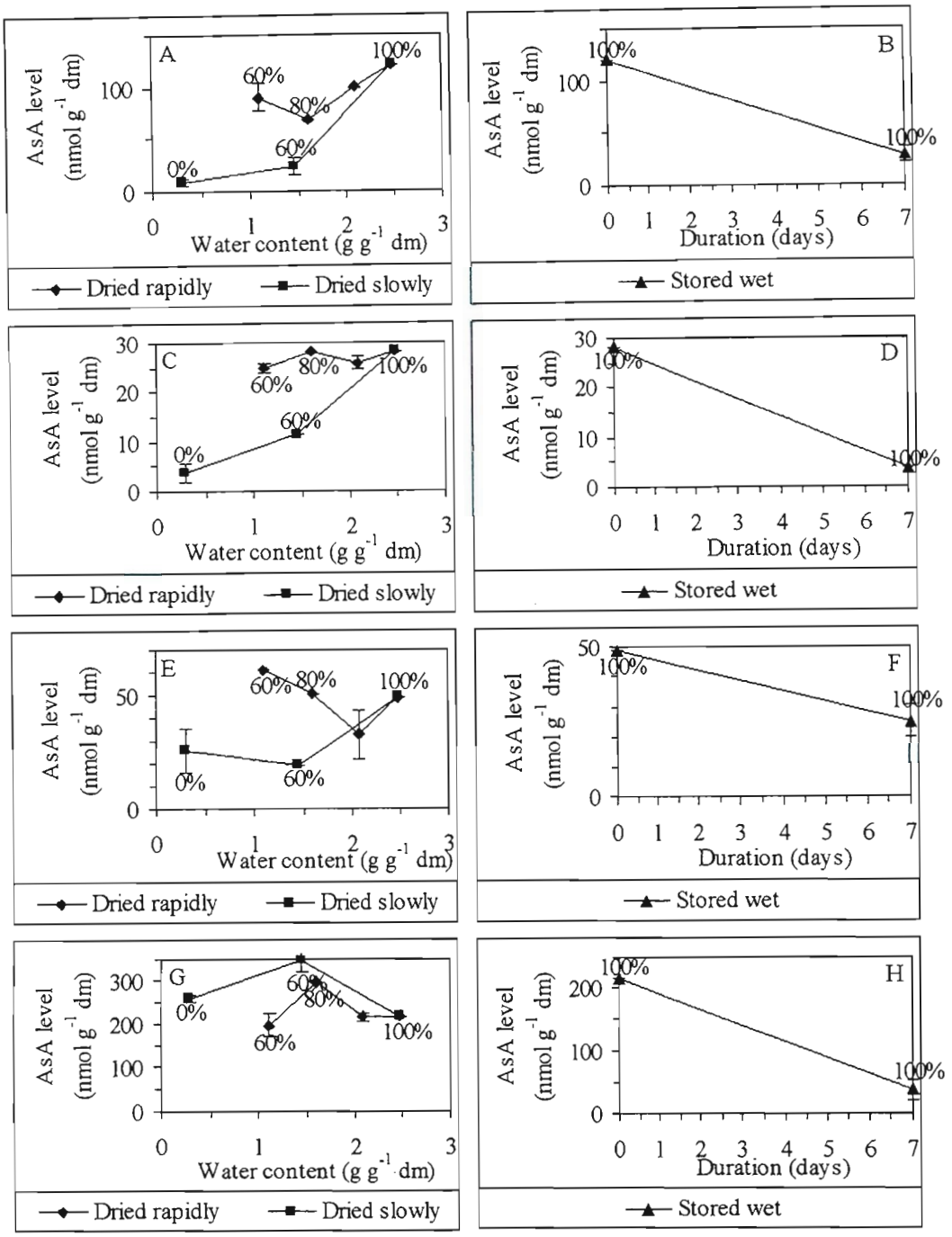

Figure 5.10 Levels of ascorbic acid in whole axes (A-B) and different axial tissues (hypocotyls [C-D], root primordia [E-F] and plumules [G-H]) of *A. marina* during rapid or slow drying or wet storage. Data points represent means of three replicate extractions. Error bars, in some cases hidden within the data symbols, show standard errors. Percentages above below data symbols indicate axis viability. Data points without percentages are unchanged over the previous values.

5.4 Discussion

An increase in the levels of hydroperoxides was seen in embryonic axes of several recalcitrant species during drying (Hendry *et al.*, 1992; Finch-Savage *et al.*, 1994; Chaitanya and Naithani, 1994; Chandel *et al.*, 1995; Li and Sun; 1999; Greggains *et al.*, 2001). Similarly, Greggains *et al.*, 2000b observed an enhancement in free radical activity and/or lipid peroxidation in *Q. robur* axes and *A. marina* plumules and root primordia although the elevation did not change with storage conditions of temperature and oxygen concentration. The results of the present study are generally in good agreement with those findings. An elevation in the hydroperoxide levels in *P. sativum*, *Q. robur* and *T. dregeana* axes was observed upon dehydration and/or wet storage such that the hydroperoxide levels were higher during slow than rapid desiccation of axes of *P. sativum*, *Q. robur* and *T. dregeana*. It is suggested that this event may be a result of metabolic imbalance and degradative processes in damaged and dead tissue, respectively.

However, the level of hydroperoxides in *A. marina* axes did not change during drying and wet storage. It is proposed that this observation may be a consequence of physical damage being more important than metabolic imbalance in these highly desiccation-sensitive axes. In addition, this event may be largely a result of degradative processes in dead tissue as a consequence of physical damage. In this regard, it is noteworthy that free radical processes in living and dead tissue differ both quantitatively and qualitatively (Hendry, 1993).

Nonetheless, an increase in hydroperoxide levels took place upon dehydration in the hypocotyls of *A. marina* such that it was higher upon rapid than slow desiccation. This observation supports those of Greggains *et al.* (2001) who showed that whole axes and different axis tissues of *A. marina* exhibit dissimilar responses to drying and possibly wet storage.

Rapid drying caused a decrease in the levels of hydroperoxide in axes of *P. sativum* and *T. dregeana*. It is suggested that the decline in the hydroperoxide levels may be a result of an increase in the activities and/or levels of free radical processing enzymes and/or antioxidants not studied in the present investigation such as guaicol peroxidase and/or tocopherol isomers and/or yet to be identified, respectively.

The activity of SOD in axes of *P. sativum* decreased during rapid drying. In contrast, it increased upon slow dehydration and wet storage. As a result, SOD activity

was higher during slow dehydration and hydrated storage than rapid drying. Thus, rapid desiccation had an adverse effect on SOD activity whereas slow drying and moist storage enhanced it. In this regard, it should be remembered *P. sativum* axes were shown to be minimally desiccation-sensitive and may be responding more like a desiccation-tolerant system, which withstand slow dehydration better than rapid desiccation, than a desiccation-sensitive one, where the converse is true.

The activity of SOD was diminished during slow drying of axes of *Q. robur*. In this regard, it is noteworthy that slow dehydration may be more damaging on the basis of the duration of the stress. These findings support the hypothesis that the differential effects of the three treatments may vary within system among species.

The activity of SOD in axes of *T. dregeana* was diminished during dehydration and such that it was higher upon slow than rapid dehydration. In contrast, SOD activity was enhanced by wet storage. The decrease in the activity of SOD is attributed to the adverse effect of water-stress on that enzyme. The increase is proposed to be a result of mild water-stress during hydrated storage being less damaging.

The activity of SOD in whole axes, but not the three axial tissues studied, of *A. marina* was elevated during rapid desiccation and moist storage such that it was higher upon rapid than slow drying. These findings concur with those of Greggains *et al.* (2001) which showed that various *A. marina* tissues respond differently to water-stress.

The activity of CAT decreased during slow drying and wet storage of axes of *Q. robur*. Similarly, CAT activity in *T. dregeana* axes declined during dehydration such the activity of CAT was higher upon rapid than slow desiccation. The reduction in CAT activity is attributed to the adverse effect of water-stress on that enzyme. In contrast, the activity of CAT in axes, but not the three axial tissues studied, of *A. marina* was enhanced during slow drying such that it was higher during rapid than slow dehydration.

The activity of GR in axes of *P. sativum* axes increased during drying and wet storage such that it was higher upon rapid than slow dehydration. This event may explain the decrease in the level of hydroperoxide in *P. sativum* upon rapid desiccation.

Conversely, a decrease in the activity of GR in axes of *Q. robur* was seen during slow drying and wet storage. Similarly, a decline in GR activity was observed upon

hydrated storage of *T. dregeana* axes. The activity of GR in axes of *A. marina* was also diminished during dehydration and moist storage such that GR activity was higher upon rapid rather than slow desiccation. Similarly, the activity of GR was reduced in *A. marina* plumules. In contrast, an enhancement in the activity of GR occurred in hypocotyls of *A. marina*.

Ultimately, no significant changes in the levels of AsA in axes of *Q. robur* were seen during drying and wet storage. While the AsA levels in axes of *P. sativum*, *T. dregeana* and *A. marina* were diminished during rapid and/or slow desiccation, the levels of AsA remained constant upon wet storage of axes of all species investigated. It is suggested that lower levels of AsA during dehydration may be a result of higher activities of GR and/or APOD and/or its greater involvement in the reduction of α-tocopherol and/or glutathione.

In conclusion, the previously-made observation of an increase in free radical activity and/or lipid peroxidation during desiccation and moist storage was confirmed by the results of the present study. In addition, free radical processes differed both between species and tissues. This difference is both quantitative and qualitative. For instance, there was a decrease in hydroperoxide level in *T. dregeana* and *P. sativum* axes with desiccation in contrast to all other species studied. Furthermore, free radical processes may differ quantitatively and qualitatively in living and dead tissues. For example, higher levels of hydroperoxides in axes of *A. marina* were seen upon rapid than slow desiccation, in which dead axes may have predominated, in contrast to all other species investigated.

Moreover, free radical processing enzymatic and low-molecular-weight non-enzymatic antioxidants were predominantly adversely affected by drying and wet storage in axes of *T. dregeana* and *Q. robur*. In contrast, no such effect was seen in *P. sativum* and *A. marina* axes. It is suggested that the latter phenomenon may be a result of germinating axes of *P. sativum* being largely at the desiccation-tolerant phase of early germination and those of *A. marina* overcome by physical damage at relatively high water contents (≥ 0.9 g g^{-1} dm). In addition, it appears that the differential effects of drying and hydrated storage may differ with species, system and enzyme.

Chapter 6

Overview and conclusions

6.1 Overview

The rate of water loss could be described by both an exponential and a modified inverse function for all species studied. However, the fit was better with the modified inverse relationship during drying of axes of *P. sativum*, *Q. robur* and *T. emetica*. In contrast, a better fit was obtained with the exponential function upon dehydration of *T. dregeana* and *A. marina* axes. Whereas the plot of water content against time fitted an exponential function better during rapid desiccation of axes of *S. madagascariensis*, the fit was better with a modified inverse function upon slow drying of *S. madagascariensis* axes.

In this regard, it is noteworthy that it was suggested that, as a generalisation, if tissue is dried relatively slowly, the relationship between water content and drying time is exponential (Pammenter *et al.*, 2002). In the present study, this statement may be true for *A. marina* axes, which dried at the slowest rate, but is not for axes of *T. dregeana*, which attained the second fastest dehydration rate (Table 2.1). It is possible that the mass of the indidvidual axes and, perhaps more accurately, the total mass of the material dried are better predictors of the kinetics of desiccation of excised axes than drying rate *per se*.

Oily seeds generally dried faster than their starchy counterparts. It should be remembered that the loss of water from tissues depends on their surface area which, in turn, is influenced by the size and shape of the tissue, the amount of material to be dried and the hydraulic conductivity of the tissue (Pammenter *et al.*, 2002; Sun, 2002). The latter would be affected by the chemical composition of the tissue.

Axes of all species investigated maintained a constant water content during wet storage. This response may be expected given that the surrounding air during storage was vapour-saturated.

Plant tissues incur damage as a result of removal of water from cells. At high (≥ 0.9 g g^{-1} dm [*sensu* Vertucci, 1990]) water contents, damage consequent upon physical processes takes place. Subsequently, changes in the metabolic status of cells occur at

intermediate (between 0.5 and 0.9 g g^{-1} dm [*sensu* Vertucci, 1990]) water contents. Damage that is consequent upon loss of water that is intimately associated with surfaces of macromolecules can be considered desiccation damage *sensu stricto* (reviewed by Pammenter and Berjak, 1999; Walters *et al.*, 2001). Such an event can be seen at water contents below 0.2 – 0.25 g g^{-1} dm.

Loss of viability was observed mainly at low (≤ 0.5 g g^{-1} dm [*sensu* Vertucci, 1990]) water contents during drying of axes of *P. sativum*, *Q. robur*, *S. madagascariensis* and *T. emetica* although it took place at higher water contents upon slow than rapid dehydration (Table 6.1). As a result, it may be inferred from these observations that desiccation damage (*sensu stricto*) underlied viability loss during desiccation of the axes of these species.

On the other hand, viability loss of axes of *T. dregeana* occurred predominantly at intermediate (between 0.5 and 0.9 g g^{-1} dm) and high (≥ 0.9 g g^{-1} dm) water contents during rapid and slow drying, respectively (Table 6.1). Thus, it may be concluded that metabolic and physical damage largely caused loss of viability in *T. dregeana* axes upon rapid and slow dehydration, respectively.

Loss of viability was seen primarily at high (≥ 0.9 g g^{-1} dm) water contents during drying of axes of *A. marina* (Table 6.1). Hence, it is likely that physical damage underlied loss of viability during dehydration of axes of *A. marina*.

Consequently, it appears that desiccation-sensitive seeds can be grouped into three categories on the basis of the prevalent mechanism of viability loss during drying: (1) minimally desiccation-sensitive seeds such as *P. sativum*, *Q. robur*, *S. madagascariensis* and *T. emetica*, which die mainly as a result of desiccation damage *sensu stricto*, (2) moderately desiccation-sensitive seeds such as *T. dregeana*, which lose viability mostly due to damage associated with deranged metabolism and (3) highly desiccation-sensitive seeds such as *A. marina*, which are essentially killed by physical damage. Irrespective of the mode of dying, it is noteworthy that the differential effect of the rate of drying is apparent.

As was noted in previous studies (e. g. Ntuli *et al.*, 1997; reviewed by Pammenter *et al.*, 2002), the tetrazolium (TZ) test overestimated the viability of axes of *A. marina*, *T. emetica* and *S. madagascariensis* in relation to the germination test (Table 6.1). This discrepancy is usually attributed to the need for extensive experience of the

evaluation of each species (reviewed by Pritchard, 1996). In contrast, the results of the TZ test of *P. sativum*, *Q. robur* and *T. dregeana* were in agreement with those of *in vitro* culture.

Similarly, the germination and TZ tests were in concurrence with regards to the lifespans of axes during wet storage. Hence, it appears that the TZ test may be a better indicator of viability during hydrated storage than drying, It is suggested that the longer period of exposure and the consequent longer measuring intervals during moist storage than dehydration may explain this anomaly. Nonetheless, the lifespans during wet storage seem to be a poor discriminator of the degree of desiccation sensitivity as assessed by the 'critical water content' during drying. In this regard, it is worth remembering that hydrated storage is equivalent to long-term and low-intensity water-sress.

In addition, it appears that both viability tests gave lower than expected survival of the axes of *Q. robur* during slow drying and wet storage (Table 6.1). It is suggested that this discrepancy is a result of the high temperature (20°C) at which these treatments were conducted for this temperate species. However, temperature was not a major factor on longevity upon rapid dehydration, presumably because of the limited duration (24 h) of this procedure. Consequently, rapid desiccation is recommended as the method of choice in determining the 'critical water content'.

The relationship between the conductivity of electrolyte leachate and water content during drying of axes of all species studied did not show the typical pattern of a constant leakage to a 'critical water content', at which point a sudden increase is observed. Rather, there was a gradual and progressive increase in leakage as dehydration proceeded. Pammenter *et al.* (1998) observed a similar pattern during desiccation of whole seeds of *Ekebergia capensis*. This observation suggest that there was progressive deterioration of cellular membranes during drying and wet storage of the axes of all species investigated, possibly as a result of oxidative attack.

Nonetheless, less electrolyte leakage took place during rapid, than slow drying of axes of all species studied (Table 6.1). It is suggested that less membrane damage occurred during rapid than slow dehydration because of the limited duration those structures were subjected to stress during rapid desiccation.

In contrast, the axes of *T. dregeana* from seeds harvested in 2001 showed the classic pattern. This apparent anomaly, as with initial water contents, drying curves and viability, is attributed to interseasonal variability in post-harvest behaviour of recalcitrant seeds (reviewed by Berjak and Pammenter, 1997a,b; 2001; 2004).

It appears that vigour as assessed by TZ, germination and conductivity tests may play a role in determining whether the responses of axes of desiccation-sensitive seeds to desiccation and wet storage show a typical pattern or not. For instance, axes of *T. dregeana* from seeds harvested in 1999, which did not show the classical pattern, displayed lower vigour than those for seeds harvested in 2001, which demonstrated the typical pattern in the present study. It is suggested that this phenomenon is a consequence of the ability of the more vigorous axes to better withstand stress to a certain point (the 'critical water content'), thereafter abruptly failing should the stress persists.

The activity of phosphofructokinase (PFK) in axes of *P. sativum* remained constant during drying and wet storage (Table 6.1). Similarly, PFK activity did not change significantly upon wet storage of *A. marina* axes. However, the activity of PFK in axes of *T. dregeana* and *A. marina* was reduced upon drying such that higher activity was seen during rapid than slow dehydration of *T. dregeana* axes. Similarly, a decrease in PFK activity in axes of *T. dregeana* occurred during hydrated storage. There was also a decline in the activity of PFK upon slow desiccation of *Q. robur* axes. In contrast, an increase in the activity of PFK took place upon rapid dehydration and moist storage of *Q. robur* axes. These observations generally support those of Leprince *et al.* (1993a) who described PFK activity as only slightly sensitive to desiccation.

No significant changes in malate dehydrogenase (MDH) were seen in axes of *P. sativum* for all treatments. Similarly, MDH activity remained constant during wet storage of *T. dregeana* axes. The activities of MDH in axes of *T. dregeana* and *A. marina* decreased upon drying such that higher activities were observed during rapid dehydration in comparison to slow desiccation of *T. dregeana* axes (Table 6.1). There was also a decline in MDH activity in axes of *T. dregeana* upon hydrated storage. In contrast, an increase in the activity of MDH was recorded in axes of *Q. robur* during drying and hydrated storage such that it was higher upon slow than rapid dehydration. These findings mainly agree with those of Leprince *et al.* (1993a) who described the activity of MDH as mildly sensitive to desiccation.

An increase in the level of the oxidised form of nicotinamide dinucleotide (NAD) was observed during rapid drying of axes of *P. sativum* (Table 6.1). Similarly, there was an enhancement in NAD level upon wet storage of *T. dregeana* axes. In contrast, a decrease in the levels of NAD occurred in axes of *Q. robur* and *A. marina* during slow dehydration. In addition, there was a decline in NAD level upon hydrated storage of *Q. robur* axses. It is suggested that the decreases in the level of NAD are a result of the impairment of the NADH dehydrogenases of NADH-ubiquinone (coenzyme Q) reductase (complex I) and NADH-cytochrome c reductase (complex IV) of the electron transport chain. It should be remembered that Leprince *et al.* (1993a) described NADH dehydrogenase in maize as highly sensitive to drying.

A decrease in the levels of hydroperoxides in axes of *P. sativum* and *T. dregeana* was seen as rapid drying proceeded, such that they were higher in axes during slow dehydration compared to those which were desiccated rapidly (Table 6.1). In contrast, hydroperoxide levels increased during hydrated storage of *P. sativum* and *T. dregeana* axes. The level of hydroperoxides in axes of *A. marina* remained constant during drying and moist storage such that it was higher upon rapid than slow dehydration. The increases in hydroperoxide levels is suggested to be a result of an enhanced leakage of electrons from electron transport chain due to water-sress. It is possible that the decreases in the level of hydroperoxides in axes of *P. sativum* and *T. dregeana* may be associated with an elevation in antioxidants not studied here (such as guaicol peroxidase and tocopherol) and/or yet to be identified.

The activity of superoxide dismutase (SOD) in axes of *P. sativum* decreased during rapid drying (Table 6.1). However, it increased upon slow dehydration and wet storage. Whereas SOD activity was elevated during rapid desiccation and hydrated storage of *Q. robur* axes, it was reduced upon slow drying. The activity of SOD in axes of *T. dregeana* was diminished during dehydration such that it was lower upon rapid than slow desiccation. In contrast, the activity of SOD was enhanced during rapid drying and wet storage of *A. marina* axes such that it was higher upon rapid than slow dehydration. There was no pattern that was common to more than one species. It appears that the response of SOD activity may vary highly between species. The reason for this behaviour is unknown.

The activity of catalase (CAT) did not change significantly during drying and during wet storage of axes of *P. sativum*. However, CAT activity in *Q. robur* axes was

reduced during slow dehydration and hydrated storage. Similarly, the activity of CAT was diminished upon desiccation of axes of *T. dregeana* such that CAT activity was higher upon rapid rather than slow drying. In contrast, it increased in *A. marina* axes during slow desiccation.

The reduction in the activities of SOD and CAT are suggested to be a result of adverse effects of water-sress on those enzymes. In contrast, the elevation of the activities of those enzymes in axes of *A. marina* may be a result of some undefined "unblocking of inhibitors" of those enzymes in dead tissue.

Whereas the activity of glutathione reductase (GR) in axes of *P. sativum* increased during drying and wet storage, GR activities decreased in *Q. robur* and *A. marina* axes such that they were higher upon rapid than slow dehydration in all three cases. The activity of GR was diminished during wet storage of axes of *T. dregeana*. However, it remained constant upon rapid and slow drying. The reason for the elevation of GR activity in *P. sativum* axes is thought to be a defence mechanism against oxidative stress. In contrast, the decrease in the activity of GR could be attributed to the adverse effect of water-sress on that enzyme.

Finally, the level of ascorbic acid (AsA) in axes of *Q. robur* remained constant during drying and wet storage. However, a decrease in AsA levels occurred upon dehydration and hydrated storage of *P. sativum*, *T. dregeana* and *A. marina* axes. The lack of an effect of water-sress on AsA levels in *Q. robur* axes is proposed to be related to the degree of desiccation sensitivity of that species. It is suggested that higher levels of AsA upon desiccation and wet storage may be a result of higher activities of dehydroascorbate reductase (DHAR).

Table 6.1 Summary of the responses of axes of various species to fast (**F**) or slow (**S**) drying or wet storage (**W**). Arrows indicate a decrease (↓), an increase (↑) or no apparent change (→). Double arrows denote changes that were discernibly larger than that of the other treatment(s) (↓ or ↑), respectively. – represents no decrease in viability. Viability is expressed in terms of 'critical water content's' or storage lifespans.

Species Response	Treatment	*P. sativum*	*Q. robur*	*S. madagascariensis*	*T. emetica*	*T. dregeana*	*A. marina*
"Critical water content" ($g\ g^{-1}$ dm) **or storage lifespan** (days) as determined by TZ staining	**F**	-	0.27	0.14	0.22	0.8	-
	S	0.26	0.8	0.4	0.4	1.4	1.4
	W	15	0	7	7	7	7
'Critical water content' ($g\ g^{-1}$ dm) **or storage lifespan** (days) as determined by germination	**F**	–	0.27	0.37	0.4	0.8	1.1
	S	0.26	0.8	0.6	0.6	1.4	1.4
	W	15	0	7	7	7	7
Leakage ($mS\ cm^{-1}\ g^{-1}$ dm)	**F**	↑	↑	↑	↑	↑	↑
	S	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
	W	↑	↑	↑	↑	↑	↑
PFK activity (U g^{-1} dm)	**F**	→	↑	not determined	n. d.	↓	↓
	S	→	↓	(n. d.)		↓↓	↓
	W	→	↑			↓	→
MDH activity (U g^{-1} dm)	**F**	→	↑	n. d.	n. d.	↓	↓
	S	→	↑↑			↓↓	↓
	W	→	↑			→	↓
NAD level ($\mu mol\ g^{-1}$ dm)	**F**	↑	→	n. d.	n. d.	→	→
	S	↑	↓			→	↓
	W	→	↓			↑	→
Hydroperoxide level ($A_{505}\ g^{-1}$ dm)	**F**	↓	↑	n. d.	n. d.	↓	→
	S	→	↑↑			→	→
	W	↑	↑			↑	→

Species Response	Treatment	*P. sativum*	*Q. robur*	*S. madagascariensis*	*T. emetica*	*T. dregeana*	*A. marina*
SOD activity ($U\ g^{-1}$ dm)	**F**	↓	→	n. d.	n. d.	↓↓	↑
	S	↑	↓			↓	→
	W	↑	→			↑	↑
CAT activity ($U\ g^{-1}$ dm)	**F**	→	→	n. d.	n. d.	↓	→
	S	→	↓			↓↓	↑
	W	→	↓			→	→
GR activity ($U\ g^{-1}$ dm)	**F**	↑↑	→	n. d.	n. d.	→	↓
	S	↑	↓			→	↓↓
	W	↑	↓			↓	↓
AsA level (nmol g^{-1} dm)	**F**	↓	→	n. d.	n. d.	↓	→
	S	↓	→			→	↓
	W	→	→			→	→

6.2 Conclusions

The responses of axes of different species to desiccation vary among harvests, tissues and species. It appears that while differences among harvests may be related to vigour, those among species may be determined by the level of desiccation-sensitivity. It also seems that changes in metabolism and the failure of the free radical processing system may underlie seed desiccation-sensitivity. In addition, the differential effects of water-sress appeared to vary with enzyme, tissue, system and species.

6.3 Further studies

It would be worthwhile to study further other antioxidants, such as tocopherol, in axes of *P. sativum* and *T. dregeana* to ascertain if any of them may explain the decrease in lipid peroxidation in axes of that species as rapid drying proceeded. In addition, it would to interesting to follow the control, at molecular level, of the activities of respiratory enzymes, PFK, MDH and NAD dehydrogenase, in *Q. robur* axes, where an increase in the activities was seen. Moreover, it would be worthwhile to study further the activity of NADH dehydrogenase in axes of *A. marina* in which there were decreases in the levels of NAD. A closer look at the activity of CAT in *A. marina* axes is recommended as it increased during slow drying.

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