# ASPECTS OF THE INFLUENCE OF TEMPERATURE ON THE DESICCATION RESPONSES OF SEEDS OF *Zizania palustris* (WILD RICE)

by

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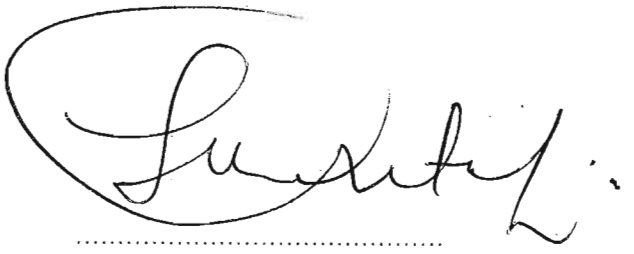
## PREFACE

The experimental work described in this dissertation was carried out in the Cell Biology Research Laboratory in the Department of Biology, University of Natal, Durban, under the supervision of Professors P. Berjak and N. W. Pammenter.

This 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

Seeds of wild rice (*Zizania palustris* var. *interior*) have been reported to show highest survival when dehydrated at 25 °C. It has also been reported that axis cells sustained least damage at this drying temperature.

In the present study, a linear relationship between drying rate and dehydration temperature was established. Whereas highest positive tetrazolium staining and lowest leakage were recorded for seeds that were dehydrated at 25 °C, maximum germination was recorded for seeds dried at 20 °C. A proportion of seeds showed the presence of glasses, irrespective of the dehydration temperatures used. Parameters of the glass to liquid transition, however, correlated with neither water content nor sugar profiles. The ratio of raffinose to sucrose was similar among all the treatments. A hydroperoxide test revealed a linear relationship between peroxide levels and temperature of drying although the levels of fatty acids were not correlated with hydroperoxide levels. Butanal levels and total aldehydes evolved, on the other hand, showed a high negative correlation with peroxide levels. Electron microscopy showed that the variability and relative abundance of peripheral membrane complexes (PMCs) was the highest for cells of embryonic axes dehydrated at 25 °C and the lowest for embryonic axes of seeds dried at 10 °C. Furthermore, intramembrane particles (IMPs) were evenly distributed in cells of axes dried at 25 or 37 °C. In contrast, membranes of cells of axes dehydrated at 10 °C showed large IMP-free areas. The relative abundance of IMPs was the highest for cells of embryonic axes dried at 25 °C, and the lowest for cells of axes dehydrated at 10 °C. From these observations, it is suggested that membrane phase transition, with the concomitant elimination of proteins, accompanies

dehydration of *Z. palustris* seeds at 10 °C, whereas at 37 °C peroxidation may predominate.

## KEY TO SYMBOLS AND ABBREVIATIONS

ABA = abscisic acid

DMSO = dimethyl sulfoxide

DSC = differential scanning calorimetry

EM = electron microscopy

ENDOR = electron nuclear double resonance

EPR = electron paramagnetic resonance

ESR = electron spin resonance

FFA = free fatty acid

FTIR = Fourier transform infrared (spectroscopy)

GA = gibberellic acid

GC = gas chromatography

$g\ g^{-1}$ d/ww = g $H_2O$ / g dry/wet weight

HPAEC = high performance anion exchange chromatography

HPLC = high pressure liquid chromatography

HRSEM = high resolution scanning electron microscopy

IAA = indole-3-acetic acid

IBPGR = International Board for Plant Genetic Resources

IMP = intramembrane particle

LEA = late embryogenic accumulated (proteins)

PAD = pulsed amperometric detector

PGR = plant growth regulator

PC = phosphatidylcholine

PE = phosphatidylethanolamine

PL = phospholipid

PMC = peripheral membrane complex

RH = relative humidity

SEM = scanning electron microscope

TEM = transmission electron microscope

$T_g$ = glass to liquid transition temperature

$T_m$ = liquid crystalline to gel phase transition temperature

TTC = 2,3,5-triphenyltetrazolium chloride

TTT = topographical tetrazolium test

TZ = tetrazolium

WC = water content

ZR = zeatin riboside

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# CHAPTER ONE

# GENERAL INTRODUCTION

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## 1.1 General background

Traditionally, seeds have been classified as orthodox (desiccation-tolerant) or recalcitrant (desiccation-sensitive) depending on their sensitivity to desiccation and chilling and their storage lifespans (Roberts, 1973). Orthodox seeds tolerate dehydration and chilling to very low water contents [5% or less, wet weight basis (Ellis *et al.*, 1990b)] and temperatures {-18 °C or less [ International Board for Plant Genetic Resources (IBPGR), 1976]} and can survive in storage at -18 °C for long periods [up to a 100 years or more (IBPGR, 1976)]. Recalcitrant seeds, however, show variability in their sensitivities to desiccation and chilling and storage lifespans and hence have been categorized as minimally, moderately and highly recalcitrant (Farrant *et al.*, 1988). Recently, a third category of seeds - intermediate - has been identified (Ellis *et al.*, 1990a; 1991). They tolerate dehydration to low water contents but become sensitive to low temperatures in this state.

In an attempt (which was not subsequently pursued) to use more scientific terminology, Berjak *et al.* (1989) used the term poikilohydrous to refer to orthodox seeds, 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). Recalcitrant seeds, on the other hand, 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 (Pammenter *et al.*, 1991). 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 aquatic or marshy environments and humid forests, usually where there is no temperature constraint (Roberts and King, 1980). In such environments, there can be little selective advantage to maturation drying and dormancy. The lack of maturation drying and dormancy and the inability to tolerate much post-shedding desiccation might be viewed as an evolutionary 'hangover' on the assumption that the acquisition of desiccation tolerance occurred subsequent to the evolution of the seed habit (Berjak *et al.*, 1989). In this regard, it is interesting to note that many of the extant gymnosperms of tropical/sub-tropical distribution are apparently recalcitrant (Berjak *et al.*, 1989; von Teichman and van Wyk, 1994).

### 1.2 Seed development and germination

The differences in post-shedding behaviour must arise as a result of differences in pre-shedding development. In contrast to orthodox seeds, 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, 1991) and *Quercus robur* L. (Finch-Savage, 1992; Finch-Savage *et al.*, 1992; Grange and Finch-Savage, 1992). This scenario makes comparison of the acquisition or non-acquisition of desiccation tolerance between the two seed types in terms of their development difficult.

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

mangrove, *Avicennia marina* (Berjak *et al.*, 1984; Farrant *et al.*, 1985; 1992a; b; 1993a; b; c). It is not unreasonable to expect that other recalcitrant seeds as well 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.

### 1.2.1 Histodifferentiation and embryogenesis

In common with orthodox seeds, recalcitrant seeds undergo the process of growth by both cell expansion and reserve deposition (Farrant *et al.*, 1993b). Those authors contend that the processes of histodifferentiation and embryogenesis are under the same plant growth regulator (PGR) control and are essentially similar in the two seed types. Essentially, indole-3-acetic acid (IAA), cytokinins and gibberellic acid (GA) are thought to promote the formation of embryonic structures and reserve repositories.

### 1.2.2 Reserve accumulation and utilization

The nature and the quantity of the reserves accumulated have been related to the germination characteristics, especially 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 not surprising that recalcitrant seeds show variability in the nature and quantity of reserves accumulated. While *A. marina* accumulates 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. Kutze (Devey *et al.*, 1987),

*Podocarpus henkelii* (Dodd *et al.*, 1989) and *Landolphia kirkii* Dyer (Berjak *et al.*, 1992)] do accumulate various complex reserves similarly to orthodox seeds.

Control of reserve accumulation appears to differ in the two seed types. For example, Farrant *et al.* (1993c) report "exceptionally" high levels of cytokinins, especially zeatin riboside (ZR) in embryos of *A. marina* during reserve accumulation. This is thought to promote a sink for the import of assimilates. Those authors further report a decline in the levels of this cytokinin towards the end of development. The high levels of cytokinins are thought to promote reserve utilisation in these metabolically active seeds (Farrant *et al.*, 1992a).

A further difference between the two seed types as regards control of reserve accumulation and utilisation concerns abscisic acid (ABA). This PGR is widely believed to play a prominent rôle in reserve accumulation, particularly in the accumulation of proteins, in orthodox seeds (e.g. Quatrano, 1987; Kermode, 1990). Recalcitrant seeds, once more, show variability as regards levels of ABA during development. While embryonic axes of *A. marina* show insignificantly low levels of this PGR, together with a lack of storage proteins (Farrant *et al.*, 1992a; 1993c), cotyledons of *Quercus robur* maintain high levels of ABA almost until shedding (Finch-Savage *et al.*, 1992). Peak ABA levels have subsequently been shown to be significantly lower than those reported for many orthodox seeds (W. E. Finch-Savage[1] and J. M. Farrant[2], pers. comm.).

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[2]Jill M. Farrant, Department of Botany, University of Cape Town, Rondebosch 7700, South Africa.

### 1.2.3 Developmental stasis

For many orthodox seeds, it has been shown that isolation of the embryo from covering tissues allows germination to occur precociously. This has been shown for a variety of gramineous seeds (Bartels *et al.*, 1988; Bochicchio *et al.*, 1988; Rasyad *et al.*, 1990), legumes (Quebedeaux *et al.*, 1976; Long *et al.*, 1981; Ackerson, 1984), castor bean (Kermode and Bewley, 1988) and cotton (Ihle and Dure, 1972). Application of ABA, however, prevents the germination of isolated 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 thought to maintain the embryo in the developmental disposition (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 (Farrant *et al.*, 1993b), although the ABA is extra-embryonically located, in the pericarp. In non-dormant orthodox seeds, levels of ABA decline during maturation drying (King, 1976; Kermode, 1990) and the desiccated state prevents germination.

### 1.2.4 Acquisition of germinability

The acquisition of germinability is similar in both orthodox and recalcitrant seeds. 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 (Oberndorf *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). Gibberellins and/or other PGRs are also necessary for

seedling establishment in orthodox seeds. It is worth noting 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).

### 1.2.5 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).

### 1.2.6 Acquisition of desiccation tolerance

A number of theories have been proposed to explain the mechanism of desiccation tolerance. Presently however, none of them seems to explain the phenomenon fully.

#### a. The role of oligosaccharides

The water replacement hypothesis (Clegg, 1986; Crowe and Crowe, 1986) is one of the earliest theories attempting to account for desiccation tolerance. It proposes that during dehydration sugars replace water on macromolecular surfaces, so enabling stabilization of membranes in the desiccated state. High levels of sugars, particularly sucrose, raffinose and stachyose, have been suggested to afford such a mechanism in

orthodox seeds (Leopold and Vertucci, 1986; Koster and Leopold, 1988; Chen and Burris, 1990; Leprince *et al.*, 1990a). These observation are, however, confounded by the fact that high levels of soluble sugars, particularly stachyose, occur in the highly recalcitrant seeds of *A. marina*. Also, sucrose comprises 45% of the total sugar content of the highly recalcitrant *Camellia sinensis* (tea) seeds, consisting of 11% of the dry mass of those seeds (Berjak *et al.*, 1989). Nevertheless, seeds of both *A. marina* and *C. sinensis* are highly desiccation-sensitive.

There is a further complication; in *C. sinensis* and other recalcitrant seeds, dehydration-related damage occurs at hydration levels far higher than those at which water would be removed from membrane surfaces (Berjak *et al.*, 1989; 1992; Pammenter *et al.*, 1991). In this regard, some authors (Williams and Leopold, 1989; Koster, 1991) have postulated that, perhaps, vitrification is the major mechanism by which desiccation tolerance is achieved in orthodox seeds. This contention is based on evidence that indicates the formation of the high-viscosity, glassy (vitrified) state when a critical proportion of oligosaccharides to water is reached. Those authors suggest that in this state intracellular movement of both molecules and chemical species, is virtually halted, thus imposing a stasis on metabolic and deleterious reactions. However, considering the high water contents at which slowly-dried recalcitrant seeds die (Pammenter *et al.*, 1993) it is unlikely that achievement of the glassy state is a feasibility, except perhaps when excised axes survive very rapid dehydration to appropriate water contents.

**b. Accumulation of complex reserves and the degree of vacuolation**

It has been suggested that the accumulation of complex reserves and the consequent low level of vacuolation might limit the mechanical disruption caused by dehydration and so contribute to tolerance of desiccation (Kermode and Bewley, 1986; Kermode, 1990). This contention is supported by the reverse scenario in *A. marina* seeds, which remain highly desiccation-sensitive. Those 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 include, among others, *Quercus alba* (Bonner, 1976), *Auracaria 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 loss of water than those of *A. marina* (Farrant *et al.*, 1985; 1986; 1993a), all are notably desiccation-sensitive.

**c. Late embryogenic accumulated (LEA) proteins**

As the name so succinctly suggests, a variety of orthodox seeds accumulate a set of dehydration- and/or ABA-inducible hydrophilic proteins during late stages of embryogenesis (Aspart *et al.*, 1984; Williamson *et al.*, 1985; Bartels *et al.*, 1988; Bochicchio *et al.*, 1988; Goday *et al.*, 1988; Rosenberg and Rinne, 1988; 1989; Blackman *et al.*, 1991; Bradford and Chandler, 1992). Some of these proteins have been implicated in the mechanism of seed desiccation tolerance (Kermode, 1990; Bewley and Olivier, 1991; Blackman *et al.*, 1991; Bradford and Chandler, 1992) or in protection against dehydration in seedlings (Gómez *et al.*, 1988; Mundy and Chua, 1988; Close *et al.*, 1989). They have been suggested to bind to macromolecular

surfaces in much a similar way as suggested for oligosaccharides (Dure *et al.*, 1989). The fact that seeds of *A. marina* do not produce these particular proteins (Farrant *et al.*, 1992b) lends credence to the hypothesis of Bradford and Chandler (1992) that lack of such proteins might be an inherent feature of desiccation sensitivity.

There are, however, complications with the hypothesis that LEA proteins confer desiccation tolerance on orthodox seeds. For example, Finch-Savage *et al.* (1994) have shown LEA proteins in several recalcitrant seeds of temperate trees. Also, Blackman *et al.* (1991) showed that LEA proteins accumulated before desiccation tolerance developed in soybean.

**d. Antioxidative systems**

The idea that antioxidant systems play a rôle in desiccation tolerance is not far-fetched. This is particularly so in the light of the evidence which suggests 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).

Among the few studies conducted in this area, there appears to be a trend toward presence and effectiveness of antioxidants and desiccation tolerance (Vertucci and Farrant, 1995). As an example, the level of tocopherol (vitamin E), a lipid soluble compound that slows the initiation of autoxidation of lipids, is about ten times more in orthodox embryos of maize and soybean than in the recalcitrant counterparts, *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).

### 1.2.7 Dormancy

Many authors have identified seed dormancy with the absence of a germination response under conditions that should facilitate this process (e.g. Harper, 1959; Simpson, 1990; Murdoch and Ellis, 1992). Recently, however, Vleeshouwers *et al.* (1995) have proposed that we make a distinction between dormancy release and germination stimulation as well as 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 (Baskin and Baskin, 1989), (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); Karssen (1982) cited in Vleeshouwers *et al.* (1995)]. 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 of prolonged unfavourable conditions (reviewed by Vleeshouwers *et al.*, 1995). These authors, however, contend that dormancy is a device for surviving short periods of

favourable conditions. They argue that during unfavourable conditions, the lack of germination-stimulating factors will prevent germination. Dormancy, on the other hand, prevents germination during favourable conditions when it is likely that the seedling that originates from the seed will not survive.

Hilhorst (1993) 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 gibberellic acid (GA) play a pivotal rôle in the release of dormancy and stimulation of germination in seeds. It is worth mentioning that Karssen (1982) has implicated ABA in the induction of dormancy and inhibition of germination. Vleeshouwers *et al.* (1995), however, caution that as yet, there is only circumstantial evidence to support the model. They admit, though, that it structures and integrates a large number of observations on dormancy and germination in a concise and comprehensible way.

### 1.2.8 Germination

As pointed out above, germination in orthodox seeds is under PGR control. By way of an example, ABA is thought to prevent precocious germination prior to maturation drying and might promote a disposition to 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 negates the effect of ABA (Kermode, 1990; Bewley and Oliver, 1991). It is associated with metabolic quiescence (Kermode, 1990) and hence acts as a punctuation between development and

germination, enabling seeds to tolerate adverse environmental conditions. This pattern of events may, however, not be universal. 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 include, among others, production of catabolic and anabolic enzymes which mobilize reserves and the synthesis of structural components, respectively. The process sees its completion with the elongation of the radicle.

The situation in recalcitrant seeds is considerably different. Such seeds show no maturation drying, no quiescence and no definite punctuation between development and germination. 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), *Q. robur* (Finch-Savage *et al.*, 1992)].

Upon shedding, seeds of *A. marina* do, however, show an amplification of metabolism (Farrant *et al.*, 1992a; b) akin to that observed in orthodox seeds. These events are not, however, accompanied by qualitative changes in protein synthesis [at least in *A. marina* (Farrant *et al.*, 1992b)], even though cell division occurs. This has 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. In *A. marina* it is difficult to identify any metabolic process that could be considered to be associated specifically with germination. Development appears to grade imperceptibly into germination (Farrant *et al.*, 1992a).

## 1.3 Deteriorative changes associated with loss of viability in seeds

While it might seem of little relevance to discuss deteriorative mechanisms of orthodox seeds in storage in the present treatise, this has been done for two reasons. Firstly, there may be marked similarities between deterioration of orthodox seeds in storage and that of recalcitrant seeds during dehydration and in wet storage at least at the molecular level (Smith and Berjak, 1995). Secondly, *Zizania palustris* seeds, used in this study, are *sensu* Roberts (1973) neither orthodox nor recalcitrant (see pp. 25-30).

### 1.3.1 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 relative humidity (RH) and temperature.

*i. Genetic factors*: It is logical to assume that seed longevity has a genetic basis. This had already been demonstrated for maize some 50 years ago by Lindstrom (1942) [cited in Smith and Berjak (1995)]. These basic genetic differences among species and cultivars could, however, 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 (e. g. Scott, 1981; Bewley and Black, 1982; Moreno Martinez *et al.*, 1988; Ramamoorthy, 1989; Diojode, 1990). Such basic genetic differences have been equated to biochemical parameters, such as levels of polyamines, putrescine and spermidine (Lozano and Leopold, 1988; Lozano

*et al.*, 1989) 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 viability as well (Smith and Berjak, 1995). This is probably the basis for the differences in viability characteristics among harvests of the same cultivar of the same species. It is interesting to note that while many studies have shown that heavier or larger seeds generally show superior viability and longevity characteristics, Olaridan and Mumford (1990) have shown that smaller seeds of *Amaranthus* species showed superior germination and storage characteristics.

*iii. Mechanical factors*: It is not very difficult to see the advantages of mechanisation in commercial practices during harvesting and threshing. These practices, however, may inflict damage on seeds, especially those that are excessively dry and this mechanical damage can contribute to loss of viability in storage.

*iv. Storage environment*: As early as 1972, Harrington proposed a generalization 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" he 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.

**b. Changes associated with viability loss**

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

Many events have been suggested to be basic to, or associated with, deterioration in seeds. These can be conveniently grouped into two categories: (1) biochemical changes and (2) ultrastructural changes.

*i. Biochemical changes*: These include: (1) damage to DNA, (2) changes in RNA and protein synthesis, (3) changes in enzymes and reserves and (4) changes in respiratory activity and ATP production.

*(1) Damage to DNA*: Osborne and co-workers have shown that molecular dysfunction at DNA level can be correlated with declining viability (Roberts and Osborne, 1973; Osborne, 1983). Repair enzymes may also suffer damage during seed storage (Elder and Osborne, 1983) and 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 is followed by cell death.

*(2) Changes in RNA 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 and protein synthesis in the deteriorative process. Studies which have investigated the rôle 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 the protein synthesis machinery is concerned, Roberts *et al.* (1973) have shown progressive degradation of ribosomes. Those authors also report loss of, and reduction in, activities of elongation factors 1 and 2, respectively.

*(3) Changes in enzymes and reserves*: A number of changes in enzymes and reserves during the deterioration of seeds have been documented in the literature. These include increased free fatty acid (FFA) production as a result of lipolytic activity, increased hydrolysis of phytin by phosphatases, increased proteolysis by proteases, crosslinking of proteins [(including enzymes), Ching and Schoolcraft, 1968] and carbonyl-amine reactions [(including the Maillard reaction), Feeney *et al.*, 1975] between carbohydrates and amino acids and proteins.

*(4) Changes in respiratory activity and ATP production*: Since germination involves energy-dependent cell division and growth, many investigators have examined respiratory changes during seed deterioration. It appears that seed deterioration is accompanied by changes in respiratory activity. These changes include reduced coupling, greater oxygen consumption and a reduced P:O ratio (Abu-Shakra and Ching, 1967).

As far as ATP production is concerned, the picture that emerges is far from clear. The studies which have shown a correlation between ATP content and viability in

seeds of several unrelated species (Ching and Danielson, 1972; Ching, 1973; Lunn and Madsen, 1981) have been confounded by others that have indicated that ATP levels are not good indicators of viability (Styler *et al.*, 1980; Mazor *et al.*, 1984).

*ii. Ultrastructural changes*: These can be grouped into three categories: (1) chromosome aberrations, (2) membrane changes and (3) general cytoplasmic and nuclear deterioration.

*(1) Chromosome aberrations*: According to Smith and Berjak (1995), the idea that chromosome aberrations underlie seed deterioration is the oldest. 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 Postolski, 1992). These changes include: 1. abnormalities of mitochondrial and plastid inner and outer membranes, 2. lobbing of the nuclear envelope, 3. fragmentation or 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, nucleolar abnormality and loss of ribosomes (including polysomes) (Smith and Berjak, 1995). These changes would impact on the DNA, RNA and protein synthetic systems and consequently on repair systems.

**c. Free-radical theory of ageing**

The free-radical theory originated in the medical sciences nearly half-a-century ago (Harman, 1956). This theory soon found its way into seed biology when Kaloyereas (1958) suggested that lipid oxidation may underlie loss of viability in seeds.

Since then, evidence to support the free-radical theory in seed ageing has been accumulating. First, it was Koostra and Harrington (1969) who analyzed 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 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 head-space 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).

There are, however, studies which contrast with the picture painted above. For instance, Fergusson *et al.* (1990) did not find a decline in total and polar FFA levels in

aged soybean axes during deterioration. Smith and Berjak (1995), however, attributed this contradiction to differences in methodology.

A number of factors determine the extent of peroxidation in ageing seeds. These factors include composition and saturation of FFAs (Priestley, 1986; Ponquett *et al.*, 1992), level of hydration (Karel and Yong, 1981), relative humidity (RH) and temperature (Smith and Berjak, 1995) oxygen availability (Ohlrogge and Kernan, 1982) and efficiency of antioxidative systems (e.g. Pokorný, 1987).

**d. Two-stage model of seed ageing**

A seminal feature of the two-stage model of seed ageing (Smith and Berjak, 1995) is the realization 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) are distinguished following the convention of Fennema (1976) and correspond to equilibrium RH ranges (0-25%, 25-80% and 80-99%). It is proposed 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 would have us believe 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).

### 1.3.2 Desiccation-sensitive (recalcitrant) seeds

#### a. Changes associated with desiccation

Water plays a plethora of rôles in cells. It gives cells structure, allows for the diffusion of substrates to the active sites of enzymes, stabilizes macromolecular conformations through hydrophilic and hydrophobic interactions, allows for the sequestering of cellular constituents and acts as a reactant or product in many important reactions (Vertucci and Farrant, 1995). It therefore goes without saying that removal of water from cells that are not desiccation tolerant, will have adverse consequences.

*i. Metabolic stresses*: Removal of water from cells will result in an increased concentration of solutes and a reduction in the fluidity of the aqueous medium. These changes affect the metabolic status of cells. The changes in metabolic activity are believed to occur at specific moisture levels (Clegg, 1978; Leopold and Vertucci, 1989). In this regard, it important to note that cells may be sensitive to the rate of desiccation in addition to loss of water *per se* (Pammenter *et al.*, 1991; Pritchard, 1991; Berjak *et al.*, 1993).

*ii. Mechanical stresses*: One of the first obvious symptoms of water stress is loss of turgidity. This may eventually lead to cell collapse. A number of factors, which influence the effects of mechanical stress during water loss in cells, have been suggested. They include the size of the cell, vacuolar space (Levitt, 1980a; b), plasma membrane phoshpolipid (PL) composition (Uemura and Steponkus, 1989) and organelle geometry (Bewley, 1979; Levitt, 1980a; Bergtrom *et al.*, 1982; Kaiser, 1982; Oliver and Bewley, 1984; Oertli, 1986).

*iii. Lipid phase transitions*: Lipid phase transitions can be induced by at least two processes. They are: (1) a decrease in water content and (2) peroxidation.

When water is removed from cells, proteins, nucleic acids and polar lipids can undergo conformational changes as a consequence of weakened hydrophilic and hydrophobic interactions (Leopold, 1986; Crowe *et al.*, 1987; Carpenter and Crowe, 1988). This can result in lipid phase transitions.

Two types of phase transitions have been identified. They are: (1) lamellar liquid crystalline to gel phase transitions in which the membrane bilayer configuration is maintained and (2) lamellar liquid crystalline to hexagonal phase transitions in which a non-bilayer structure is formed.

According to Vertucci and Farrant (1995), since lipid phase transitions are reversible, they may not be lethal in themselves. Lipid phase transitions may, however, result in irreversible, hence lethal, membrane protein elimination and/or clustering (Quinn, 1985; Crowe *et al.*, 1986; 1989; 1992; Bryant and Wolfe, 1989; 1992; McKersie *et al.*, 1989; Hoekstra *et al.*, 1992; Steponkus and Webb, 1992).

A number of factors determine the possibility of the occurrence, as well as the nature, of phase transitions. They include molecular geometry of the liposome formed during membrane contraction, the nature and quantities of its polar lipid components, presence or absence of other membrane constituents (such as proteins and sterols) and the temperature and degree of dehydration (e.g. Quinn, 1985; Small, 1986; Bryant and Wolfe, 1989; 1992; Steponkus and Webb, 1992; Webb *et al.*, 1993).

**b. Changes associated with hydrated storage**

*i. Events occurring in embryonic axis cells*: There may be no clear marker event between the end of development and initiation of germination in some recalcitrant seeds (Farrant *et al.*, 1993b) and all the tropical and subtropical species so far examined initiate germination in wet storage (Berjak *et al.*, 1989; Farrant *et al.*, 1989). Recalcitrant seeds of temperate species, however, can tolerate months of wet storage as they are shed either dormant and/or immature. The latter continue development and/or growth prior to the initiation of germination in a phenomenon that Tompsett (1987) has described as after-ripening.

The situation for seeds that initiate germination in wet storage is exemplified for *Camellia sinensis* (tea). Embryonic axes from those seeds in wet 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). However, unless more water is supplied thereafter, extensive intracellular degeneration rapidly follows with concomitant viability loss.

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

## 1.4 *Zizania palustris* - a current overview

North American wild rice [Poaceae, *Zizania palustris* var. *interior* (Fasset) Dore] is an aquatic grass native to shallow lakes and streams of northern United States and southern Canada (Warwick and Aiken, 1986). It was originally a staple food of native Americans in those areas (Aiken *et al.*, 1988).

Currently, wild rice is harvested from the native lake populations and is also grown commercially in paddies in Minnesota and California. The paddies re-seed naturally under Minnesota conditions, but, in California, seed must be stored over winter and replanted the following spring (Kovach and Bradford, 1992a).

Under natural conditions, seeds (caryopses) of *Z. palustris* abscind at relatively high water content ($\geq 0.3$ g $g^{-1}$ ww) and fall into the water, sinking to the bottom and remaining dormant until the next spring. A number of early studies concluded that wild rice seeds must be stored submerged at near-freezing temperatures to maintain viability [Brown and Scofield (1903); Duvel (1906); Muenscher (1936); Simpson (1966) loc. cit. Kovach and Bradford, 1992a].

However, this conclusion is complicated by the presence of a deep dormancy at maturity that requires up to six months of hydrated cold storage (stratification) before all viable intact seeds will germinate (Simpson, 1966; Cardwell *et al.*, 1978; Atkin *et al.*, 1987; Kovach and Bradford, 1992b). Total viability of unstratified or partially stratified seeds must be assessed by methods such as the tetrazolium test or by scraping or slitting the pericarp over the embryo (perhaps with the addition of gibberellin or fusicoccin) to break the dormancy (Woods and Gutek, 1974; Cardwell *et al.*, 1978; Kovach and Bradford, 1992a).

It has been noted that in a number of studies of the desiccation tolerance of wild rice seeds, the dried seeds had not been stratified; failure to germinate might, therefore, have been due to maintenance of dormancy rather than to loss of viability during storage (Ellis *et al.*, 1985). Simpson (1966) also noted that the seed coat of wild rice is highly impermeable to water, the seeds therefore requiring scarification or long periods of submersion to hydrate fully before germination is possible. Consequently, the germination test period in some studies may not have been extended enough to assess viability accurately. The apparent recalcitrant behaviour (e.g. Probert and Brierley, 1989; Probert and Longley, 1989) of seeds of *Z. palustris* in storage might, therefore, be due to dormancy or delayed imbibition rather than to intolerance to desiccation. On the other hand, Probert and co-workers (Probert and Brierley, 1989; Probert and Longley, 1989) found that viability of both stratified and dormant seeds declined when the embryonic water content declined below 0.45 g $g^{-1}$ ww with 50% of the embryos failing to survive dehydration to 0.3 g $g^{-1}$ ww (*c.* 0.2 g $g^{-1}$ seed WC). Since those authors used techniques to break dormancy and overcome seed coat impermeability, they concluded that wild rice seeds are truly recalcitrant.

There are, however, some conflicting data on the desiccation tolerance of wild rice seeds. For example, Fyles (1920), cited in Kovach and Bradford (1992a) reported that freshly harvested wild rice seeds could be stored in air at ambient temperatures for up to 25 days and still retain approximately 50% viability. Simpson (1966) found 77% viability in wild rice seeds air-dried at room temperature for 14 days, although viability was lost after 90 days under these conditions. Unfortunately, seed water contents were not reported in these studies, so the extent of dehydration attained is unknown. Oelke and Stanwood (1988), however, found that wild rice seeds dried to *c.* 0.12 g $g^{-1}$ ww at

22 °C and then stored at -2 to 2 °C for 6 months were still highly viable after a further 6 months of stratification in cold water. A subsequent study (Oelke *et al.*, 1990) found that *c.* 50% of wild rice seeds survived when dehydrated under ambient conditions to 0.09 g $g^{-1}$ ww, stored up to 9 months at 3 °C, and imbibed and stratified at 3 °C for 3 months. Thus, survival of wild rice seeds of dehydration to relatively low water content may occur under some conditions.

The previous studies of wild rice seed dormancy and storage have established some general principles, but have also left a number of inconsistencies and gaps in our knowledge. There is evidence that the embryos are unable to survive desiccation to very low water content, at least under some conditions, but a moderate level of dehydration below full imbibition may be tolerated. These seeds do not germinate immediately upon maturation, as do many recalcitrant seeds, but rather have an extended dormancy period. In their native temperate habitat, the seeds survive near-freezing or subzero temperatures while overwintering, and, in fact, require long-term stratification to break dormancy. These properties put together would classify wild rice seeds as being 'minimally recalcitrant' according to a scheme proposed by Farrant *et al.* (1988).

In a recent study (Kovach and Bradford, 1992a), it was reported that wild rice seeds can survive dehydration to low water content (0.06-0.08 g $g^{-1}$ ww). However, maximal survival of desiccation to low water content was possible only if dehydration occurred at temperatures ≥25 °C and a long imbibition period (3 weeks minimum) was allowed at temperatures between 10 and 25 °C prior to stratification or dormancy-breaking treatments. A reduction in survival of dehydration at temperatures <25 °C appeared to occur when embryonic axis water contents were reduced below 0.08 g $g^{-1}$

ww. Those results have led Berjak *et al.* (1994) to conclude that seeds of *Z. palustris* do not belong to the recalcitrant category *sensu* Roberts (1973), but certainly indicate that their behaviour is far from orthodox.

Despite their temperature-dependent dehydration responses, seeds of *Z. palustris* share many properties with orthodox seeds, and direct comparisons have been made with the highly desiccation-tolerant caryopses of paddy rice, *Oryza sativa* by Bradford and co-workers (Bradford and Chandler, 1992; Still *et al.*, 1994). Seed development in wild rice seeds is similar to that of orthodox grass seeds until the dehydration phase of development [LaRue and Avery (1938) loc. cit. Kovach and Bradford (1992a)]. Bradford and co-workers also found that embryos of *Z. palustris* and *O. sativa* accumulated a dehydrin protein during development, and ABA and sucrose contents in *Z. palustris* were about twice those in *O. sativa*. Furthermore, embryos of both species contained oligosaccharides at *c.* 10% of the maximum sucrose concentrations. Thus, despite marked similarities and enhanced production in *Z. palustris* of some components that are considered intrinsic to desiccation tolerance, seeds of *Z. palustris* are sensitive to temperature of dehydration and do not tolerate water contents below 0.07 g $g^{-1}$ ww in any case (Still *et al.*, 1994).

In a recent study, Berjak *et al.* (1994) examined the ultrastructural condition of embryonic axes of *Z. palustris* after dehydration at different temperatures, and following reimbibition and stratification. Those authors reported that axis cells sustained least damage when dehydration was carried out at 25 °C; viability assessed by tetrazollium (TZ) test (after 15 months of dry storage at -18 °C) was 85%. Ultrastructural deterioration was more severe with lower temperatures during water loss. Damage sustained as a result of unfavourably low dehydration temperatures was

visible when seeds were fixed from the dried state and was generally exacerbated during fully imbibed stratification; viability assessed by TZ test (after 14 months of dry storage at -18 °C) was 42% for seeds dried at 10 °C. Seeds that had been dried at higher temperatures also showed considerable ultrastructural disturbance when fixed from the dry state, but this was largely reversed during fully imbibed stratification although signs of damage that had been sustained still persisted; viability assessed by TZ test (after 16 months of dry storage at -18 °C) was 84% for seeds dried at 30 °C. It was hypothesized that at temperatures above 25 °C, damage by free-radical-mediated events may predominate, whereas, at temperatures below the optimum, irreversible lipid phase transitions may be the major factor resulting in membrane damage; at the dehydration temperature of 30 °C viability assessed by the TZ test was 84%. The hypothesis stresses that the two mechanisms of membrane deterioration are probably not mutually exclusive.

In another recent study, Vertucci *et al.* (1994) measured the changes in the physical properties of water in excised embryos during the late stages of seed development of *Z. palustris*. Those authors found that the water content of embryos at high water activities decreased with maturation, as did the temperature at which freezing transitions [determined by differential scanning calorimetry (DSC)] were not observed. While the temperatures of freezing and melting transitions decreased as embryo water content decreased, there were no discernible differences among embryos at different developmental stages with respect to temperature of freezing and melting transitions for similar water contents. It was observed that the properties of water in maturing *Z. palustris* embryos approached those for orthodox seeds as determined from the strength of water sorption, the enthalpy of the melting transition and the moisture

content at which water is unfreezable. The orthodox condition was, however, "never achieved".

More recently, Vertucci *et al.* (1995) examined the interaction between water content and temperature on freezing and desiccation damage of *Z. palustris* embryos at different stages of maturity. They found that viability of embryos decreased on drying below a critical water content. The critical water content was the greatest for the least mature embryos. Critical water contents also increased with decreasing temperature. It was noted, however, that even though the critical water content varied with developmental status and temperature, the water activity corresponding to the critical water content appeared to be constant.

### 1.5 The purpose and scope of the present study

The fundamental aim and objective of the present study was to test the hypothesis of Berjak *et al.* (1994). The hypothesis states that, for *Z. palustris* seeds, two different mechanisms of membrane deterioration may predominate depending on the temperature of dehydration. That is, at dehydration temperatures >25 °C free radical-mediated damage may predominate, whereas at decreasing temperatures <25 °C, membrane phase transitions may become increasingly significant.

To achieve the objectives of the present study, a number of tests were performed. Firstly, seeds of *Z. palustris* were dehydrated at a range of temperatures. They were then imbibed at 20 °C for five weeks. Following imbibition, they were stratified at 5 °C for five months. Imbibition and stratification are supposed to break the dormancy of *Z. palustris* seeds (Kovach and Bradford, 1992a). During dehydration, imbibition and stratification, water contents (WCs) were monitored periodically to assess if moisture

plays any rôle during viability loss and if so, to determine if there was any interaction with temperature at which seeds were dehydrated. Seed vigour was measured by conductivity, tetrazolium and germination tests. To understand the mechanism of desiccation tolerance in seeds of *Zizania palustris*, the possible rôle of vitrification (glass formation) was investigated by differential scanning calorimetry (DSC). Also, the possible rôle of sugars came under the spotlight using high pressure liquid chromatography (HPLC). The occurrence of lipid phase transitions was assessed by freeze fracture electron microscopy (EM) and high resolution scanning electron microscopy (HRSEM). Other tests included a simple peroxide test for the formation of hydroperoxides during dehydration. This test was performed in conjuction with gas chromatography (GC) to monitor the levels of free fatty acids (FFAs), reactants in peroxidation, and head-space analysis to monitor levels of aldehydes, breakdown products of hydroperoxides (products of peroxidation).

### 1.6 Importance of the present study

The importance of the present study is, perhaps, two-fold. As pointed out above, *Z. palustris* is grown commercially in the United States. In the words of Kovach and Bradford (1992a) "... It would be extremely valuable for germplasm preservation and breeding programmes and for storage of commercial seed for planting to determine whether conditions do exist to circumvent the apparent recalcitrance of wild rice seeds and preserve viability at low moisture contents ...". It is not over-ambitious to state that this study may make a significant contribution in this regard.

At a second level, development in wild rice seeds is similar to that of orthodox counterparts [LaRue and Avery (1938) loc. cit. Kovach and Bradford (1992a)].

Kovach and Bradford (1992a) therefore contend: " ... Wild rice may, therefore, provide a good model system to study the mechanism of desiccation tolerance in plant tissues ...". This study may, therefore, shed more light on our understanding of the mechanism of desiccation tolerance.

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# CHAPTER TWO

# DEHYDRATION AND REHYDRATION CHARACTERISTICS

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## 2.1 Introduction

Water has physical properties that make it an ideal biological solvent. It is, therefore, hardly surprising that it plays many rôles in cellular metabolism. Because it is virtually incompressible, it fills cells and gives them structure. The fluid environment it provides allows the diffusion of substrates to active sites of enzymes. Hydrophilic and hydrophobic interactions stabilize 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 (Vertucci and Farrant, 1995).

To appreciate the important rôle 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, which was briefly done in Chapter 1 (pp. 22-24). Cells may, however, 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; Pritchard, 1991; Finch-Savage, 1992; Berjak *et al.*, 1993). The reason for this phenomenon is thought to be that when cells are held at moisture levels where only catabolic activities occur, they may suffer damage because they have been subjected to the stress of unregulated metabolism for a longer period than when dried rapidly (e.g. Pammenter *et al.*, 1991). When dried rapidly, in effect inadequate time is allowed for aqueous-based deleterious reactions (Pammenter *et al.*, 1991). Because of the many rôles 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). 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 moisture 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, with increasing levels of hydration, water becomes progressively capable of fulfilling the particular functions required for specific metabolic processes. 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.

Water relations are central to understanding seed biology. Water plays a significant rôle in regulating developmental processes and preventing precocious germination (Adams and Rinne, 1980; Kermode, 1990; 1995; Bradford, 1995; Karssen, 1995). Most seeds (i.e. orthodox types) undergo maturation drying and subsequently become quiescent or perhaps, dormant. Dormancy, which is a key adaptation of seeds, allows them to survive long periods in adverse conditions and re-establish plant communities under more favourable conditions. This scenario, in turn, allows the spread of plants

into increasingly hostile climates (Bradford, 1995). The intricate and varied dormancy mechanisms exhibited by seeds are the evolutionary outcome of selection for seeds that can successfully interpret environmental information so as to initiate growth at a time favourable for seedling establishment (Bradford, 1995).

As the availability of water is one of the most critical factors determining survival of seedlings, seeds are closely attuned to their hydric environment as well as to temperature, light, oxygen and nutrient availability (Corbineau and Côme, 1995). In seeds that undergo maturation drying, the germination process is initiated by imbibition of water by the dry seed. This process consists of three phases: (1) imbibition, (2) activation of germination and (3) growth. The initial rate of imbibition is primarily determined by three factors: (1) seed coat permeability, (2) seed/substratum contact area and (3) substratum medium (Hadas, 1982; Koller and Hadas, 1982; Vertucci, 1989). The initial rate of imbibition is important, as rapid imbibition by dry seeds can be damaging in itself, particularly at low temperatures (Kovach and Bradford, 1992a; Taylor *et al.*, 1992).

The acquisition of desiccation tolerance and the possible rôle of oligosaccharides in this regard were discussed briefly in Chapter 1 (pp. 9-11). It is interesting to note that, for both recalcitrant and orthodox seeds, the relative level of desiccation tolerance changes with development; seeds tend to become more tolerant of desiccation as they mature and less so as they germinate (reviewed by Vertucci and Farrant, 1995).

The objectives of the phase of the work described in this chapter, were: (1) to ascertain the characteristics of the water relations of seeds of *Zizania palustris* during dehydration at different temperatures and rehydration (imbibition and stratification),

and (2) to ascertain whether dehydration temperature had any effects on the seed water content attained after dehydration and rehydration.

## 2.2 Materials and methods

### 2.2.1 Plant material

Seeds (caryopses) of *Zizania palustris* var. *interior* were mechanically harvested from a commercial production field near Davis, California. The seeds were brought to the laboratory, thoroughly mixed and major débris removed. The seeds were then packed on ice and dispatched to South Africa by air.

In South Africa, the outer coverings of the seeds were removed and the seeds were separated into three maturity stages based on the texture of the endosperm and colour of the pericarp (Vertucci *et al.*, 1994). Grains were classified as either soft and green (most immature), hard and green (intermediate maturity) or hard and brown (most mature). Throughout the present study, only seeds from the last-mentioned category were used. During the separation of seeds into maturity classes, they lost some of their moisture. It was decided, therefore, to rehydrate the seeds to moisture levels representative of the fresh state (Kovach and Bradford, 1992a) before dehydration. This was achieved by placing beakers coated with paper towel and partially filled with water in the container with the seeds until the requisite whole seed water content had been attained.

### 2.2.2 Dehydration and rehydration of seeds

#### a. Dehydration of seeds

Fresh seeds were partitioned into six samples. Each of five samples was placed in a desiccator over a saturated solution of $MgCl_2$ (33 ± 1% RH). In each of the desiccators an electric fan was mounted to circulate air continuously around the seeds. The desiccators were placed in one of: (1) an incubator at 10 °C, (2) constant temperature rooms at 20 and 25 °C or (3) incubators at 29 and 37 °C. The salt solutions in the desiccators were replenished as needed to maintain a fully saturated condition as water was absorbed from the seeds. The last sample was submerged in water at 20 °C, a storage condition that maintains viability but should not break dormancy (Kovach and Bradford, 1992b). This sample acted as the control material.

#### b. Rehydration of seeds

Following dehydration, seeds from each experimental sample were imbibed in water at 20 °C for five weeks. The seeds were then imbibed in water at 6 °C (stratified) for five months. The control material was maintained at 20 °C throughout.

### 2.2.3 Water content determinations

Whole seed and excised embryonic axis water contents (WCs) were determined by oven-drying at 80 °C for 48 hours. Means for the whole seed and excised embryonic axis water contents are based on five individual replicate seeds or axes, respectively. Water contents were determined weekly during dehydration and imbibition, and monthly during stratification.

### 2.2.4 Statistical analysis

Measures of rates of dehydration, imbibition and stratification were determined by linear regression of log transformed WCs. The significance of the differences between treatments were assessed according to Sokal and Rohlf (1981).

## 2.3 Results

### 2.3.1 Dehydration period

It appears that both dehydration (prior to and during separation of seeds from the outer coverings) and the subsequent rehydration to compensate for the loss of moisture as a result of that dehydration affected only the whole seed water contents but not those of the excised embryonic axes water contents (Table 2.1).

**Table 2.1** Water contents of mature *Zizania palustris* seeds and excised embryonic axes after separation into maturity classes and after rehydration to compensate for moisture loss during the separation.

| Water contents (g $g^{-1}$ dw) | | |
|---|---|---|
| **Seed part** | **After separation** | **After rehydration** |
| Whole seeds | 0.15±0.05 | 0.23±0.05 |
| Embryonic axes | 0.54±0.07 | 0.57±0.06 |

It took at least three weeks to reach equilibrium water contents (Fig. 2.1). When assessed immediately after drying, the final water contents of excised embryonic axes and whole seeds were 0.36 g $g^{-1}$ dw and 0.08 g $g^{-1}$ dw, respectively. These values were independent of dehydration temperature ($r = -0.36$, $p = 0.55$ and $r = -0.55$, $p = 0.34$, respectively). For both whole seeds and embryonic axes, the rates of water loss were higher during the initial stages (first one to two weeks) of dehydration but decreased as the equilibrium seed water contents were approached. The initial rates of dehydration

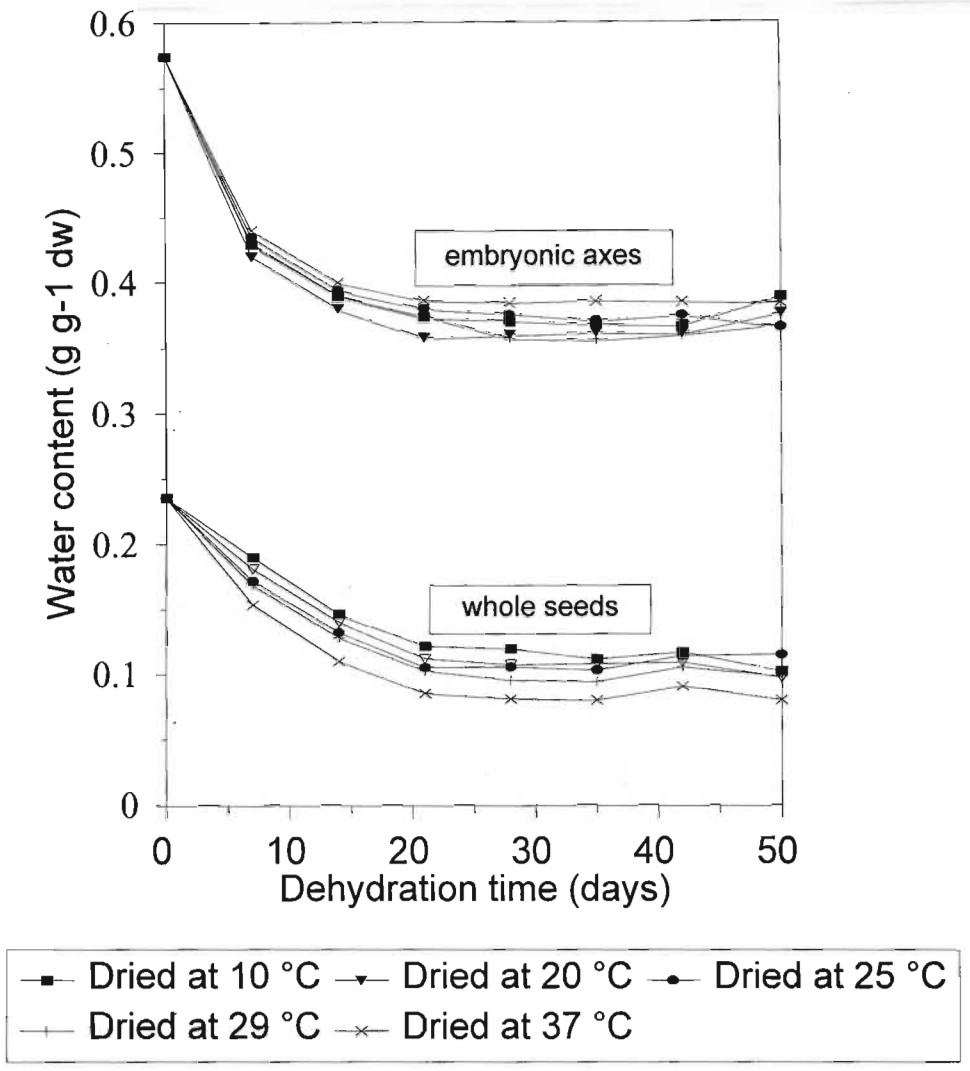


**Figure 2.1** Water contents of whole seeds and embryonic axes during dehydration at a range of temperatures. (Error bars omitted for clarity).

of whole seeds and embryonic axes were affected differently. For whole seeds, there was a highly significant linear increase in rates of water loss (determined by linear regression of log transformed WCs) with increasing dehydration temperature ($r = -0.97$, $p = 0.01$). In contrast, the correlation between initial rates of water loss by embryonic axes and dehydration temperature was not significant ($r = 0.57$, $p = 0.32$). The water contents of the axes was consistently higher than that of the whole seeds. The relationship was linear and highly significant (e.g. at a dehydration temperature of 25 °C, $r = 0.97$, $p = 0.004$).

### 2.3.2 Imbibition period

The final water contents of both the whole seeds (about 0.40 g $g^{-1}$ dw) and embryonic axes (about 2.5 g $g^{-1}$ dw) after imbibition (Fig 2.2) seemed independent of the temperature at which seeds were dehydrated ($r = 0.43$, $p = 0.47$ and $r = 0.23$, $p = 0.71$, respectively). The kinetics of imbibition appeared to be a reversal of the events during dehydration. Initially, the rates of water uptake were relatively high and decreased as equilibrium water contents were approached (Fig. 2.2). Dehydration temperature, however, did not influence the initial rates of imbibition significantly ($r = 0.37$, $p = 0.53$ and $r = 0.32$, $p = 0.62$ for whole seeds and embryonic axes, respectively). Also, as with drying, whole seed water contents were consistently less than embryonic axis water contents over a wide range of water contents (e.g. at a dehydration temperature of 25 °C, $r = 0.74$, $p = 0.09$).

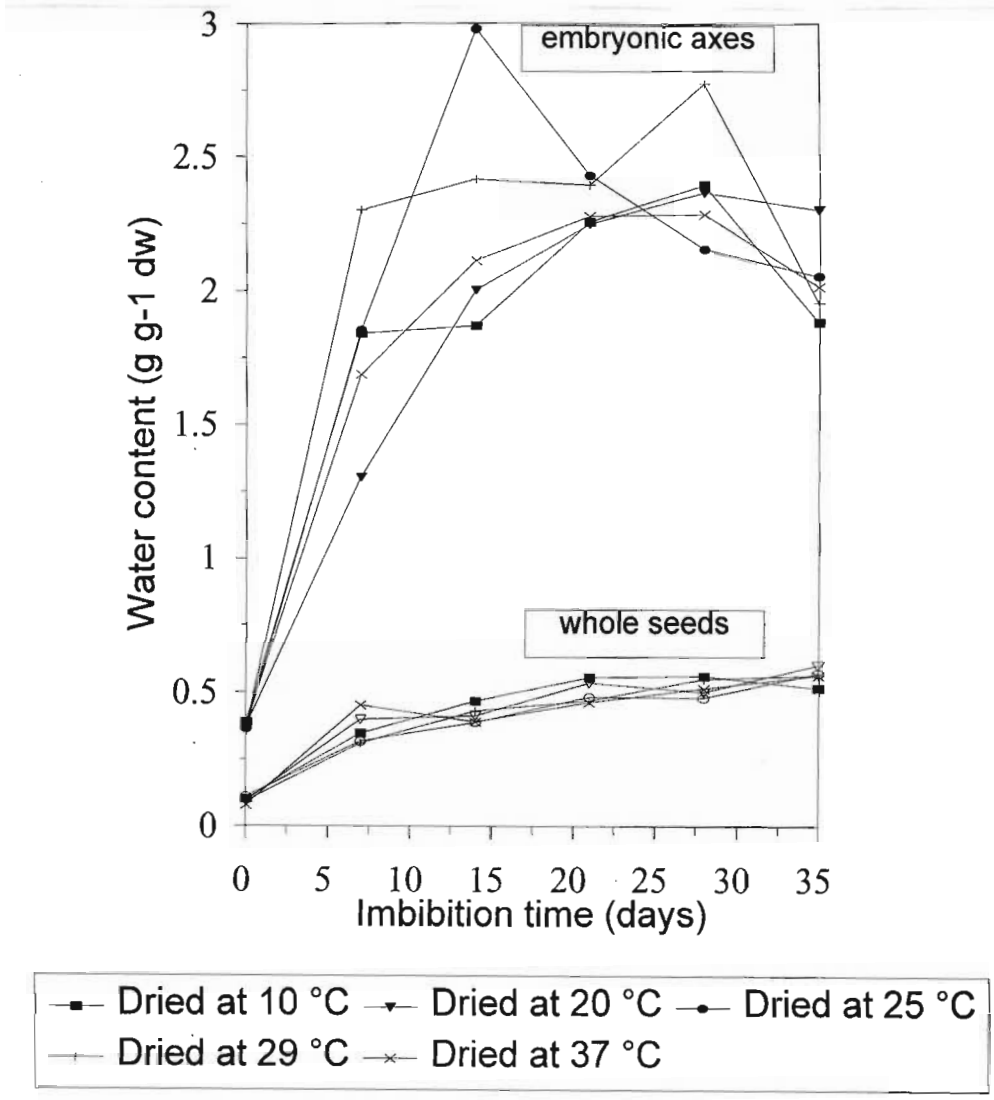


**Figure 2.2** Water contents of the whole seeds and embryonic axes during imbibition at 20 °C. (Error bars were omitted for clarity).

### 2.3.3 Stratification period

During stratification, whole seed water contents fluctuated around 0.60 g $g^{-1}$ dw (Fig. 2.3). Those fluctuations were not significantly influenced by the temperature at

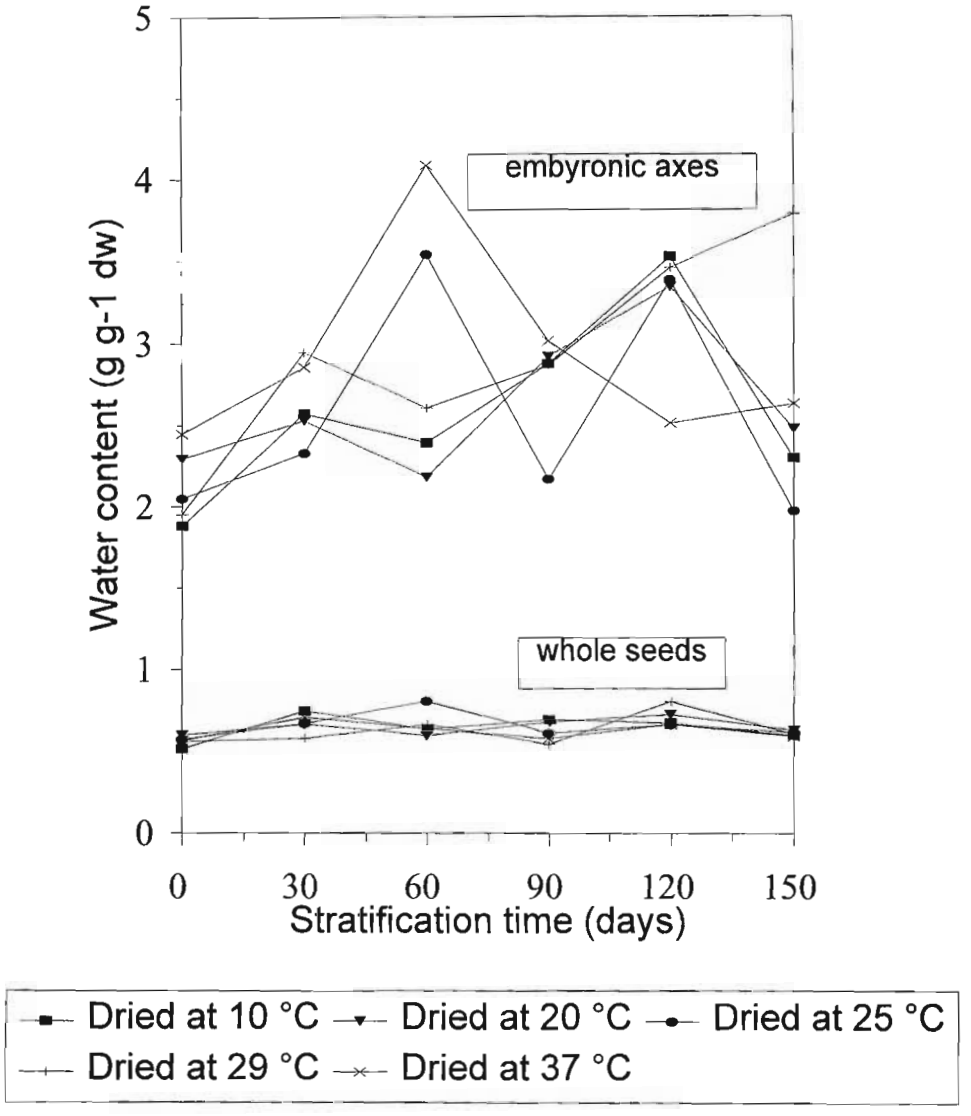


**Figure 2.3** Water contents of whole seeds and embryonic axes during stratification at 5 °C. (Error bars were omitted for clarity).

which seeds were dried ($r = -0.36$, $p = 0.54$), nor were there any consistent changes with time. Here again, no relationship was apparent between dehydration temperature and the final water contents of whole seeds or embryonic axes after stratification ($r = 0.38$, $p = 0.53$ and $r = 0.77$, $p = 0.12$, respectively). Embryonic axis water contents fluctuated around 2.50 g $g^{-1}$ dw and, as for whole seeds, were also not significantly influenced by the temperature of dehydration ($r = 0.31$, $p = 0.61$). The fluctuations of embryonic axis water contents showed no consistent changes with time. Once again, the linear relationship between whole seed water contents and the higher embryonic axis water contents was highly significant (e.g. at a dehydration temperature of 25 °C, $r = 0.81$, $p = 0.05$).

## 2.4 Discussion

The results of the present study as regards water relations are in good agreement with those of Kovach and Bradford (1992a). The observations made, included: (1) higher initial rates of dehydration at all dehydration temperatures for both whole seeds and embryonic axes, (2) dehydration temperature dependence of initial rates of dehydration for whole seeds, (3) linear relationship between higher water contents of embryonic axes and whole seed water contents over a wide range of water contents regardless of dehydration temperature, during dehydration, imbibition and stratification, (4) relatively high initial rates of imbibition irrespective of dehydration temperature for both whole seeds and embryonic axes, (5) dehydration temperature independence of initial rates of imbibition and final water contents attained after imbibition and stratification (6) similar durations, irrespective of the temperature, to achieve equilibrium water contents during dehydration, imbibition and stratification for

both whole seeds and embryonic axes and (7) linear relationship between the rates of moisture loss and dehydration temperature for whole seeds.

There were, however, two areas in which the results of the present study differed from those of Kovach and Bradford (1992a). In the first case, those authors found the final water contents after dehydration of both embryonic axes and whole seeds to be temperature dependent. That observation was not confirmed by the present study. Also, Kovach and Bradford (1992a) found a linear relationship between the rates of moisture loss and dehydration temperature for embryonic axes. This study could not establish such a relationship between dehydration temperature and initial rates of dehydration for embryonic axes.

In the present study, seeds of *Z. palustris* were found to have survived dehydration at 25 and 29 °C better than at either higher (37 °C) or lower (10 and 20 °C) temperatures (see Chapter 3). While whole seed water contents declined most rapidly at 37 °C, thus minimizing the period during which presumably only catabolic metabolism occurred (e.g. Pammenter *et al.*, 1991), it must be assumed that opposing deleterious factors came into play. One of these might have been free radical generation and its consequences, as suggested by Berjak *et al.* (1994). This could be considered tantamount to an accelerated ageing effect which is known to be positively correlated with temperature and moisture content (e.g. Harrington, 1972).

However, what is intriguing is that the rate of water loss by embryonic axes was not significantly correlated with dehydration temperature. As free radicals (or the consequences of other deleterious processes) are far more likely to have an adverse effect on the embryo tissue than on the (relatively) inert endosperm, one must speculate that such effects, if exacerbated by dehydration temperature(s) at critical

moisture contents, may have profoundly influenced the aleurone layer and/or possibly the scutellum which was not excised with the axis in the present study.

Whole seed dehydration at the lower temperatures (10 and 20 °C) occurred at significantly lower rates than at the higher temperatures where best survival was obtained. While this was not the case for the embryonic axes, it might be suggested that extending the time at which critical tissues - again perhaps the aleurone layer and/or the scutellum - were maintained in a state where catabolism predominated (e.g. Pammenter *et al.*, 1991), might have been the underlying cause for the poor viability retention, especially at 10 °C.

In agreement with Simpson (1966) and Kovach and Bradford (1992a), periods of three weeks were required to achieve full dehydration and imbibition. This high resistance to water loss and uptake is consistent with the low permeability to water of the wild rice pericarp.

In the present study, it was established that the initial rates of water loss (regressions of log-transformed WCs) are linearly related to dehydration temperature. It follows that WC is exponentially related to temperature of drying. In this regard, it is interesting to note that the rate of water loss depends on the vapour pressure deficit (Morris, 1974). Vapour pressure, on the other hand, is dependent on the temperature and external pressure. But, the relationship between temperature and vapour pressure is exponential. It is suggested that this relationship explains the observation made in the present study that WCs are exponentially related to dehydration temperature.

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# CHAPTER THREE

# VIGOUR AND VIABILITY TESTS

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## 3.1 Introduction

The primary function of seeds is to produce seedlings, and, to achieve this, seeds must germinate. Seed quality can, therefore, be equated to its ability to germinate (germinability or viability). Germination, however, may be considered as activation of the embryo (e.g. Côme and Corbineau, 1989) on one hand, or establishment of the seedling, on the other. Also, there is a lack of consistent relationship between laboratory germination tests and field emergence (Perry, 1973). In an attempt to rectify this anomaly, the concept of seed vigour has been developed. Vigour is simply defined as a concept describing several seed performance characteristics, and 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, uniform emergence under field conditions and/or describes the storage potential of a seed lot (Hampton and Tekrony, 1995). The past 40 years have seen many different vigour testing methods proposed, but only very few are in international usage (Hampton, 1992). These include, among others, the tetrazolium (TZ), conductivity and cool germination tests.

The conductivity test provides measurement of electrolyte leakage from plant tissues. It was first recognized for seeds of several crop species by Hibbard and Miller (1928) (cited in 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 (Steere *et al.*, 1981; Hepburn *et al.*, 1984; the present study). 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).

The tetrazolium (TZ) test has been used to estimate seed vigour as well as seed viability. According to Hampton and Tekrony (1995), the primary difference between the two evaluations is that certain seed conditions which are not critical in assessing seed viability can be important in a seed vigour assessment. 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 (TTC) interacts with reduction processes of the living cells and accepts hydrogen from dehydrogenases. Following hydrogenation, it forms a red, stable and non-diffusible substance called triphenyl formazan. The topographical tetrazolium test (TTT), used in the present study, is an extension of the TZ test as described in Chapter 6 of the International Rules for Seed Testing (ISTA, 1993).

Another test of vigour is the cool germination test which assesses vigour at temperatures that are suboptimal for the species concerned (ISTA, 1993). It was developed for some warm-season crops and provides sufficiently severe test conditions

to enable the separation of vigorous from less vigorous seeds (Hampton and Tekrony, 1995). It is based on the observation that different species have different optimal temperature ranges for germination.

The objective of the work descibed in the present chapter was to assess the effects of dehydration, dehydration temperature and rehydration (imbibition and stratification) on vigour, viability and dormancy of seeds of *Z. palustris*.

## 3.2 Materials and methods

### 3.2.1 Seed material

Seeds of *Z. palustris* were either dehydrated at a range of temperatures and then stored dry at 5 °C or stored in water at 20 °C. Some of the dried seeds were then imbibed individually at 20 °C or imbibed at 20 °C and then stratified at 5 °C under sterile conditions.

### 3.2.2 Conductivity tests

Electrolyte leakage of individual seeds was measured using a conductivity meter (CM100) over 24 h for 5 replicates. While for the fresh and dried material, conductivity of the leachate was measured with seeds immersed in distilled water, for imbibed and subsequently stratified seeds, water in which they had been rehydrated was used during the measurements. The seeds were then transferred to a water bath at 100 °C for 10 min. Following boiling, the conductivity of the leachate was measured again. Percentage leakage was determined as the highest conductivity before boiling as a fraction of the highest conductivity after boiling.

### 3.2.3 Tetrazolium (TZ) tests

Apparent seed viability was determined by the TZ tests. Twenty seeds which had been premoistened for 18 h were cut longitudinally through the embryo, soaked in 0.5% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC) solution for 24 h at 20 °C in the dark, and scored according to intensity and location of staining using criteria as outlined in International Rules for Seed Testing (ISTA, 1993).

### 3.2.4 Germination tests

Germination tests were conducted for 20 days with seeds submerged under water at 20 °C under constant light. Seeds were scored as germinated when they showed protrusion of the epiblast and swelling of the coleoptile and mesocotyl (Aiken *et al.*, 1988) or had a normal green shoot and seminal roots (Kovach and Bradford, 1992).

## 3.3 Results

### 3.3.1 Electrolyte leakage

Within each dehydration temperature, there was a significant difference in the electrolyte leakage of seeds after dehydration, imbibition and stratification (F-ratio = 14.39, $p<0.001$, Fig. 3.1). Electrolyte leakage was the highest after stratification and the lowest after imbibition. The differences in electrolyte leakage among seeds dried at different temperatures was significant after dehydration and subsequent stratification (F-ratio = 11.22, $p<0.001$ and F-ratio = 11.22, $p = 0.01$, respectively). After imbibition, however, there were no differences in leakage (F-ratio = 1.70, $p = 0.28$). Electrolyte leakage was the highest for seeds dried at 10 °C after dehydration and stratification. Seeds dried at 25 °C showed the lowest leakage after dehydration and stratification.

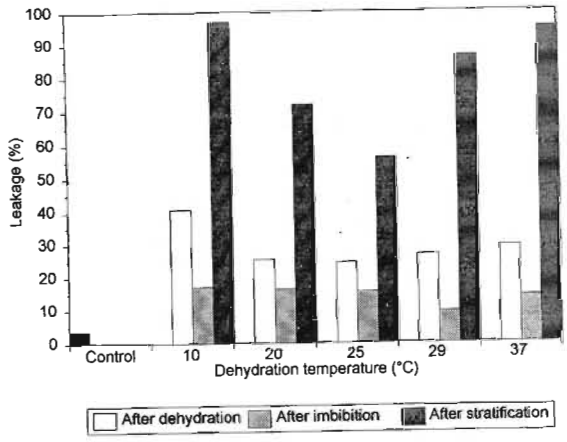


**Figure 3.1** Electrolyte leakage, expressed as % maximum (induced by boiling) of *Zizania palustris* seeds after dehydration, imbibition and stratification.

### 3.3.2 TZ tests

Freshly-harvested seeds showed a 100% positive TZ staining (Fig. 3). As with electrolyte leakage, within any one dehydration temperature there was a significant difference in positive TZ staining recorded after dehydration, imbibition and stratification (F-ratio = 2.7, p = 0.002). Highest positive tetrazolium staining was generally recorded immediately after dehydration, although tetrazolium staining had declined to between 65 and 25%. These values might be artificially low as a consequence of the damage associated with rapid imbibition that occurs when dried material is prepared for TZ testing (Kovach and Bradford, 1992a). Seeds showed the lowest positive tetrazolium staining after imbibition. Also, there was a significant difference in TZ staining among drying temperatures after dehydration, imbibition and stratification (F-ratio = 1.72, p = 0.01), with seeds dehydrated at 25 °C showing the highest positive staining and those dehydrated at 10 °C showing the lowest. TZ staining in the control material declined to 85% after 13 months of hydrated storage at 20 °C. No such decline was recorded in positive TZ staining for dehydrated seeds after dry storage at 6 °C for 9 months (F-ratio = 16.33, p = 0.15).

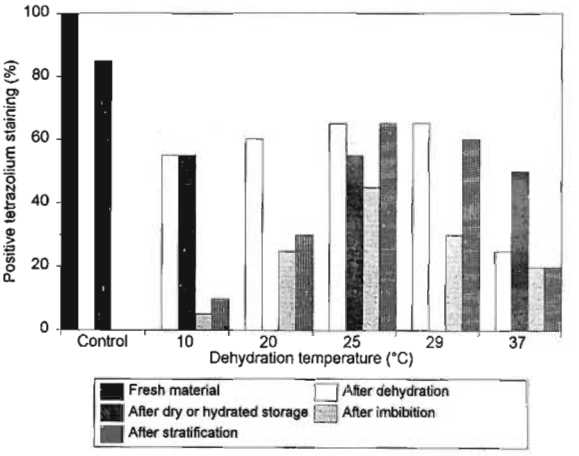


**Figure 3.2** Viability, as assessed by positive TZ staining, of seeds of *Zizania palustris* after harvest, dehydration, imbibition stratification or 13 months hydrated (control) or 9 months dry storage (in the case of seeds dehydrated at 10, 25 or 37 °C). Note that the TZ test was not performed on seeds after storage at 20 or 29 °C.

### 3.3.3 Germination tests

The highest germination was recorded at 20 °C, followed by seeds that were dehydrated at 25 °C (Table 3.1). None of the seeds dried at 10, 29 or 37 °C germinated after 20 days of submersion in distilled water. It is noteworthy that close to a third (32%) of seeds that had never been dehydrated, but had been stored in water at 20 °C for 12 months, germinated without any stratification whatsoever.

**Table 3.1** Germination of seeds of *Zizania palustris* dried at different temperatures or stored in water at 20 °C.

| Treatment | Germination (%) |
|---|---|
| **Dehydrated at 10 °C** | 0 |
| **Dehydrated at 20 °C** | 20 |
| **Dehydrated at 25 °C** | 5 |
| **Dehydrated at 29 °C** | 0 |
| **Dehydrated at 37 °C** | 0 |
| **Stored hydrated at 20 °C** | 32 |

### 3.4 Discussion

In general, the results of the present study with regard to electrolyte leakage are what one would expect in relation to membrane damage as observed by Berjak *et al.* (1994). Those authors observed that least damage was sustained by axis cells when they were dehydrated at 25 °C. Ultrastructural damage was more severe with lower temperatures. In the present study, electrolyte leakage after dehydration was the least at 25 °C and was the most severe at 10 °C. This agreement between the two studies is not surprising as electrolyte leakage is thought to assess the integrity of cell membranes (Powell, 1988).

Electrolyte leakage was higher after dehydration than after subsequent imbibition. It is now a well-established phenomenon that dried seeds undergo imbibitional damage, in which they leak solutes, during the initial stages of rehydration (e.g. Crowe *et al.*, 1989). Additionally, in a previous study prolonged rehydration was found to reverse ultrastructural damage accrued at higher temperatures and exacerbate it after

dehydration at lower temperatures (Berjak *et al.*, 1994). It is, therefore, not surprising that at least for the material dried at higher temperatures, electrolyte leakage was diminished after imbibition. What is surprising, however, is that electrolyte leakage was not exacerbated after dehydration at lower temperatures followed by imbibition. It is suggested that the deteriorative processes observed by Berjak *et al.* (1994) after stratification might have not proceeded far enough after only imbibition.

It was unexpected that electrolyte leakage increased after stratification and that the significant differences in leakage observed after dehydration were lost after imbibition. The former anomaly, particularly, is attributed to microbial growth, especially fungal growth. Microbial growth was observed in the present study and that of Kovach and Bradford (1992a), despite attempts in the current work to curb it by using sterile plant material and equipment.

The results of the present study with regard to viability as assessed by the TZ test agree with those of previous studies of Bradford and co-workers (Kovach and Bradford, 1992a; b; Berjak *et al.*, 1994). Those authors reported viabilities of approximately 90% for the freshly harvested seeds. In the present study, a viability of 100% was observed for the seeds in the fresh state. Also, the highest viability of the dried seeds was recorded after dehydration at 25 °C. When seeds were dried at 30 °C, the viability was similar to those dehydrated at 25 °C (Berjak *et al.*, 1994), much the same way as it was at 29 °C in the present study. Viability was lower after dehydration at reduced temperatures and lowest when seeds had been dried at 37 °C. Also, dried seeds showed no marked decrease in viability after storage at low temperatures for longer than a year, in fact, showing an apparently higher proportion of TZ staining relative to the situation after stratification, in the case of material dried at 10 or 37 °C.

Kovach and Bradford (1992b) have shown that seeds of *Zizania palustris* can be stored in water for longer than 12 months, especially at temperatures <15 °C. In the present study, seeds were stored in water at 20 °C. It is suggested that the 100 to 85% decline in viability, as measured by positive TZ staining, observed in the present study could be explained by the fact that seeds were stored at less than optimum temperature conditions (20 vs. <15 °C). It must also be noted that TZ staining may be misleading in the case of infected seeds (see below), thus even the indicated 85% viability was probably a considerable over-estimation. This view was strongly supported when, on the final assessment of the hydrated control material after 13 months of storage, about 50% of the seeds were scored as being visibly fungally deteriorated.

Apparent viability, as assessed by the TZ staining, declined during imbibition at all dehydration temperatures. This could be explained in terms of imbibitional damage as outlined above. In contrast, during stratification an increase in seeds testing positive with TZ was observed irrespective of the temperature at which seeds were dried except for the 37 °C material. These observations suggest the operation of repair mechanisms following dehydration and/or imbibitional damage, and that a proportion of the seeds testing negative with TZ staining after imbibition were, in fact, viable but relatively inactive at that stage. The anomaly that after dry storage a higher proportion of seeds dehydrated at 10 or 37 °C showed positive tetrazolium staining than after stratification, may be a spurious result, explicable in terms of the metabolic activity of associated micro-organisms. Heightened microbial activity would be expected in seeds that had been more damaged as a result of events occurring during dehydration at unfavourable temperatures.

With regard to the germination test, the highest viability was observed after dehydration at 20 °C. While this observation has been made in at least one other study (Bradford, pers. comm.[1]), it highlights the value of using more than one vigour test to assess seed quality as has been suggested by Smith and Berjak (1995) and Hampton and Tekrony (1995). Also, the germination test results were considerably lower than the TZ test results. Kovach and Bradford (1992a) have attributed a similar discrepancy to the presence of dormant seeds. That observation was borne out by a later study of Vertucci *et al.* (1995) where it was shown that germination increased with an increase in the period of stratification from 4 to 9 months. In the present study, stratification was done for 5 months only.

The most unexpected observation of this phase of the present study was the observation that almost a third (32%) of seeds stored at 20 °C for 12 months germinated without any stratification. This figure is actually in excess of 60%, if obviously deteriorated seeds (probably degraded by associated micro-organisms) were excluded. Simpson (1990) has mentioned a number of factors which influence both the induction and termination of dormancy. They obviously vary from species to species. It is suggested that, contrary to previously-held views (Simpson, 1966; Cardwell *et al.*, 1978; Atkins *et al.*, 1987; Kovach and Bradford, 1992a; Berjak *et al.*, 1994), low temperature is just one factor amongst several that facilitate the termination of dormancy in seeds of *Zizania palustris*. Low temperature stratification, therefore, may not be an absolute necessity for dormancy-breaking as is widely assumed. Possible mechanisms involved in dormancy-breaking in submerged *Z. palustris* seeds at 20 °C

[1]K. J. Bradford, Department of Vegetable Crops, University of California, Davis, CA, CA 95616-8361, USA.

could include environmental factors such as water, radiation (e.g. light and photo-period), and gases (e.g. $O_2$ , $CO_2$, and volatile organics such as ethylene and ethanol), internal factors such as growth regulators [e.g. gibberellic acids (GAs) and abscisic acid (ABA)] and mutual influences of inflorescence and caryopsis parts (Simpson, 1990).

From the fact that a significant proportion of the non-dried, control seeds germinated spontaneously after 12 months, it may be suggested that the actual germinability of the experimental seed samples (particularly those dried at 20 - 29 °C) might be considerably higher than indicated. It is possible that stratification for five months only, is not sufficient of a dormancy-breaking mechanism to realize the full germinability potential of *Z. palustris* seeds.

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# CHAPTER FOUR

# DESICCATION TOLERANCE

## Table of contents

## 4.1 Introduction

The ability to tolerate desiccation is an unusual feature in life (Vertucci and Farrant, 1995). This ability is, however, not an exclusive preserve of orthodox seeds. In spermatophytes, for instance, other propagating structures such as pollen, dormant buds and somatic embryos also possess this unusual feature. Desiccation tolerance of vegetative tissues is common among lower orders such as mosses and algae (Bewley, 1979; Levitt, 1980; Bewley and Oliver, 1992; Crowe *et al.*, 1992) although in these tissues desiccation tolerance is based on repair rather than protection, as is hypothesized for seeds (Bewley and Oliver, 1992).

Despite its widespread occurrence, very little is known about the mechanisms of desiccation tolerance (Vertucci and Farrant, 1995). Because not all tissues possess the ability of desiccation tolerance, those authors have concluded that it is difficult to achieve and is energetically costly.

In seeds, desiccation tolerance is acquired during development and is lost after germination (reviewed by Vertucci and Farrant, 1995). Many investigators have shown that, for both recalcitrant and orthodox seeds, embryos become more tolerant as they mature and less tolerant as they germinate (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; Berry and Bewley, 1991; Pritchard, 1991; Finch-Savage, 1992; Sun and Leopold, 1993; Tompsett and Pritchard, 1993).

The critical moisture content to which mature embryos can be dried without inducing desiccation damage has been suggested to be species-dependent (Levitt,

1980). These critical moisture levels 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 (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 Farrant, 1995).

At least five types of water can be distinguished from calorimetric and motional properties (Clegg, 1978; Rupley *et al.*, 1983; Vertucci, 1990). Type 5 water is dilute solution water and occurs at water contents between 0.6 to 0.9 g $g^{-1}$ dw. Type 4 water is believed to be concentrated solution water and is detected at water contents between 0.45 and 0.7 g $g^{-1}$ dw. Type 3 water is thought to form bridges over hydrophobic moieties on macromolecules and is detected at water contents between 0.25 and 0.45 g $g^{-1}$ dw. Type 2 water has glassy characteristics and is believed to have strong interactions with polar surfaces of macromolecules and hydroxyl groups of solutes. It is detected at water contents between 0.08 and 0.25 g $g^{-1}$ dw. Type 1 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 of 0.08 g $g^{-1}$ dw or less.

It appears that the different water types in plant cells perform different functions. For example, type 5 water is probably required for turgor in seeds (Vertucci, 1990). Whilst removal of type 3 water is associated with membrane structural changes (Vertucci and Farrant, 1995), on one hand, removal of type 1 water appears lethal to intermediate seeds on the other (Ellis *et al.*, 1990a; b; 1991; Kovach and Bradford,

1992), and may affect the long-term viability of some orthodox seeds and pollen (Vertucci and Roos, 1990; 1993; Hoekstra *et al.*, 1992).

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 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.

A number of hypotheses have been proposed in an attempt to elucidate the mechanism of desiccation tolerance (pp. 9-13; reviewed by Vertucci and Farrant, 1995). However, none of these hypotheses seems to account for desiccation tolerance fully. These hypotheses consider phenomenon at the ultrastructural and biochemical levels.

At the ultrastructural level, at least three general observations have been made. These include: (1) the observation that reduced vacuolation (as a result of intravacuolar protein accumulation or vacuole fragmentation into small bodies) increases desiccation tolerance (reviewed by Vertucci and Farrant, 1995), (2) the correlation between dedifferentiation of organelles (during the latter stages of reserve deposition) and desiccation tolerance (Bewley, 1979; Bergtrom *et al.*, 1982; Hetherington *et al.*, 1982; Schneider *et al.*, 1993) and (3) the observation that reduced membrane surface helps obviate lethal bilayer-bilayer interactions (at water contents greater than those at which sugars might be effective) (Vertucci and Farrant, 1995).

The observations of events at the biochemical level can be grouped into three categories - observations about proteins, carbohydrates and lipids, antioxidative systems and abscisic acid (ABA). As far as reserves are concerned, three further sub-categories can be identified - proteins, carbohydrates and lipids.

Besides intravacuolar accumulation of reserve proteins, which fulfil a space-occupying rôle, two types of non-reserve protein have been associated with desiccation tolerance. Late embryogenic accumulated (LEA) proteins have been associated with increased desiccation tolerance (Gomez *et al.*, 1988; Mundy and Chua, 1988; Close and Chandler, 1990; Kermode, 1990; Blackman *et al.*, 1991; 1992; Bewley and Oliver, 1992; Bradford and Chandler, 1992; Ried and Walker-Simmons, 1993). It has also been suggested that heat shock proteins, which are believed to stabilize macromolecule conformation (Lindquist and Craig, 1988), play a rôle in desiccation tolerance in seeds (Vertucci and Farrant, 1995).

Among all the groups of compounds that have been implicated in desiccation tolerance, carbohydrates, perhaps, have received the most attention. Whilst the "water replacement hypothesis" (Clegg, 1978; Crowe *et al.*, 1992) states that the hydroxyl groups of sugars substitute for water and provide the hydrophilic interactions required for membrane stabilization, the "glass formation hypothesis" argues that the formation of glasses (by non-specific interaction of hydroxyl groups of solutes and water during drying) confers desiccation tolerance (see p. 10). Vertucci and Farrant (1995) have also pointed out that the 'proper' location of sugars is crucial for efficient protection against dehydration. It has also been suggested that sucrose and other sugars may protect other cellular constituents by scavenging free radicals (Smirnoff and Cumbes, 1989). It is noteworthy that the production of free fatty acids (FFAs) by free radicals

may render sugars ineffective protectants against membrane disorganisation (McKersie *et al.*, 1989).

There is a view that increased oligosaccharide production (which lowers monosaccharide content) results in a reduction in respiratory substrates, thus limiting the source of free radicals (Rogerson and Matthews, 1977; Leprince *et al.*, 1992). Also, monosacharides may contribute to Maillard reactions that disrupt the protein structure (Karel, 1975; Loomis *et al.*, 1979; Koster and Leopold, 1988; Wettlaufer and Leopold, 1991).

With regard to lipids, it has been argued that high ratios of phosphatidylcholine (PC) to phosphatidylethanolamines (PEs), which can undergo lethal hexagonal phase transitions with drying (Liljenberg and Kates, 1985; Hoekstra *et al.*, 1989; Chen and Burris, 1990), are beneficial to desiccation tolerance. The effects of changes to the level of fatty acid saturation is unclear. On the one hand, increased levels of unsaturation can have beneficial effects by increasing tolerance to moderately low water contents (Uemura and Steponkus, 1989) and decreasing the likelihood of bilayer liquid crystalline to gel phase transitions which could lead to demixing (Quinn, 1985; Small, 1986; Crowe *et al.*, 1992). On the other hand, however, increased levels of unsaturation can have a detrimental effect by increasing the possibility of lethal hexagonal changes (Vertucci and Farrant, 1995).

As far as antioxidative systems and ABA are concerned, it is noteworthy that vegetative tissues with greater desiccation tolerance appear to have more efficient antioxidative systems (Dhindsa, 1991; Hendry *et al.*, 1992; Pastori and Trippi, 1993) and that ABA has been implicated in the response of vegetative tissues to water stress (Bewley and Oliver, 1992; Hetherington and Quatrano, 1992).

The objectives of the work reported in the present chapter were: (1) to determine if sugars and glass formation (vitrification) play any rôle in conferring desiccation tolerance on seeds of *Zizania palustris* during dehydration, and (2) if so, to establish whether such a rôle is influenced by the temperature at which seeds are dehydrated.

## 4.2 Materials and methods

### 4.2.1 Sugar extraction and analysis

One gram of embryonic axes of *Zizania palustris* seeds that had been dehydrated at 10, 25 or 37 °C were pulverized in liquid nitrogen. The seed material from each sample was then rehydrated with 5 ml of boiling distilled water for 10 minutes. The samples were then centrifuged and the supernatant was collected and filtered through a 0.45 µm nylon filter. The samples were analyzed using high performance anion exchange chromatography (HPAEC) and pulsed amperometric detection (PAD). Identification was done by means of retention time only.

### 4.2.2 Differential scanning calorimetry (DSC)

Thermal transitions were simultaneously measured for three to four embryonic axes excised from *Zizania palustris* seeds which had been stored at 6 °C for 9 months, and pooled. The seeds had been dehydrated at 10, 25 or 37 °C. Thermograms were recorded using a Perkin-Elmer differential scanning calorimeter DSC-7. The measurements were made at temperatures between -120 and +50 °C. Helium was used as the purge gas. The thermal behaviour of samples was studied during heating, after cooling to -120 °C. The heating and cooling rates were set at 10 and 20 °C $min^{-1}$, respectively.

## 4.3 Results

### 4.3.1 Sugar contents

No stachyose was detected in the extracts from embryonic axes of any of the treatments. Seeds stored hydrated at 20 °C and those dehydrated at 25 °C showed a different sugar profile from that of seeds dehydrated at 10 or 37 °C (Table 4.1). No raffinose was detected in the hydrated controls and was low in the material dried at 25 °C. The levels of monosaccharides (glucose and fructose) were higher in the control and 25 °C-dried material. Further, the amount of sucrose was lower in the embryonic axes of seeds stored hydrated at 20 °C or dehydrated at 25 °C than in those dehydrated at 10 or 37 °C. The ratios of sucrose to raffinose ranged between 14 and 16 for all the experimental treatments.

**Table 4.1** Sugar contents (mg $g^{-1}$) of embryonic axes of *Zizania palustris* dried at different temperatures or stored in water at 20 °C.

| Sugar / Treatment | Glucose | Fructose | Sucrose | Raffinose |
|---|---|---|---|---|
| **Stored hydrated at 20 °C** | 2.09 | 2.09 | 4.18 | 0 |
| **Dried at 10 °C** | 1.75 | 1.46 | 201.03 | 13.69 |
| **Dried at 25 °C** | 3.03 | 3.03 | 48.51 | 3.03 |
| **Dried at 37 °C** | 1.41 | 1.41 | 226.42 | 16.17 |

### 4.3.2 DSC

A proportion of embryonic axes showed the occurrence of glass transitions, irrespective of the temperature of drying (Table 4.2). Glass to liquid transitions were recorded across a relatively wide range of temperatures (-113.21 to -42.70). There was, however, no significant correlation between water content and parameters of the glassy state, such as the glass to liquid transition temperature ( r = 0.5, p = 0.90), specific heat capacity (r = -0.41, p = 0.32) or relaxation time [(time lapse of the transition), r = -0.13, p = 0.76].

**Table 4.2** Thermal characteristics of water in seeds of *Zizania palustris* dried at 10, 25 and 37 °C.

| Dehydration temperature (°C) | Number of samples showing glass formation | Water contents (g $g^{-1}$ DW) | Glass to liquid transition temperature (°C) | Specific heat capacity (mJ $g^{-1}$ $°C^{-1}$) | Relaxation time (s) |
|---|---|---|---|---|---|
| 10 | 2/6 | 0.072 | -88.41 | 6.12 | 14.52 |
| | | 0.029 | -63.01 | 7.48 | 15.9 |
| 25 | 2/6 | 0.020 | -106.39 | 8.65 | 15.96 |
| | | 0.060 | -105.22 | 6.56 | 14.34 |
| 37 | 4/6 | 0.021 | -113.21 | 3.92 | 15.96 |
| | | 0.031 | -84.57 | 6.77 | 15.96 |
| | | 0.042 | -62.62 | 6.08 | 17.52 |
| | | 0.017 | -42.70 | 10.00 | 12.78 |

**4.4 Discussion**

Desiccation tolerance in seeds is thought to involve multiple components. These include: (1) accumulation of non-reducing sugars (e.g. Leprince *et al.*, 1990; Blackman *et al.*, 1992), (2) accumulation of reserves and degree of vacuolation (reviewed by Farrant *et al.*, 1993), (3) synthesis of late embryogenic accumulated (LEA) proteins (e.g. Blackman *et al.*, 1991) and (4) free-radical scavenging systems (e.g. Hendry, 1993).

High levels of sugars, particularly sucrose, raffinose and stachyose, have been suggested to afford desiccation tolerance (Leopold and Vertucci, 1986; Koster and Leopold, 1988; Chen and Burris, 1990; Leprince *et al.*, 1990), and the ratio of raffinose to sucrose has been implicated in desiccation tolerance (Koster and Leopold, 1988, Chen and Burris, 1990, Blackman *et al.*, 1992; Bernal-Lugo *et al.*, 1993; reviewed by Horbowicz and Obendorf, 1994). Sugars have been proposed to confer desiccation tolerance on seeds by replacing water on macromolecular surfaces (Clegg, 1986; Crowe and Crowe, 1986) or by forming the glassy (vitrified) state (Koster, 1991; Leopold *et al.*, 1992; Bruni, 1993).

No stachyose was detected in any of the treatments, in the present study. Also, no raffinose was detected in the control material. Since, according to Becker and Lorenz (1981), raffinose is the major oligosaccharide present in wild rice, it is suggested that it may have been metabolised during hydrated storage. Sugar profiles of seeds stored hydrated at 20 °C and those dehydrated at 25 °C were similar. For instance, both showed relatively high levels of monosaccharides (glucose and fructose) and low levels of sucrose. It is most likely that oligosaccharides and sucrose are metabolized during germination (Williams and Leopold, 1989). Also, it is suggested that the high levels of

monosaccharides (respiratory substrates) in the control and 25 °C-dried material are indicative of higher metabolic activity than in the metabolically-quiescent 10 or 37 °C-dehydrated seeds (Rogerson and Matthews, 1977; Leprince *et al.*, 1992). Interestingly, 32% of the total control seed sample that had not been stratified, but had been maintained in water at 20 °C, had broken dormancy and germinated. The proportion of the germinated seeds was actually far higher if calculated as a fraction of the viable seed population (see Chapter 3). Germinative metabolism is suggested to account for the sugar composition of the control seeds.

Horbowicz and Obendorf (1994) have suggested that seeds with a sucrose to oligosaccharide ratio of <1.0 show higher desiccation tolerance and storability and *vice versa*. In the present study, all seed samples showed sucrose to oligosaccharide ratios of >1 and a degree of desiccation sensitivity (see Chapter 3). It is suggested that the seeds of *Zizania palustris* do not contradict the hypothesis of Horbowicz and Obendorf (1994).

Despite glasses being formed in similar proportions in all experimental treatments, it is noteworthy that some seeds incurred desiccation damage in all treatments. This leads one to conclude that, while glass formation in *Zizania palustris* may contribute to seed tolerance of desiccation, it does not confer full desiccation tolerance on those seeds.

In the present study, there was no significant correlation between water content and sugar composition (the sugar profiles being qualitatively similar in the experimental samples) and parameters of the glassy state. This contrasts with previous studies which have shown that the glass to liquid transition is dependent mainly on sugar composition and molecular weight and the presence of plasticizers; water is the most ubiquitous

plasticizer (reviewed by Leopold *et al.*, 1994). Also, Eisenberg (1984) has shown that as sugar solution is dried down, the formation of glass is associated with a marked decrease in heat capacity. This leads one to speculate that other plasticizers [such as temperature (Williams and Leopold, 1989; Franks *et al.*, 1991)] and/or other factors [such as crystallization (Herrington and Branfield, 1984)] may be are responsible for the relatively wide range of glass to liquid transition temperatures observed in embryonic axes of *Zizania palustris*.

In conclusion therefore, it appears that the formation of glasses cannot be equated with desiccation tolerance in seeds of *Z. palustris*, irrespective of the temperature at which dehydration occurred. Additionally, the ratios of sucrose to oligosaccharides was found to be unfavourable in terms of protection against the effects of dehydration, notwithstanding the temperature at which this took place.

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# CHAPTER FIVE

# LIPID PEROXIDATION

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## 5.1 Introduction

Free radicals are molecular species which contain an unpaired electron (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 causes the formation of yet another free radical and a self-propagating chain reaction ensues.

Until relatively recently, interest in free radical reactions was confined to the chemical sciences. This scenario changed about half-a-century ago when Harman (1956) introduced the free-radical theory of ageing to medical sciences. Soon thereafter, Kaloyereas (1958) introduced that theory to the seed sciences when it was suggested that lipid oxidation underlies the loss of viability in (orthodox) seeds.

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

The tight control of the processes that involve free radical formation can and does, however, become upset [e.g. by environmental stresses (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. It is perhaps, the biggest irony of life that oxygen, the primary agent of aerobic respiration, is often the primary promotor of the deleterious free radical reactions in biological systems.

The biochemical events which lead to free radical production are complex (Benson, 1990). Also, 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 (Benson, 1990; Hendry and Crawford, 1994).

Among the many targets of free radical attack, membranes are one of the most important (Benson, 1990). Membrane lipids contain unsaturated bond systems and are, thus, 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 (Smith and Berjak, 1995).

Free radicals have been implicated in a number of processes in seed biology. These include: (1) ageing of orthodox seeds (pp. 21-22), (2) dehydration of recalcitrant seeds (p. 23-25) and deterioration of recalcitrant seeds in hydrated storage (p. 25-26). In this chapter, however, we concentrate on the involvement of free radicals during dehydration of recalcitrant seeds.

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 to lipid peroxidation, and accumulate stable free radicals under moisture stress conditions (Finch-Savage *et al.*, 1994). As an example, Hendry

*et al.* (1992) have shown that loss of viability during drying in the desiccation-sensitive seeds of *Quercus robur* L. 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 an identical free radical in a range of species subjected to dehydration.

The direct detection of free radicals requires sophisticated equipment (Benson, 1990). 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 this occurs depends on the free radical species and the applied magnetic field.

Because of the inhibitory cost and availability of EPR 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 phospholipid content and measuring the release of free fatty acids (FFAs), and the production of lipid peroxides and their breakdown products (e.g. volatiles such as hydrocarbons, aldehydes and alcohols).

The aim of the work reported in the present chapter was to test whether free radical-mediated damage predominates in *Z. palustris* seeds that had been dehydrated at temperatures >25 °C as hypothesized by Berjak *et al.* (1994).

## 5.2 Materials and methods

### 5.2.1 Seed material

Seeds of *Z. palustris* that had been dehydrated at a range of temperatures or stored in water at 20 °C were ground in liquid nitrogen. The material was then freeze-dried for 72 h.

### 5.2.2 Lipid extraction

Lipids were extracted from ground seeds using dichloromethane/methanol (2:1 v/v) containing butylated hydroxytoluene (50 mg $l^{-1}$). Following centrifugation at 1500 × g in a benchtop centrifuge, the upper solvent phase was removed, taken to dryness under nitrogen at 45 °C and treated as indicated in 5.2.4 to produce fatty acid methyl esters.

### 5.2.3 Hydroperoxide determination

Twenty µl of 0.014M ferrous chloride ($FeCl_2$) were added to 5 ml of the lipid extract in dichloromethane/methanol (2:1 v/v) and shaken. Twenty µl of 30% potassium thiocynate (KSCN) were then added with shaking. The absorbance was read with a spectrophotometer at 505 nm, against a blank containing reagents and solvents only.

### 5.2.4 Lipid esterification and fatty acid analysis

Methyl esters of the fatty acids were obtained using the base-catalyzed technique of Metcalfe and Wang (1981). The dried lipid extract was resuspended in 1.5 ml of diethyl ether, and 200 µl of 1M tetramethylammonium hydroxide in methanol were added. The mixture was shaken and allowed to stand for 1 min before addition of 2 ml

of distilled water. The mixture was shaken and then centrifuged at 1500 × g in a benchtop centrifuge. The upper ether layer was aspirated and dried with anhydrous sodium sulphate.

Samples were analyzed on a Varian 3700 Gas Chromatograph fitted with a flame-ionization detector. One µl was injected onto a 1.8 m × 2 mm internal diameter glass column packed with 10% Silar 5CP on Supercoport 100/120 mesh. The injector temperature was set at 220 °C, the detector at 250 °C and the column operated isothermally at 200 °C. Nitrogen was used as a carrier gas at 25 ml $min^{-1}$, and three replicate injections were made of each sample.

### 5.2.5 Lipid fractionation

Lipids were fractionated by silicic acid column chromatography (Beutelmann and Kende, 1977). A total lipid extract, prepared as described above, was dissolved in 1 ml dichloromethane and applied to a Bondelut® syringe column containing 0.5g silica gel. Neutral lipids and glycolipids were eluted successively, with 4 ml each of dichloromethane and acetone. The phospholipid fraction was eluted with 4 ml of methanol.

The fractions were dried down under nitrogen at 45 °C, resuspended in diethylether, and esterified as described above. One µl was injected onto the gas chromatograph. Fatty acid methyl esters were identified by co-chromatography of authentic standards (Sigma item 189-1).

### 5.2.6 Head-space analysis

One-and-a-half grams of intact seeds were placed in 14 ml serum vials, sealed with teflon lined septa and heated in a sand bed at 80 °C for 100 min. A 250 µl sample of head-space gas was taken with a gas-tight syringe, pre-heated to 80 °C. The head-space volatiles were separated on a fused silica (PLOT) column of 25m × 0.53 µm internal diameter coated with Poraplot Q (Chrompak, The Netherlands). The column was operated isothermally at 150 °C, injector and detector temperatures were 200 °C and 240 °C, respectively. The column head pressure was 40 kPa and was operated in splitless mode.

Tentative identification of head-space volatiles was made by co-chromatography of authentic aldehyde standards (Polysciences, USA).

## 5.3 Results

### 5.3.1 Hydroperoxide levels

The hydroperoxide levels were linearly correlated with dehydration temperature, albeit marginally significantly ($r = 0.89$, $p = 0.10$, Fig. 5.1). A comparison of individual treatments, however, revealed a highly significant difference in the levels of hydroperoxide between seeds stored hydrated at 20 °C (control) and those dehydrated at 37 °C (F-ratio = 1.70, $p = 0.02$).

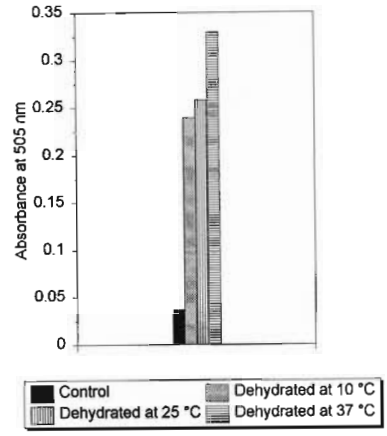


**Figure 5.1** Hydroperoxide levels of *Zizania palustris* seeds stored hydrated at 20 °C or dehydrated at 10, 25 or 37 °C.

### 5.3.2 Fatty acid levels

There were no differences in amounts of individual fatty acid methyl esters, expressed as an area percent, derived from the neutral and glycolipid fraction of lipids (i.e. storage and other lipids) among the different treatments (Fig. 5.2). An examination of the fatty acids of the neutral and glycolipid fraction revealed that linoleate (18:2) was the predominant fatty acid at *c.* 38%. Levels of palmitate (16:0), oleate (18:1) and linolenate (18:3) were in the range 16 - 22% each.

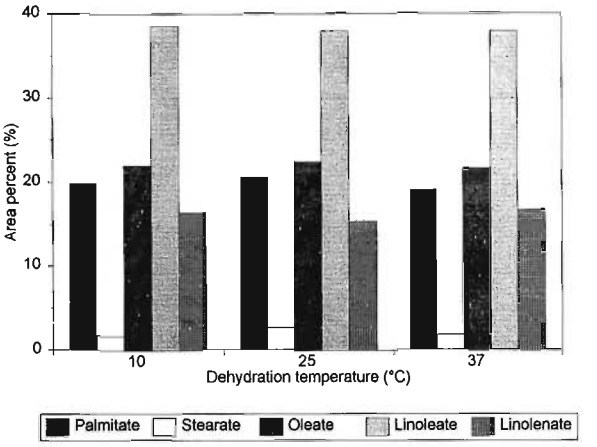


**Figure 5.2** Area percent of chromatogram of fatty acids derived from the neutral and glycolipid fraction of lipids of seeds of *Zizania palustris* dehydrated at 10, 25 and 37 °C.

A comparison of the individual fatty acid methyl esters of the phospholipid (PL) fraction (i.e. membrane lipids, Fig. 5.3) revealed that palmitate, stearate, oleate, linoleate and linolenate occurred in similar levels in seeds dried at 10, 25 or 37 °C.

In comparing the PLs and the neutral / glycolipid fractions, the following should be noted: (1) the linoleate levels were similar; (2) palmitate levels were 30% of the total fatty acid fraction of phospholipids, compared with the 20% in the neutral/glycolipids and (3) there were corresponding lower levels of oleate (18:1) [17%] and linolenate (18:3) [12%] in the PLs compared with the neutral / glycolipids (22% and 18%, respectively for 18:1 and 18:3).

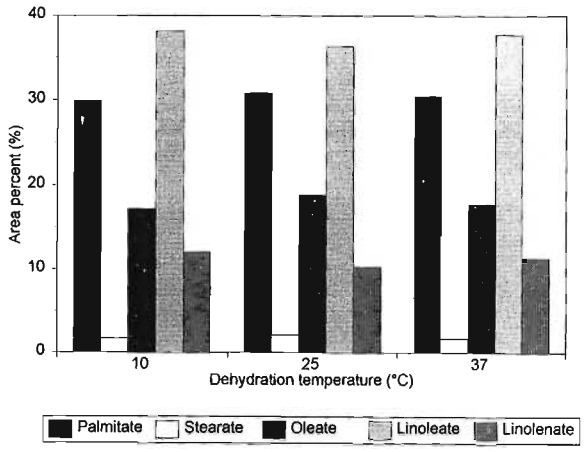


**Figure 5.3** Area percent of chromatogram of free fatty acids derived from the phospholipid fraction of lipids of seeds of *Zizania palustris* dried at 10, 25 and 37 °C.

### 5.3.3 Aldehyde levels

Five aldehydes were identified on the basis of co-chromatography as propanal, pentanal, methylbutanal, hexanal and butanal and there were four unknown volatiles. Methylbutanal showed a marginally significant correlation with dehydration temperature ($r = 0.78$, $p = 0.06$) and butanal was negatively correlated with dehydration temperature ($r = -1.00$, $p = 0.00001$). A comparison of individual treatments revealed that seeds dried at 37 °C produced considerably less aldehydes, as expressed by total integrated count, than seeds dried at 10 or 25 °C (Figure 5.4). Also, total integrator count was significantly correlated with dehydration temperature ($r = -0.81$, $p = 0.05$).

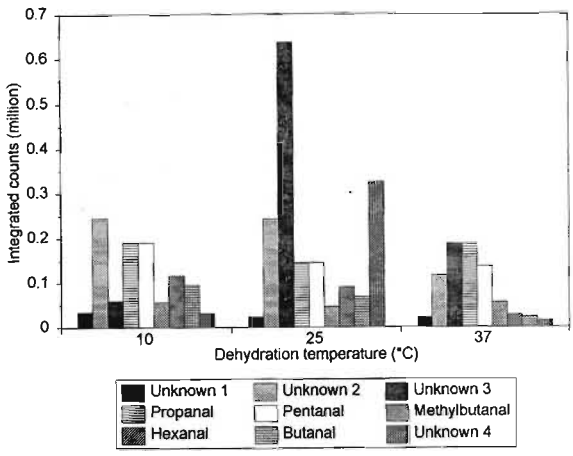


**Figure 5.4** Integrated counts of aldehydes evolved from the seeds of *Zizania palustris* dried at 10, 25 and 37 °C.

## 5.4 Discussion

Evidence for the involvement of free radicals in seed ageing is equivocal (reviewed by Smith and Berjak, 1995). While some studies have shown a 'strong' correlation between hydroperoxide [products of oxidation of unsaturated free fatty acids (FFAs) by free-radicals] levels and viability loss (e.g. Hailstones and Smith, 1988), others have failed to establish such a relationship (e.g. Fergusson *et al.*, 1990). Berjak and Smith (1995), however, attribute the contradictions to methodology. For instance, the former investigators looked at viability loss whilst the latter looked at vigour loss.

Traditionally, lipid peroxidation has been shown to occur in orthodox seeds in dry storage (e.g. Hailstones and Smith, 1988; 1989; Leprince *et al.*, 1995). More recently, however, lipid peroxidation has been demonstrated in recalcitrant seeds during dehydration (e.g. Wood *et al.*, 1995).

In the present study, a marginally significant linear relationship was established between hydroperoxide levels and temperature of dehydration of *Zizania palustris* seeds. Temperature has been established as one of the factors which determine the extent of peroxidation in at least deterioration of orthodox seeds in dry storage (reviewed by Smith and Berjak, 1995; Leprince *et al.*, 1995). It is suggested that the results of the present study show the influence of temperature in promoting catalytic activity. That is, the rates of reactions increase with a rise in temperature.

Hailstones and Smith (1988) further supported the suggestion of the involvement of peroxidation in orthodox seed ageing by showing a decline in the levels of unsaturated fatty acids. The linear relationship between hydroperoxide levels and dehydration temperature, established in the present study, could however, not be supported by a decline in the levels of unsaturated fatty acids from either the phospholipid (membrane) or the neutral and glycolipid (storage and other) fractions. One possible explanation for this result could be that other components [e.g. proteins (Smith and Berjak, 1995)] could be responsible for the differences in measured hydroperoxide levels.

The involvement of lipid peroxidation in seed ageing has been further supported by an increase in the levels of aldehydes (breakdown products of hydroperoxides) evolved (e.g. Wilson and McDonald, 1986; Hailstones and Smith, 1989). While methylbutanal showed a marginally significant correlation with dehydration and hence peroxide levels (which are marginally correlated with dehydration temperature), butanal showed a highly significant negative correlation with dehydration temperature (and hence peroxide levels). In this regard, it is noteworthy that Hailstones and Smith (1989) showed that butanal (along with hexanal and pentanal) was highly correlated with peroxide levels and seed vigour. Those authors, however, showed a positive

correlation between butanal and peroxide levels. In contrast, in the present study a negative correlation was observed. Other differences in the levels of individual aldehydes among the treatments were observed. This is to be expected considering that the formation of aldehydes from hydroperoxides is a thermal process and hence is temperature-dependent (Frankel, 1982). Also, the total amount of aldehyde evolved was lower at the higher (37 °C) than at the lower (10 °C) temperatures. This observation could explain the negative correlation between butanal and peroxide levels in the present study as more butanal and/or other aldehydes could be evolved during dehydration at 37 °C.

Most of the studies on lipid peroxidation have used whole seeds (e.g. Hailstones and Smith, 1988; 1989; Leprince *et al.*, 1995). This practice may, however, lead to masking of the events of lipid peroxidation, which presumably occur predominantly in the cellular embryo, by larger reserves in the storage tissues. This situation could explain the lack of significant differences in the levels of aldehydes and fatty acids in the present study.

In conclusion, it is suggested that seeds of *Zizania palustris* show different levels of peroxidation depending on the temperature of dehydration. This relationship is, however, marginally statistically significant and could not be supported by a decline in fatty acid levels or an increase in the production of volatile aldehyde breakdown products of hydroperoxides.

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# CHAPTER SIX

# SOME MICROSCOPICAL EFFECTS OF DEHYDRATION ON MEMBRANES

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## 6.1 Introduction

According to Hoekstra *et al.* (1989), in recent times there have been numerous efforts to understand the mechanism of desiccation tolerance in living tissues (e.g. Crowe and Clegg, 1978; Leopold, 1986). Among the various research areas of focus in this regard, have been the biochemistry and biophysics of membrane lipids, which form the basis of the present chapter of the present treatise.

Two primary stresses have been hypothesized to destabilize membrane bilayers during dehydration: (1) fusion and (2) lipid phase transitions (reviewed by Crowe *et al.*, 1987; Crowe *et al.*, 1988; Crowe and Crowe, 1991). Some *in vitro* studies reveal, however, that a small amount of trehalose or other sugars is sufficient to inhibit fusion between vesicles completely, but that inhibition of fusion alone is not sufficient to preserve dry vesicles (Crowe *et al.*, 1986; Womersley *et al.*, 1986). In view of those results, many investigators have turned to the study of lipid phase transitions (see below) as an alternative explanation for the mechanism of stabilization or its lack (reviewed by Crowe *et al.*, 1992).

*In vivo*, membrane phospholipids (PLs) are hydrated. As an example, phosphatidylcholine (PC) is hydrogen-bonded to 10-12 water molecules around each phosphate (Crowe *et al.*, 1992). It is, therefore, hardly surprising that water constitutes approximately 20% of the weight of the hydrated bilayers.

When water is removed, the packaging of the head groups increases, which leads to increased van der Waals' interaction among the hydrocarbon chains. This results in a dramatic increase in the phase transition temperature ($T_m$) (Hoekstra *et al.*, 1989; Crowe *et al.*, 1992). For instance, lipids used in liposome studies will undergo a -10 to 60 °C increase in their $T_m$ during dehydration. This leads to these lipids assuming a gel

state at room temperature, unlike their liquid crystalline phase in the hydrated state (Crowe *et al.*, 1988). When the dry lipids are placed in water, they undergo the reverse phase transition to form the liquid crystalline state (Crowe *et al.*, 1992).

It is a well established phenomenon that membranes become leaky as they pass through phase transitions (Blok *et al.*, 1975; Crowe *et al.*, 1989). In this regard, it is to be expected that dry membranes would leak on rehydration during what has come to be known as imbibitional damage. This leakage is particularly acute if rehydration is carried out at low temperatures (Crowe *et al.*, 1992). The rates of leakage may be reduced by slow imbibition (Hoekstra *et al.*, 1989; Crowe *et al.*, 1992). Hoekstra (1984) has also shown that leakage can be reduced by performing imbibition at higher temperatures than the $T_m$ which ensures the establishment of the liquid crystalline state before water is introduced.

Observations of leakage from dry seeds prompted Simon and co-workers (Simon and Harun, 1972; Simon, 1974; 1978; Simon *et al.*, 1976) to hypothesize that the site of imbibitional damage is the plasma membrane and that the damage can be explained in terms of phase changes of PLs, particularly (according to those authors) the formation of non-bilayer hexagonal II phases. The formation of such phases as a common phenomenon, however, does not seem to be the rule (Crowe *et al.*, 1989). Those authors have shown that membrane leakage during imbibition can be explained in terms of lipid phase transitions from gel to liquid crystalline phase during which PLs become more permeable.

From *in vitro* studies, it has been shown that trehalose can depress the $T_m$ of lipids well below that of hydrated systems and thus prevent the leakage of membranes associated with lipid phase transitions (Crowe *et al.*, 1992). Those authors argue that

trehalose achieves this stabilizing effect on membrane lipids by way of interaction between its -OH groups with the phosphate of membrane PLs (Crowe *et al.*, 1985a; b; 1986; 1987; 1988; 1989; Madden *et al.*, 1985; Lee *et al.*, 1986; 1989; Strauss and Hauser, 1986; Strauss *et al.*, 1986; Caffrey *et al.*, 1988; Crowe and Crowe, 1988; Quinn *et al.*, 1988; Quinn, 1989; Tsvetkov *et al.*, 1989; Harrigan *et al.*, 1990). Models based on X-ray crystallography studies suggest that trehalose fits well between the polar head groups of PLs with multiple sites for interaction (Brown *et al.*, 1972; Taga *et al.*, 1972; Chandrasekhar and Gaber, 1988; Rudolph *et al.*, 1990). Further, those models demand that the membrane bilayer be expanded to accommodate trehalose, an effect that would reduce the chances of interaction between the hydrocarbon chains (Crowe *et al.*, 1992).

Trehalose does not, however, occur in seeds (Koster and Leopold, 1988). It is noteworthy, though, that disaccharides such as sucrose and other oligosaccharides such as raffinose and stachyose, which are present in considerable quantities in seeds (Koster and Leopold, 1988), are capable of performing the rôle of trehalose outlined above, albeit not as efficiently (Crowe *et al.*, 1992). Green and Angell (1989) and Leopold and co-workers (Caffrey *et al.*, 1988 and Williams and Leopold,1989) have suggested that the greater efficiency of trehalose may be related to its reluctance to crystallize and its propensity to vitrify. It is presumed that a crystallized sugar would be unavailable to interact with PLs (Crowe *et al.*, 1992).

The most common method for measuring phase transitions is differential scanning calorimetry (DSC). This method is, however, not often useful in detecting transitions in the complex mixtures of lipids found in native membranes since cooperativity of transitions in such mixtures is low, with resulting diminished enthalpy (Crowe *et al.*,

1992). Consequently, it has been necessary to turn to other methods. Crowe *et al.* (1992) have, therefore, adapted Fourier transform infrared (FTIR) spectroscopy for this purpose and have shown that the elevation in vibrational frequency in $CH_2$ groups in membrane lipids can be used to detect phase transitions in native membranes. Attempts to measure phase transitions in membrane lipids in *Zizania palustris* axes using FTIR spectroscopy have, however, been thwarted by the high background level of nonpolar storage lipids (K. J. Bradford and J. H. Crowe cited in Berjak *et al.*, 1994). This is rather surprising considering the low oil content of those seeds [< 1% weight:weight (White and Jayas, 1996)].

In this chapter, the possible occurrence of lipid phase transitions during dehydration of *Z. palustris* seeds was investigated microscopically. It was thought that, if membrane PL phase transitions were at all involved, they would probably predominate at low temperatures as has been observed in other studies (reviewed by Crowe and Crowe, 1986).

## 6.2 Materials and methods

### 6.2.1 High resolution scanning electron microscopy (HRSEM)

Embryonic axes of *Z. palustris* seeds that had been dehydrated at 10, 25 or 37 °C or stored in water at 20 °C were sampled for small pieces of tissue (*c.*1 × 1 × 1 mm). Five axes were sampled per treatment. These were then pre-fixed in 1% osmic solution ($OsO_4$) buffered at pH 7.2 with 0.05 M sodium cacodylate buffer for 2 h. After rinsing with a buffer solution, specimens were cryoprotected by successive immersion in 5, 30 and 50% dimethyl sulfoxide (DMSO) for 30 min in each solution. The specimens were then frozen in liquid nitrogen and fractured using a razor blade. The split pieces were

thawed in 50% DMSO solution at room temperature. They were then repeatedly rinsed in buffer until DMSO was completely removed and were then etched in 0.1% $OsO_4$ buffered at pH 7.2 with cacodylate. The specimens were held at room temperature for 3, 6, 9 or 12 days. (It should be noted that on viewing with the HRSEM, no discernible differences were evident). Following the etching process, specimens were post-fixed in 1% $OsO_4$ for 1 h. To enhance the electrical conductivity of the specimens, they were rinsed in buffer containing 2% tannic acid for 1 h and then with 1% $OsO_4$ for 1 h. The specimens were dehydrated in a graded ethanol series, then dried in a critical point dryer and coated with platinum in an ion coater. Metal coated specimens were viewed in a JEOL 6000 field emission high resolution SEM.

### 6.2.2 Freeze-fracture electron micrcoscopy (EM)

Embryonic axes of *Z. palustris* seeds that had been dehydrated at 10, 25 or 37 °C were examined. Five seeds per treatment were sampled and fragments of the embryonic axes were pooled. These were sub-sampled, mounted on pure glycerol on 3 mm gold hats (Balzers, 3 mm gold specimen carrier) and frozen by plunging into liquid propane in a liquid nitrogen reservoir. The embryonic axis samples of seeds that had been stored in water at 20 °C were first cryoprotected in 20% dimethyl sulfoxide (DMSO) and plunge frozen in the same fashion as the dehydrated counterparts. The samples were then fractured in JEOL 9010C freeze fracture instrument at -180 °C. Replicas were made by 60 ° rotary carbon deposition, coated at 60 ° with platinum for 3.6 sec followed by carbon for 22 sec. Replicas were then coated with 1% polycarbonate plastic [Lexan, (General Electric, Pittsfield, Massachussets, USA)] in dichloromethane to keep them intact and the biological material digested in 3%

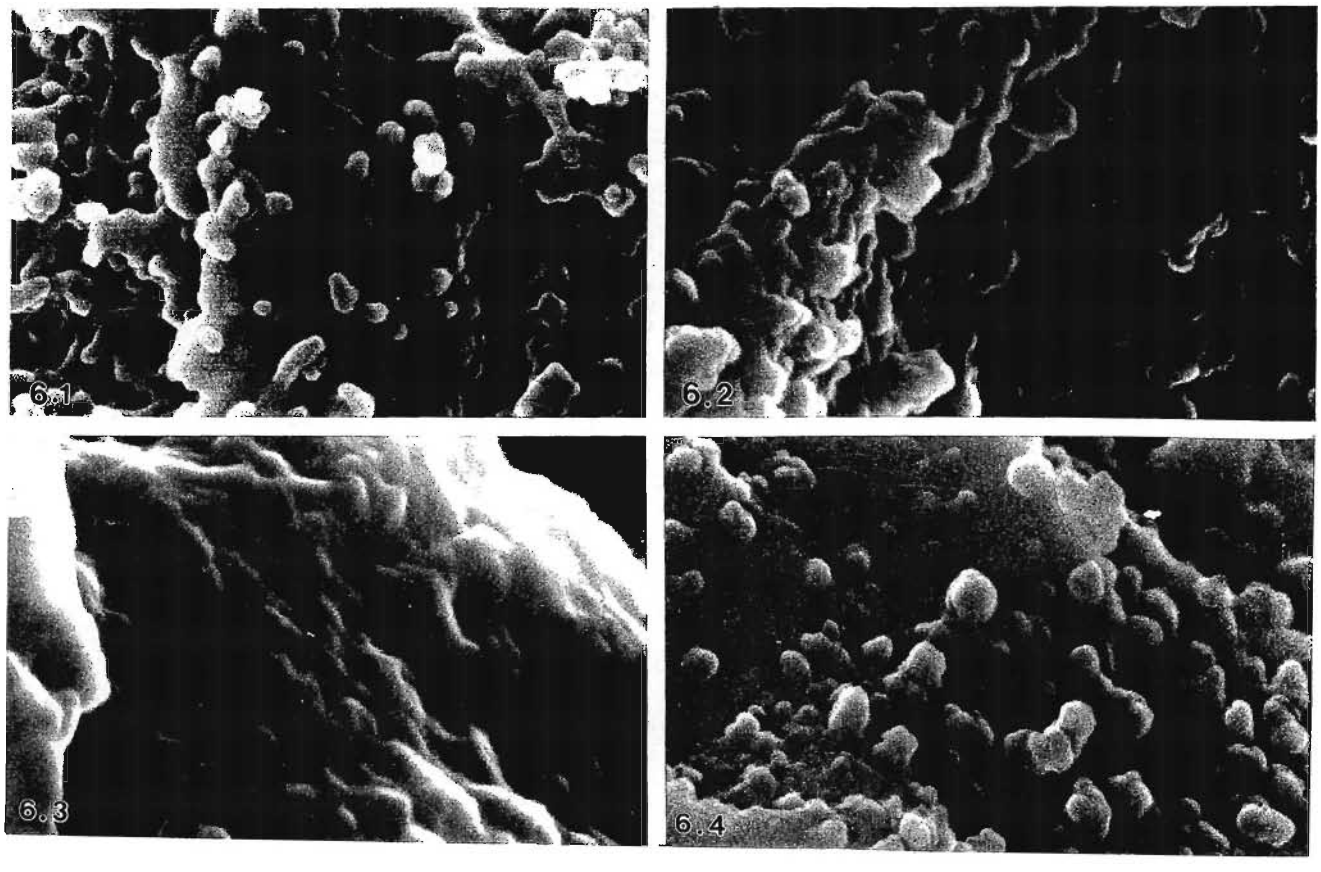
hydrogen peroxide for 3-5 h followed by rinsing in distilled water. Replicas were then placed in Chomerge (Fisher Scientific) for 1-2 days to complete the digestion. They were rinsed and Lexan was removed with dichloromethane. The replicas were viewed in a JEOL 1010 transmission electron microscope (TEM), using 10 ° of tilt.

## 6.3 Results

### 6.3.1 HRSEM

Peripheral membrane complexes (PMCs) presumed to be multi-protein particles on membrane surfaces of cells of embryonic axes of seeds stored hydrated at 20 °C (control) showed a high degree of variability both in terms of sizes and shapes (Figure 6.1). The variability of shapes and relative dimensions of PMCs on membrane surfaces of cells of embryonic axes of seeds dried at 25 °C resembled that of the control (Figure 6.2). They were, however, much more clustered. In contrast, PMCs on membrane surfaces of cells of embryonic axes of seeds dried at other temperatures showed less variability both in terms of sizes and shapes. For instance, PMCs on membrane surfaces of cells of embryonic axes of seeds dehydrated at 10 °C were relatively homogeneous. In these specimens the incidence of PMCs could be seen to be very reduced. Also, there were many areas which appeared devoid of such associated particles (Figure 6.3). The variability of PMCs on membrane surfaces of embryonic axes of seeds dried at 37 °C appeared intermediate between those of seeds dried at 10 or 25 °C (Figure 6.4). The incidence of PMCs in 37 °C specimens relative to material that had been dehydrated at 25 °C, appeared somewhat reduced, but nowhere near the extent that occurred in 10 °C-dried specimens.

A comparison of relative abundance of PMCs in material dried at different temperatures revealed that membrane surfaces of cells of embryonic axes of seeds dried at 25 °C showed the highest number of PMCs per unit area. On the other hand, membrane surfaces of cells of embryonic axes of seeds dried at 10 °C showed the least abundance of PMCs per unit area. Membrane surfaces of embryonic axes of seeds dried at 37 °C showed a density of PMCs apparently intermediate between those of seeds dried at 10 or 25 °C.




**Figures 6.1-6.4.** HRSEM images showing the distribution of peripheral membrane complexes (PMCs) associated with surfaces in control material (6.1) and in that dried at 25, 10 and 37 °C. (6.2, 6.3 and 6.4, respectively). (Magnification × 150,000).

### 6.3.2 Freeze-fracture EM

As the samples of *Z. palustris* embryonic axes prove to be extremely difficult to digest from the replicas (Yasumura, pers. comm.[1]), coherent impressions of integrated subcellular structure were not obtained. For consistency of interpretation, therefore, membrane surfaces of similar-appearing flattened saccules were chosen for close examination. It should be noted, however, that there was a general consistency in the distribution of intramembrane particles (IMPs) (or their absence) among replicas of most membranes viewed for any one treatment.

The membrane surfaces of cells of embryonic axes of seeds stored in water at 20 °C showed a homogeneous distribution of IMPs (Figure 6.5). As with The PMCs viewed by HRSEM, membrane surfaces of cells of embryonic axes of seeds dehydrated at 25 °C, showed the closest resemblance to that of the control in terms of the distribution of the IMPs (Figure 6.6). Membrane surfaces of cells of embryonic axes of seeds dried at 10 °C showed large areas devoid of IMPs (Figure 6.7). Membrane surfaces of cells of embryonic axes of seeds dried at 37 °C showed a distribution of IMPs which was not substantially different from that typifying material dried at 25 °C (Figure 6.8).

An investigation of the relative abundance of IMPs among the treatments revealed that membrane surfaces of cells of embryonic axes of seeds dried at 25 °C showed the most abundance. Membrane surfaces of cells of embryonic axes of seeds dried at 10 °C had generally retained few or no IMPs. The abundance of IMPs on membrane surfaces of cells of embryonic axes of seeds dried at 37 °C, once again, was somewhat diminished compared with that of seeds dried at 25 °C. However, there did not appear

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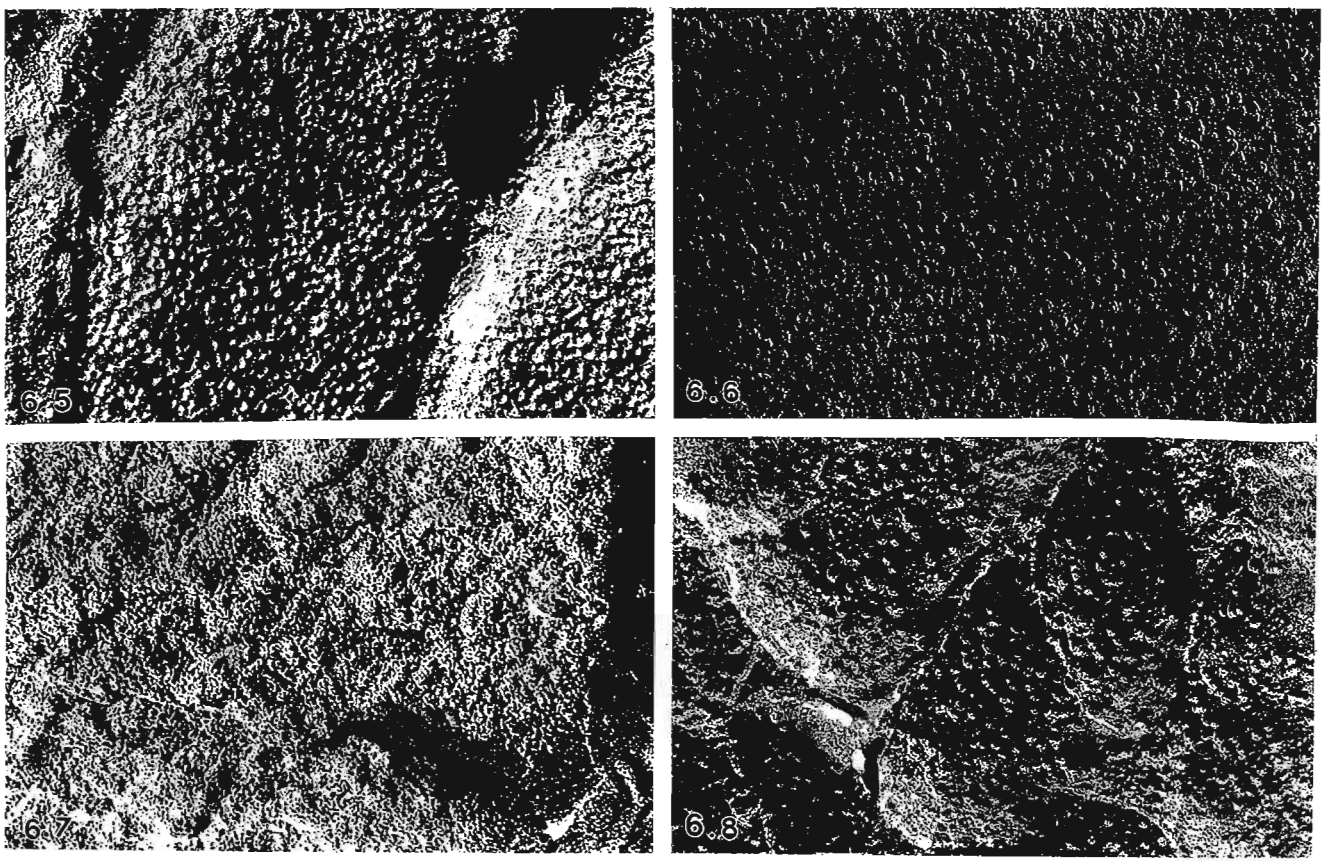
to be major variation in the distribution or the abundance of IMPs between the two treatments.




**Figures 6.5-6.8.** Transmission electron micrographs of membrane surfaces revealed from replicas of freeze-fractured material. Figure 6.5 shows the distribution of intramembrane particles (IMPs) in material stored hydrated at 20 °C while Figs 6.6, 6.7 and 6.8 show the situation prevailing in material dehydrated at 25, 10 and 37 °C, respectively. Note particularly that after drying at 10 °C, large areas of membrane were devoid of IMPs. (Magnification × 150,000).

## 6.4 Discussion

The results of the present chapter show a differential abundance, composition and distribution of PMCs among the experimental treatments and between the control and the experimental treatments. Differences were also indicated in IMP frequency and distribution. Both PMCs and IMPs are presumed to be proteins associated with membranes. The PMCs are assumed to be large peripheral protein complexes that are usually bound to membrane surfaces by interactions with protruding portions of integral proteins or directly with PL polar head groups. Neither the size nor the morphology of the PMCs is suggestive of ribosomal structure. IMPs, on the other hand, are taken to be integral membrane proteins (e.g. Crowe and Crowe, 1986).

The seeds stored hydrated at 20 °C (control) and seeds dried at 25 or 37 °C showed an even distribution of IMPs protruding from membrane surfaces. Cells of embryonic axes of seeds dried at 10 °C, however, showed large IMP-free areas on the membrane surfaces. Also, the relative abundance of IMPs on membrane surfaces was the lowest for seeds dehydrated at 10 °C. This leads one to conclude that IMPs were lost during dehydration at 10 °C.

A number of studies have shown the occurrence of lipid lamellar liquid crystalline to gel phase transitions during dehydration of membranes, particularly at lower temperatures, which are associated with protein clustering and/or elimination (reviewed by Crowe and Crowe, 1986). It is suggested that the loss of IMPs from membranes of cells of embryonic axes of seeds dehydrated at 10 °C was a result of a lipid phase transitions occurring at some critically low water content. From the freeze-fracture replicas presently studied, it appears that there was a large-scale elimination of

IMPs (by implication integral proteins) from the bilayer concomitant with the suggested transition to the gel phase.

The control material, which had never been dehydrated, showed a greater heterogeneity of sizes and shapes of PMCs than the experimental treatments. Also, there were different relative abundances of PMCs on membrane surfaces among treatments. This leads one to conclude that peripheral protein loss occurred during dehydration, particularly when dehydration occurred at 10 °C. Again, it is suggested that lipid lamellar liquid crystalline to gel phase transitions could have led to loss of particle association with membrane surfaces, especially during low-temperature dehydration.

Although membrane surfaces of cells of embryonic axes of seeds dried at 25 °C showed the closest resemblance to the control in terms of variability of PMCs, the distribution of these particles on membrane surfaces of cells of embryonic axes of seeds dried at 25 °C was much more compacted. It is suggested that the observed clustering of PMCs was a result of membrane shrinkage due to water loss rather than to lipid phase transitions (see above).

If the differences in the abundance, composition and distribution of PMCs and IMPs among the treatments could be explained in terms of lipid phase transitions as a result of dehydration at low temperatures only, one would expect no differences to occur in the abundance, composition and distribution of PMCs and in frequency and distribution of IMPs associated with membranes of cells of embryonic axes of seeds dried at 25 or 37 °C. However, there were differences between the 25 and 37 °C treatments. It is suggested that the obvious reduction in PMCs and the apparent diminution of IMPs on membranes of cells of embryonic axes of seeds dehydrated at

37 °C compared with those dried at 25 °C could be explained by a higher level of lipid peroxidation in seeds dried at 37 °C (see Chapter 5). Aside from immediate resultant membrane abnormalities, it should be noted that lipid peroxidation can also induce phase transitions (Vertucci and Farrant, 1995).

In conclusion, it is suggested that the different responses of cells of embryonic axes of seeds dried at 10 °C from those dried at 25 or 37 °C with respect to abundance, composition and distribution of PMCs and frequency and distribution of IMPs in association with membranes, is a result lipid phase transitions as result of dehydration at low temperatures. On the other hand, the differences between cells of embryonic axes of seeds dried at 25 °C from those dried at 37 °C with respect to abundance, composition and distribution of PMCs and frequency and distribution of IMPs on membrane surfaces could be explained in terms of lipid peroxidation, and also a measure of consequent phase transition.

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# CHAPTER SEVEN

# OVERVIEW AND CONCLUSIONS

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## 7.1 Overview

There was a larger difference in water contents of embryonic axes and whole seeds immediately after dehydration in the present study than in that of Kovach and Bradford (1992a). Barnes (1979) showed that if dry maize seeds are hydrated and then immediately dehydrated to the original whole seed water content, the embryo water content after the dehydration was different from that prior to hydration. Seeds used in the present study were hydrated before dehydration. It is suggested that the phenomenon described by that author may explain the discrepancy between this study and that of Kovach and Bradford (1992a). During storage subsequent to the drying treatments further equilibration of water content between embryo and endosperm appeared to occur. When calorimetric studies were studies were undertaken (9 months after drying) embryo water content had declined to less than 0.1 g $g^{-1}$. A similar phenomenon was described by Barnes (1979) for maize seeds.

Electrolyte leakage was higher after dehydration than after subsequent imbibition. This might be explained in terms of imbibitional damage during which cells leak solutes during the initial stages of rehydration (e.g. Crowe *et al.*, 1989). It is surprising that electrolyte leakage increased after stratification and that the significant differences in leakage observed after dehydration were lost after imbibition. These anomalies are attributed to microbial activity, especially fungal growth, which was clearly visible. The fact that post-imbibitional electrolyte leakage is highest for the two extremes of drying temperature used, implies a higher degree of inherant deterioration exacerbated by greater fungal activity. Microbial growth was also observed in the study of Kovach and Bradford (1992a) during rehydration.

Dried seeds generally showed no marked decrease in viability after storage at low temperatures for longer than a year (except for the sample dehydrated at 20 °C). Seeds stored in water at 20 °C showed only a modest decline in viability, as measured by positive TZ staining (100 to 85%). These observations suggest that seeds of *Z. palustris* may be stored for at least one year under water at 20 °C. It is suggested that the 15% viability decline was largely a consequence of storing seeds at higher than the optimum temperature [20 °C presently vs <15 °C (Kovach and Bradford, 1992b)], and also because of fungus-mediated deterioration.

In agreement with electrolyte leakage results, apparent viability, as assessed by TZ staining, declined during imbibition. It is argued that this result is also a consequence of imbibitional damage. In contrast, following stratification the proportion of seeds testing positive with TZ was increased. This observation suggests the operation of repair mechanisms following dehydration and/or imbibitional damage, and that a proportion of the seeds testing negative with TZ staining after imbibition was, in fact, viable but relatively inactive at that stage. It must also be remembered though, that micro-organism activity in association with the seeds could contribute significantly to a positive (and therefore spurious) TZ result.

Seeds dehydrated at 20 °C showed the highest germination percentage. This result highlights the value of using more than one vigour test to assess seed quality as has been suggested by Smith and Berjak (1995) and Hampton and Tekrony (1995). Also, the germination test results were considerably lower than the TZ test results. This discrepancy may be attributed to the presence of dormant seeds. Vertucci *et al.* (1995) have shown that germination increased with an increase in the period of stratification from 4 to 9 months, which is held to break the dormancy (Kovach and Bradford,

1992b). In the present study seeds were stratified for 5 months only. Most indicators of seed health indicated a higher viability after dehydration at 20 or 25 °C than at temperatures below or above this. This in accord with previous results for this species (Berjak *et al.*, 1994).

A third of all the seeds stored at 20 °C for 12 months germinated without any stratification. Realistically this represents >60% of the viable seed sample, as about half of the control seeds had rotted during hydrated storage. Simpson (1990) has mentioned a number of factors which influence both the induction and termination of dormancy. It is possible that, contrary to the previously-held view (Simpson, 1966; Cardwell *et al.*, 1978; Atkin *et al.*, 1987; Kovach and Bradford, 1992a; Berjak *et al.*, 1994), low temperature is just one factor amongst several that facilitate the termination of dormancy in seeds of *Zizania palustris*. Low temperature stratification, therefore, may not be an absolute necessity for dormancy-breaking in seeds of *Zizania palustris* as widely assumed. In this regard, it is interesting to note that seeds stored hydrated at 20 °C showed relatively high levels of monosaccharides (glucose and fructose) and low amounts of sucrose. It is most likely that oligosaccharides and sucrose are metabolized during germination (Williams and Leopold, 1989). Also high levels of monosaccharides (respiratory substrates) in the control material are indicative of higher metabolic activity than in the metabolically-quiescent experimental counterparts (Rogerson and Matthews, 1977; LePrince *et al.*, 1992).

Vertucci and Farrant (1995) have pointed out that respiratory metabolism occurs down to seed water contents of 0.25 g $g^{-1}$ dw. As the embryonic axes of *Z. palustris* were at a water content of 0.36 g $g^{-1}$ dw after dehydration, it is suggested that respiratory metabolism might have been ongoing until the hydration level precluded

this in seeds dehydrated at 25 °C. This would account for the low levels of sugars in these embryos, while the high levels in axes from seeds dehydrated at either 10 or 37 °C might well be correlated with loss of respiratory capacity (ref. TZ results).

No raffinose was detected in seeds stored in water at 20 °C, in contrast to the situation in all dehydrated material. Since according to Becker and Lorenz (1981), raffinose is the major oligosaccharide present in wild rice seeds, it is suggested that that it may have been metabolized during hydrated storage. It appears that sucrose, which occurred in greater amounts in all treatments relative to the control material, may be produced in larger quantities in response to desiccation. Horbowicz and Obendorf (1994) have suggested that seeds with a sucrose to oligosachharide ratio of <1.0 show higher desiccation tolerance and storability and *vice versa*. In the present study, all seed samples showed sucrose to oligosaccharide ratios of >1 and some loss of viability. These responses of seeds of *Zizania palustris*, therefore, do not contradict the hypothesis of Horbowicz and Obendorf (1994).

Although the seeds responded differently to the treatments, the proportion of seeds showing aqueous glasses did not correlate with the viability results. Also no correlation was observed between water content and sugar composition and molecular weight and parameters of the glassy state. This contrasts with previous studies which have shown that the glass-to-liquid transition is dependent mainly on sugar composition and molecular weight and the presence of plasticizers such as water (reviewed by Leopold *et al.*, 1994). This leads one to speculate that other plasticizers [such as temperature (Williams and Leopold, 1989; Franks *et al.*, 1991)] and/or other factors [such as crystallization (Herrington and Branfield, 1984)] may be responsible for the relatively wide range of glass-to-liquid transition temperatures observed in

embryonic axes of *Zizania palustris*. Overall, the ability to form glasses, or the properties of the glasses, did not appear to be important in terms of the response of the seeds to dehydration at different temperatures.

A marginally significant linear relationship was established between hydroperoxide levels and temperature of dehydration. Temperature has been established as one of the factors which determines the extent of peroxidation in deterioration of orthodox seeds in dry storage (reviewed by Smith and Berjak, 1995; LePrince *et al.*, 1995). It is suggested that the results of the present study show the influence of temperature in promoting catalytic activity.

The linear relationship between hydroperoxide levels and dehydration temperature however, was not supported by a decline in the levels of unsaturated fatty acids from either the phospholipid (membrane) or the neutral and glycolipid (storage and other) fractions. One possible explanation for this result could be that other components [e.g. proteins (Smith and Berjak, 1995)] could be responsible for the differences in measured hydroperoxide levels.

While the evolution of methylbutanal on heating showed a marginally significant correlation with dehydration and hence peroxide levels (which were related to dehydration temperature), butanal evolution showed a highly significant negative correlation with dehydration temperature (and hence peroxide levels). In this regard, it is noteworthy that Hailstones and Smith (1989) showed that butanal (along with hexanal and pentanal) was correlated with peroxide levels and vigour in soybean seeds. Those authors, however, showed a positive correlation between butanal and peroxide levels. In contrast, in the present study a negative correlation was observed. Also, the total amount of aldehyde evolved was lower at the higher (37 °C) than at the lower

(10 °C) temperatures. It is possible that at the higher drying temperature of 37 °C, the volatiles produced by peroxidation are lost during drying, giving rise to lower evolution during head-space analysis (the volatiles having been already lost). This could explain the negative correlation between butanal and peroxide levels in the present study.

Differential abundance, composition and distribution of PMCs was observed among the experimental treatments. Differences were also indicated in IMP frequency and distribution. Both PMCs and IMPs are presumed to be proteins associated with membranes. The PMCs are assumed to be large peripheral protein complexes that are usually bound to membrane surfaces by interactions with protruding portions of integral proteins or directly with PL polar head groups. IMPs, on the other hand, are taken to be integral membrane proteins (e.g. Crowe and Crowe, 1986). It is suggested that the differences in abundance and composition of PMCs and frequency and distribution of IMPs seen in the 10 °C-dried seeds may be the result of protein elimination as a consequence of lipid phase transition at lower temperatures. A number of studies have shown the occurrence of lipid lamellar liquid-crystalline-to-gel phase transitions during dehydration of membranes, particularly at lower temperatures, which are associated with protein clustering and/or elimination (reviewed by Crowe and Crowe, 1986). Further, it is argued that the differences in the abundance and composition of PMCs and the frequency and distribution of IMPs of cells of embryonic axes of seeds dried at 25 or 37 °C could be explained by a higher level of lipid peroxidation in seeds dried at 37 °C. Aside from immediate resultant membrane abnormalities, it should be noted that lipid peroxidation can also induce phase transitions (Vertucci and Farrant, 1995).

In conclusion, it is suggested that the differences in vigour of seeds of *Z. palustris* dried at different temperatures cannot be explained by their sugar profiles nor their abilities to form glass. Rather, a decline in vigour at higher dehydration temperatures could be mainly a result of lipid peroxidation and the more marked decline in vigour at lower temperatures of drying may be predominantly be due to peripheral and integral protein elimination from cellular membranes as a consequence of lipid phase transitions. Thus, the highest vigour for seeds dried at 25 °C may be ascribed to the minimal combined effect of both lipid peroxidation and protein elimination at 25 °C.

## 7.2 Conclusions

In conclusion, it is suggested that the differences in vigour of seeds of *Z. palustris* dried at different temperatures cannot be explained by their sugar profiles nor their abilities to form glass. Rather, a decline in vigour at higher dehydration temperatures could be mainly a result of lipid peroxidation and the more marked decline in vigour at lower temperatures of drying may be predominantly be due to peripheral and integral protein elimination from cellular membranes as a consequence of lipid phase transitions. Thus, the highest vigour for seeds dried at 25 °C may be ascribed to the minimal combined effect of both lipid peroxidation and protein elimination at 25 °C.

## 7.3 Further studies

In view of the equivocal results of this study as regards lipid peroxidation, it might be desirable to do a direct free radical test (see Chapter 5). It may be remembered that although there were significantly higher levels of hydroperoxides in seeds dried at 37 °C than those stored hydrated at 20 °C or dried at 10 or 25 °C, this was not supported

by a decline in fatty acid levels nor higher evolution of aldehydes in seeds dried at 37 °C.

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