

Drought and Desiccation-Tolerance and Sensitivity in Plants

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1. Introduction

As sessile organisms, plants encounter a plethora of stresses in their natural environment (reviewed by Janská *et al.*, 2010). They must withstand these stressors to survive. Stresses are abiotic or environmental and biotic. Environmental stressors include water, temperature, salt or salinity, light and metal ions. Water stress consists of both low – osmotic stress or water deficit: drought and drying or dehydration or desiccation (relative humidity [RH] < 100%) and high (RH = 100%) – flood and imbibition or rehydration - water stress. Salinity and water stress are closely related. Whereas high salinity or hyperosmolality is desiccating, low salinity and/or hypo-osmotic shock promotes hydration. Similarly, temperature stress comprises low – chilling/cold ($0\text{ }^{\circ}\text{C} > T^{\circ} < 25\text{ }^{\circ}\text{C}$), freezing ($-40\text{ }^{\circ}\text{C} < T^{\circ} \leq 0\text{ }^{\circ}\text{C}$) and cooling ($T^{\circ} < \text{original temperature [usually } < -40\text{ }^{\circ}]$) and high – warming or thawing ($T^{\circ} > \text{original temperature [usually } > -196\text{ or } -80\text{ }^{\circ}\text{C}]$), heat ($25\text{ }^{\circ}\text{C} \leq T^{\circ} 50\text{ }^{\circ}\text{C}$) and heat shock ($T^{\circ} > 50\text{ }^{\circ}\text{C}$) – temperature stress as are light and metal ions stressors. Biotic stresses comprise micro-organisms – viruses, bacteria and fungi, insects and herbivores.

Desiccation-tolerance is not synonymous with drought tolerance (Alpert, 2005)! On one hand, desiccation-tolerance of an organism is defined as the ability of a living structure to survive drying to equilibrium with low (< 50%) RH and maintain low intracellular water concentrations (WCs). Drought tolerance (*sensu stricto*) is survival of low environmental water availability while maintaining high internal water contents (WCs), on the other. A drought-tolerant organism that is not desiccation-tolerant will die if it loses much of its water, whereas a desiccation-tolerant organism will survive under the same conditions. Thus, *desiccation-tolerance is one mechanism of drought tolerance!*

Desiccation-tolerance is generally understood to pertain to organisms that will survive dehydration to an overall WC equal to, or less than, 0.1 g (water) per g dry mass (g g^{-1}) (Berjak, 2006). Anhydrobiosis is commonly used synonymously with desiccation-tolerance in literature but Berjak (2006) cautions against this habit as anhydrobiosis implies complete absence of intracellular water which is not the case at WCs of around 0.1 g g^{-1} .

Intracellular WCs of $\leq 0.1 \text{ g g}^{-1}$ are considered to represent levels at which macromolecules can no longer be surrounded by a water monolayer, thereby precluding enzymatic activity and, thus, all metabolism (Billi and Potts, 2002). Unique water properties pertain at such low WCs. In fact, water should be viewed not only as an intracellular medium but also as a structural component of macromolecules, such as proteins, which were water to be completely removed, are likely to undergo conformational changes (Billi and Potts, 2002). In solution, proteins are held to exclude small molecules from their immediate vicinity, thus being surrounded by solute-free water (Timasheff, 1982, Parsegian, 2002).

Upon extreme dehydration, but assuming a residual layer of water remains in close association with proteins, small solutes – which must include inorganic ions – are likely to perturb the residual water, where their localised effects could contribute to macromolecule denaturation (Berjak, 2006). This phenomenon may well impose one constraint on biological material being able to survive completely anhydrous conditions, thus imposing a limitation on absolute desiccation tolerance (Berjak, 2006).

2. Drought tolerance

Environmental variables, especially those affecting water availability and temperature, are the major determinants of plant growth and development (reviewed by Janská *et al.*, 2010). Drought is undoubtedly one of the prime abiotic stresses (reviewed by Ashraf, 2010). Crop yield losses due to drought stress are considerable.

Mechanisms of drought tolerance occur at four levels (reviewed by Ashraf, 2010). They include genetic factors which underlie morphological adaptations, physiological acclimation and cellular adjustments. Morphological adaptations consists of improved root length and thickness, thick and/or waxy coverings of leaves, lower leaf size and weight, higher green leaf area, delayed leaf senescence and smaller epithelial cells. Physiological acclimation comprises higher stomatal conductance and density, slower rates of transpiration, early and reduced asynchrony between male and female flowering and maturation and better production, assimilation, accumulation and partitioning of biomass and seed yield. Cellular adjustments entail higher chlorophyll content and particle numbers or harvest index, lower osmotic potential and mechanisms of desiccation-tolerance (see below).

3. Desiccation-tolerance

Desiccation-tolerance is rare but universal (Alpert, 2005)! There may be considerable commonality among the mechanisms and processes facilitating desiccation-tolerance across the spectrum of organisms that show this trait (Berjak, 2006).

3.1 Occurrence

Desiccation-tolerance occurs in organisms or life stages of species or taxa of higher – ‘resurrection plants’ and ‘orthodox seeds’ – and lower – mosses – plants, animals – nematodes and bdelloid rotifers and microorganisms - terrestrial micro-algae, lichens: symbionts of fungi (mycobiont) and algae or cyanobacteria (photobiont), bacteria and yeast (Berjak, 2006).

‘Resurrection plants’, so far known to be constituted of 330 species, have been described from nine pteridophyte and ten angiosperm families (Proctor and Pence, 2002). Their

vegetative tissues are characterised by desiccation-tolerance. The plants concerned are able to remain viable despite considerable dehydration, resuming metabolic activity when water becomes available. Desiccation-tolerance in angiospermous resurrection plants, like orthodox seeds, is based on a spectrum of mechanisms that accompany drying (Illing *et al.*, 2005). Developing 'orthodox seeds' acquire the ability to tolerate desiccation relatively early, preceding the final developmental phase of maturation drying on the parent plant by some time (Bewley and Black, 1994; Vertucci and Farrant, 1995; Kermode and Finch-Savage, 2002).

Although desiccation-tolerance appears to be far more restricted across the spectrum of animals than plants (Alpert, 2005), the phenomenon has been documented for nematodes (Solomon *et al.*, 2000; Browne *et al.*, 2002) and bdelloid rotifers (Lapriniski and Turnacliffe, 2003; Caprioli *et al.*, 2004), for example, and, classically, for the encysted embryos of the brine shrimp, *Artemia* spp. (Clegg, 1986, 2005).

Lichens represent a symbiosis between a fungus - the mycobiont and a green alga or cyanobacterium - the photobiont. The remarkable outcome of the symbiosis is that neither of the partners remains constrained to the cryptic habitats that would be obligatory for either one alone (Kranmer *et al.*, 2005).

Prokaryotes in soils, which might periodically become very dry, need to be able to protect against the consequences of dehydration (Billi and Potts, 2002). In addition, survival of bacteria in the dry state is important in health issues (Berjak, 2006).

3.2 Mechanisms

Bewley (1979) pioneered the idea that desiccation-tolerance may be protoplasmic in a landmark article! The trait of desiccation-tolerance is an outcome of the interaction of a spectrum of phenomena and properties (Pammenter and Berjak, 1999) described as intrinsic cell characteristics (Walters *et al.*, 2005a). However, the degree of expression of these characteristics and, indeed, whether all or some are present underlie the differences in responses to drying between desiccation-tolerant and variously desiccation-sensitive organisms (Pammenter and Berjak, 1999). The modes and interaction of the operation of the protective mechanisms and processes of desiccation-tolerance remain largely conjectural (Berjak *et al.*, 2007; Berjak and Pammenter, 2008). Berjak (2006) stresses the point that desiccation-tolerance involves not only the facility to survive extreme water loss, but also the ability to survive for prolonged periods in the dehydrated state.

3.2.1 Intracellular physical characteristics

The first set of major components of the suite of mechanisms of desiccation-tolerance involves intracellular physical features (Pammenter and Berjak, 1999). It was first demonstrated by Berjak and co-workers (Berjak *et al.*, 1984, 1989). They include minimization of vacuolation, protection of (the integrity of) DNA and orderly dismantling of cytoskeletal elements.

Both 'orthodox and recalcitrant seeds' deal with the problem of volume reduction by the accumulation of space-filling insoluble reserves (Berjak and Pammenter, 2008). There is the notable exception of 'highly-recalcitrant propagules' of *Avicennia marina*.

Boubriak *et al.* (2000) found DNA to be severely damaged after slight drying in embryos of *A. marina*. This event is followed by the inability for its repair after loss of 22 % of the water present at shedding. More recently, studies have shown putative role of helicases in plant abiotic stress tolerance (Owtttrim, 2006; Vashisht and Tuteja, 2006).

With respect to the cytoskeleton, Faria *et al.* (2005) used an α -tubulin antibody in an immunocytochemical (ICC) assay to show that only disassociated tubulin granules were present in the radicle cells in the dry state of 'orthodox seeds' of *Medicago truncatula*. In contrast, well-established and extensive cortical arrays of microtubules were present in germinating *M. truncatula* and 'recalcitrant' *Inga vera* seeds (Faria *et al.*, 2005 and 2004, respectively). Microtubules disassociated on drying, giving rise to tubulin granules which disappeared following further dehydration. The damaged cells appeared to have lost the capacity for microtubule reconstitution upon rehydration. These observations support earlier findings indicating failure of the reconstitution of microfilaments and, hence, a complete cytoskeleton following desiccation in embryonic axes of *Quercus robur* (Mycock *et al.*, 2000) and *Trichilia dregeana* (Gumede *et al.*, 2003).

3.2.2 Intracellular de-differentiation and 'metabolic repression or switch-off or shut-down'

De-differentiation and 'metabolic switch-off or shut-down' constitute the second set of characteristics of the acquisition of desiccation-tolerance in 'orthodox seeds' (Pammenter and Berjak, 1999). Comparisons with developing 'recalcitrant seeds' indicate that these phenomena do not occur, although the metabolic rate may be at its lowest at, or shortly before, the seeds are shed (Farrant *et al.*, 1997).

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

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

There is only a transient cessation of DNA replication at shedding, with re-entry into the S-phase soon thereafter, in embryos of *A. marina* (Boubriak *et al.*, 2000). In addition, the 4C DNA content was found to be relatively low and constant in both shoot and root apices in *I. vera* embryos from six weeks after flowering to shedding (Faria *et al.*, 2004). In contrast to the findings for *A. marina*, it did not change significantly after 13 h of imbibition of mature seeds. Reviewing previously published information for a range of 'recalcitrant-seeds species', Faria *et al.* (2004) concluded that the majority of cells appeared to be arrested in the G1 phase of the cell cycle, thus the more vulnerable 4C phase would be avoided when the seeds are shed and at the greatest risk of drying.

3.2.3 Sugars and oligosaccharides

It seems invariable that sucrose and certain raffinose series oligosaccharides or galactosyl cyclitols accumulate in 'orthodox seeds' during maturation drying as demonstrated by the

pioneering studies of Leopold and co-workers and others (e. g. Blackman *et al.*, 1995; Steadman *et al.*, 1996; Black *et al.*, 1999; reviewed by Leprince *et al.*, 1993; Horowicz and Obedendorf, 1994; Obendorf, 1997). In addition, high sucrose concentrations are also common to desiccated 'resurrection plant' tissues (reviewed by Berjak *et al.*, 1997).

Leopold and co-workers contend that the role of sucrose is dynamic in hindering the close approach of membranes to one another, and hence preventing their lateral proximity (Bryant *et al.*, 2001, Koster and Bryant, 2005; Halperin and Koster, 2006). In this regard, it is noteworthy that membrane lateral proximity promotes phase transitions of some phospholipids and even the demixing of membrane components which is accompanied by the exclusion of integral proteins (e. g. Ntuli *et al.*, 1997).

It appears that sucrose with raffinose or stachyose accumulates in the axes and cotyledons of developing 'recalcitrant seeds' (Berjak and Pammenter, 2008). 'Highly recalcitrant' embryos of *A. marina* were found to accumulate substantial amounts of sucrose and stachyose (Farrant *et al.*, 1993b). In addition, sucrose accumulation accompanied dehydration in the less 'recalcitrant' *Camellia sinensis* embryonic axes (Berjak *et al.*, 1989). Similarly, embryos of *Quercus robur*, which are more desiccation-tolerant than those of the latter counterpart, accumulate sucrose and raffinose concomitant with the later stage of reserve accumulation (Finch-Savage *et al.*, 1993; Finch-Savage and Blake, 1994). Furthermore, *Quercus alba* embryos have a high sucrose content (Connor and Sowa, 2003).

From a wide-ranging survey of sucrose accumulation among both 'orthodox and recalcitrant seeds', it seems that a variety of 'recalcitrant seeds' accumulate substantial quantities of sucrose relative to oligosaccharide (Steadman *et al.*, 1996). However, Berjak and Pammenter (2008) argue that sucrose cannot play a part in protecting against desiccation damage as conjectured for 'orthodox seeds' as upon drying in the natural environment, 'recalcitrant seeds' would have already lost viability at WCs well in excess of those at which any benefits could be derived by the contribution of this disaccharide to the 'intracellular glass(y state)' (see below) or in counteracting lateral contact between membranes, as discussed above. It is probable that hydrolysis of sucrose affords a readily available respiratory substrate required to sustain ongoing development which grades imperceptibly into germination, followed by seedling establishment under favourable conditions in 'recalcitrant seeds' (Berjak and Pammenter, 2008).

3.2.4 Late embryogenesis-abundant/accumulating, (small) heat-shock proteins, oleosins and aquaporins

Galau and co-workers were the first advocates of the role of late embryogenesis-abundant/accumulating (LEA) proteins in desiccation-tolerance (Galau *et al.*, 1986, 1987; Galau and Hughes, 1987). LEA proteins, together with sucrose, have been the focus of much recent attention in the context of the acquisition and retention of desiccation-tolerance in 'orthodox seeds' (reviewed by Buitink *et al.*, 2002; Kermodé and Finch-Savage, 2002; Berjak, 2006; Berjak *et al.*, 2007; Berjak and Pammenter, 2008).

Six groups of LEA proteins have been identified on the basis of particular peptide motifs (Cumming, 1999). These proteins generally lack cysteine residues, are composed predominantly of charged and uncharged polar amino acid and, with the exception of Group 5 LEA proteins, are highly hydrophilic and heat stable.

Buitink *et al.* (2006) have demonstrated that 18 genes coding for LEA and two heat-shock proteins (HSPs) were upregulated and identified as being common to the acquisition of desiccation-tolerance in *M. truncatula* seeds. The same situation prevailed during its experimental re-imposition in the seedlings.

It has been suggested that LEA proteins of some groups could provide a protective hydration shell around intracellular structures and macromolecules while others have been hypothesized to sequester ions during dehydration and in the desiccated state (Berjak and Pammenter, 2008). This action has been attributed to their hydrophilicity.

It has also been proposed that the lysine-rich K segment of Group 2 LEA proteins, dehydrins, might stabilize hydrophobic domains of other proteins which could become exposed as dehydration proceeds (Close, 1997). Such activity is ascribed to the propensity of dehydrins to form α -helices (Close, 1996). Such interactions could counteract inappropriate intermolecular hydrophobic associations (Cumming, 1999). A similar function has been suggested for small HSPs (sHSPs) (reviewed by Buitink *et al.*, 2002; Berjak *et al.*, 2007).

What is especially significant in terms of desiccation-tolerance is that dehydration, particularly in the presence of sucrose, induces at least some LEA proteins to assume α -helical conformation (Wolkers *et al.*, 2001). Such conformational change is suggested to be the basis of the formation and maintenance of the intracellular 'glass(y state)' (Berjak, 2006, Berjak *et al.*, 2007, Berjak and Pammenter, 2008). An additional feature linking LEA proteins to desiccation-tolerance is their concomitant appearance with abscisic acid (ABA) regulation of *lea* gene transcription (reviewed by Bray, 1993; Kermode, 1990, 1995; Cumming, 1999; Kermode and Finch-Savage, 2002; Berjak, 2007).

Berjak and Pammenter contend that although the evidence for LEA protein involvement in desiccation-tolerance is 'correlative and circumstantial rather than by direct experimental demonstration' (Cumming, 1999), it is compelling and, indeed, convincing! The appearance of LEA proteins is associated with 'orthodox seed' maturation, as it is with the imposition of a variety of stresses causing water deficits in plant cells (Cumming, 1999).

The situation regarding the occurrence of LEA proteins in 'recalcitrant seeds' is equivocal (Berjak and Pammenter, 2008). They occur in a range of species from different habitats while apparently being absent from others. Group 2 LEA proteins, dehydrins, have been identified in 'recalcitrant seeds' of some temperate trees (Finch-Savage *et al.*, 1994; Gee *et al.*, 1994), other temperate species and some of (sub-)tropical provenance (Farrant *et al.*, 1996) and in grasses typified by *Portersia coarctata*, *Zizania* spp. and *Spartina anglica* (Gee *et al.*, 1994). However, no dehydrin-type LEA proteins could be found in seeds of ten tropical wetland species (Farrant *et al.*, 1996).

Berjak and Pammenter (2008) argue that it is difficult to envisage a functional role for LEA proteins in 'recalcitrant seeds' based on the conjecture about functionality of such proteins in desiccated or desiccating 'orthodox seeds'. However, those authors maintain that the presence of LEA proteins in 'recalcitrant seeds' of particular species could facilitate more effective survival to lowered water contents following extremely rapid dehydration by flash drying necessary to enable the axes to be cryopreserved (Berjak *et al.*, 1990).

Collada *et al.* (1997) showed the abundant presence of small HSPs (sHSPs) in cotyledons of 'recalcitrant' *Castanea sativa*. Those authors thus concluded that the occurrence of sHSPs

could not be linked to desiccation-tolerance. However, a variety of unidentified sHSPs are expressed in 'recalcitrant' amaryllid embryos, most of which are amenable to cryostorage (Berjak and Pammenter, 2008).

Leprince and co-workers were the first to implicate oleosins in desiccation-tolerance (Leprince *et al.*, 1998). Oleosins in hydrated cells are held to maintain oil bodies as discrete entities. They were suggested to be lacking in or inadequate in lipid-rich 'recalcitrant seeds'. In this regard, no oleosins were detected in the 'highly recalcitrant seeds' of the tropical species, *Theobroma cacao*. However, later work involving cloning and characterization of cDNA and peptide sequencing has shown that two oleosins are present in mature *T. cacao* seeds (Guilloteau *et al.*, 2003).

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

For instance, the vacuolar membrane protein, α -TIP, a water channel protein accumulates during seed maturation in parenchyma cells of seed storage organs. Synthesis of this integral membrane protein does not appear to be related, in a quantitative manner, to storage protein deposition. A role in seed desiccation, cytoplasmic osmoregulation and/or seed rehydration has been suggested (Johnson *et al.*, 1989).

The water-channel activity can be regulated by phosphorylation. The protein assembly as a 60 Å X 60 Å square in which each subunit is formed by a heart-shaped ring comprised of α -helices (Daniels *et al.*, 1999). Homologues to PIPs and TIPs are controlled by dehydration and ABA in desiccation-tolerant resurrection plant *Craterostigma plantagineum* (Mariaux *et al.*, 1998). Members of a subset of PIPs (PIP_a) are regulated by ABA-dependent and ABA-independent pathways.

3.2.5 Intracellular 'glass(y) [/vitrified] state'

There is considerable evidence for the existence of the intracellular milieu in the 'glassy or vitrified state' in 'orthodox seeds' once sufficient water has been lost (Berjak and Pammenter, 2008). Leopold and co-workers pioneered the involvement of (the) 'glass(y) state), as a consequence of a supersaturated sugar solution, in desiccation-tolerance (Koster and Leopold, 1988; Williams and Leopold, 1989).

Later, there was a realization that there are many other intracellular molecules that must contribute to 'glass' (e. g. Walters, 1998). Koster (1991) was the first to show that certain properties of model systems, constituted to simulate intracellular sugar mixtures, differed from the situation in seeds.

In addition, Oliver *et al.* (2001) suggested that LEA proteins might underlie the stability of intracellular 'glasses' in the dry state. Existing as unordered random coils in solution, LEA proteins assume a far more ordered conformation upon dehydration as demonstrated, for example, by Wolkers *et al.* (2001) for a Group 3 LEA protein from desiccation-tolerant *Typha* pollen and Boudet *et al.* (2006) for both Group 1 and Group 5 LEA proteins from *M. truncatula* seeds.

Berjak (2006) proposed that intracellular 'glasses' in dry seeds may be based on coiled LEA proteins in interaction with sucrose and the residual water. However, a sugar-based phase might occur in narrow intermembrane spaces, with LEA proteins excluded on the basis of size as suggested by Koster and Bryant (2005).

While the relative stability of intracellular 'glassy state' is held to maintain viability albeit not indefinitely in the desiccated state of 'orthodox seeds', intracellular 'glasses' just would not normally form in 'recalcitrant seeds' as they require water contents of less than 0.3 g g^{-1} (Berjak and Pammenter, 2008). Water concentrations of approximately 0.3 g g^{-1} coincide with a marked increase in cytomatrical or cytoplasmic viscosity, indicative of 'glass formation' (Buitink and Leprince, 2004).

Under slow drying conditions which would prevail in the natural environment, 'recalcitrant seeds' die at far higher water contents (reviewed by Pammenter and Berjak, 1999; Walters *et al.*, 2002)! However, it may be possible that transient intracellular 'glasses' can be formed as a consequence of flash drying of excised embryonic axes, which is a procedure intrinsic to the cryopreservation protocol for germplasm conservation of 'recalcitrant-seeded' species (Berjak and Pammenter, 2008).

3.2.6 (Re)active oxygen species and free radical-processing antioxidants

The free-radical theory of ageing originated in the medical sciences more than half-a-century ago (Harman, 1956). It was later introduced into seed science when Kaloyereas (1958) suggested that lipid oxidation might underlie loss of viability in seeds.

There has been a particular focus on free radicals, (re)active oxygen species (R/AOS) and antioxidant systems implicated in the acquisition and maintenance of desiccation-tolerance in both 'orthodox seeds' and vegetative tissues of 'resurrection plants' of late (Berjak and Pammenter, 2008). One of the most intriguing aspects of ROS to have emerged recently is their dual role in intracellular signaling as well as intracellular destruction (reviewed by Laloi *et al.*, 2004; Foyer and Noctor, 2005; Suzuki and Mittler, 2006).

Free radicals and AOS are held to result from metabolic imbalance in cellular respiration and photosynthesis. For example, phosphofructokinase (PFK), a rate-limiting enzyme in glycolysis, malate dehydrogenase (MDH), a key enzyme in the tricarboxylic acid (TCA) cycle and dehydrogenases of complexes I and IV of the electron transport chain all of oxidative phosphorylation are slightly, midly and highly adversely affected by desiccation (reviewed by Côme and Corbineau, 1996).

AOS are formed when high-energy electrons are transferred to molecular oxygen (O_2). They include singlet oxygen ($^1\text{O}_2$), hydrogen peroxide (H_2O_2) and the superoxide (O_2^-) and the hydroxyl ($\text{OH}\cdot$) radicals. They have long been considered toxic species that can cause damage to lipids, protein and nucleic acids (e. g. Halliwell, 1987; Fridovich, 1998; Hendry, 1993; Suzuki and Mittler, 2006). Not surprisingly, the activity of a spectrum of enzymatic and non-enzymatic antioxidants is considered to be of prime importance in quenching ROS activity.

However, ROS are now considered as secondary messengers in a diversity of signal transduction cascades in metabolically active hydrated plant tissues (Foyer and Noctor

2005). Hydrogen peroxide and the superoxide radical are singled out because of their implication in many plant developmental and growth processes. Nevertheless this role does not gainsay the vital necessity of their control by a spectrum of antioxidants (Berjak and Pammenter, 2008).

While strict control of AOS is taken for granted in hydrated cells, possession and effective operation of a suite of both enzymatic and non-enzymatic antioxidants is of prime importance during dehydration of 'orthodox seeds' and desiccation-tolerant vegetative tissues (e. g. Kranner *et al.*, 2002; reviewed by Pammenter and Berjak, 1999; Bailly, 2004; Kranner and Birtić, 2005; Berjak, 2006; Berjak *et al.*, 2007; Berjak and Pammenter, 2008). This scenario prevails in the dry state and as soon as water uptake by desiccated cells commences.

Antioxidants are either enzymic or non-enzymic. Enzymic oxidants include ascorbate free radical reductase (AFRR), ascorbate and guaicol peroxidase (A/GPO[D]), catalase (CAT), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and superoxide dismutase (SOD). Non-enzymic oxidants consists of ascorbate/ic acid (AsA) (vitamin C), reduced and di-/oxidized glutathione (GSH and GSSG), retinol (vitamin A) and α , β and γ -tocopherol (vitamin E).

It is possible that certain antioxidants may be operative within localized regions of higher water activity within desiccated cells. As an example, 1-cys-peroxiredoxin (CPR) has been localized to nuclei in imbibed dormant barley embryos (Stacey *et al.*, 1999). It has been suggested to provide antioxidant protection to DNA.

However, it has been suggested that there are localized regions with water activity adequate to facilitate molecular mobility in the desiccated state - 'localised water pools' (Rinne *et al.*, 1999; Leubner-Metzger, 2005). Berjak (2006) argues that it is possible that CPR can function to protect the genome against ROS in desiccated seeds if some such regions occur in the milieu of the chromatin. In this respect, it should be remembered that the cysteinyl residue of CPR can be regenerated ultimately by electron donors such as thioredoxins and glutaredoxin (Dietz, 2003).

In this regard, Rinne *et al.* (1999) conjectured that enzyme activity continues to occur in dehydrin-associated areas of greater water activity in the otherwise dehydrated cells of buds. Leubner-Metzger (2005) showed localized β -1,3-glucanase activity in the inner testa to be instrumental in after-ripening in air-dry tobacco seeds. A similar argument may be advanced for the activity of other enzymic, as well as non-enzymic, antioxidants (Bailly, 2004) in localized regions of greater water activity within intracellular 'glasses' in dehydrated seeds (see above).

Unlike the situation in 'orthodox seeds' during the latter stages stages of development, metabolism is sustained at measurable levels in 'recalcitrant seeds' (Farrant *et al.*, 1993a, b). When water is lost, and especially when dehydration proceeds slowly, metabolism is considered unbalanced. This situation can result in considerable intracellular metabolic damage and death of seed/embryos at surprisingly high WCs (Pammenter and Berjak, 1999 and Walters *et al.*, 2002; Ntuli, 2011a) (see below). Metabolic damage in 'recalcitrant seeds' is thought to be intimately associated with the generation of AOS where the intracellular antioxidant defences are inadequate to quench them.

Recent data for 'recalcitrant' *Araucaria bidwilli*, *Quercus robur*, *Trichilia dregeana* and germinating *Pisum sativum* embryos show that there is a transient increase in antioxidant activity upon initial dehydration. However, with further water loss, activity declines, accompanied by an increase in free radicals and thio-barbituric acid-reactive substances (TBARS) (Francini *et al.* 2006; Ntuli *et al.*, 2011; Song *et al.*, 2004; Ntuli, 2011b, respectively).

3.2.7 Osmolytes/protectants

It is now established that compatible organic solutes play a central role in plant drought tolerance (Ashraf and Foolad, 2007). Overproduction of compatible organic osmotic is one of the responses of plants exposed to osmotic stress (Serraj and Sinclair, 2002; Ashraf *et al.*, 2008).

Among the many organic osmolytes known to play a substantial role in stress tolerance, glycine betaine (GB), a quaternary ammonium compound, occurs richly in response to dehydration stress (Mansour, 2000; Mohanty *et al.*, 2002; Yang *et al.*, 2003; Ashraf and Foolad, 2007). Choline monoxygenase (CMO) and betaine aldehyde dehydrogenase (BDH) are two key enzymes for the biosynthesis of GB in higher plants.

Proline, like GB, is also an important compatible organic osmolyte that plays a key role in stress tolerance (reviewed by Ashraf, 2010). Pyrroline-5-carboxylate synthetase (PCS) is the key enzyme for proline biosynthesis.

Trehalose, a nonreducing sugar, is also a potential organic osmoticum which has a substantial role in the protection of plants against stresses (reviewed by Ashraf, 2010). Trehalose-6-phosphate synthase (TPS) is a key enzyme involved in trehalose biosynthesis.

Mannitol, a polyol, is one of the most important osmoprotectants that play a vital role in plant stress tolerance (reviewed by Ashraf, 2010). Mannitol-1-phosphate dehydrogenase (MPD) is involved in mannitol biosynthesis.

3.2.8 Lipid composition

Liu *et al.* (2006) showed that the proportion of saturated fatty acids in membrane phospholipids was significantly higher in 'recalcitrant' than in 'orthodox seeds'. In addition, Nkang *et al.* (2003) found that mature seeds of *Telfairia occidentalis*, which were characterized by predominantly saturated fatty acids, increased accumulation of both mono- and polyunsaturated fatty acids when dried at 28 °C when total lipid was evaluated. In contrast, high levels of saturated fatty acids were retained and the marked decline in viability was delayed when seeds were dried at 5 °C. Interestingly, Ajayi *et al.* (2006) reported that *T. occidentalis* seeds lost viability at 6 °C within a relatively short time, suggesting chilling sensitivity, despite the retention of saturated fatty acids during desiccation at 5 °C reported by Nkang *et al.* (2003).

Lipid peroxidation has been shown to be associated with deterioration of seeds exhibiting 'intermediate' post-harvest physiology, particularly in terms of chilling sensitivity (Berjak and Pammenter, 2008). Crane *et al.* (2006) have shown that crystallization of the predominantly saturated storage lipid occurs in *Cuphea carthagenensis* seeds at both high and very low WCs after maintenance at 5 °C. Those authors showed that rehydration without a preceding melting of crystallized triglycerides was lethal. Similarly, Neya *et al.* (2004)

showed that hydrating lipid-rich 'non-orthodox seeds' of *Azadirachta indica* in warm water alleviated the effects of imbibitional stress that occurred when cold water was used.

3.2.9 Repair

Osborne and co-workers were the first to advocate the role of DNA repair in desiccation-tolerance (e. g. Osborne, 1983). 'Orthodox seeds' must apparently repair any damage accumulated in the dry state soon after imbibition is initiated. Repair occurs in the lag phase of water uptake before radical protrusion.

This requirement demands unimpaired operation of repair mechanisms and restitution of normal cell structure and function. Simultaneously, the presence and efficient operation of appropriate antioxidants is vital (reviewed by Pammenter and Berjak, 1999).

Newly harvested *A. marina* embryos were capable of repair when DNA fragmentation was induced by radiation but this ability was increasingly compromised if embryos had first been dehydrated (Boubriak *et al.*, 2000). In addition, Connor and Sowa (2003) showed that a reversible shift occurred between the gel and liquid crystalline phases on rehydration after initial dehydration of *Q. alba* acorns from Fourier transform infra-red analyses of membrane lipids.

3.2.10 Endogenous amphiphilic substances

Hoekstra and co-workers pioneered the implication of amphiphilic substances in desiccation-tolerance (Golovina *et al.*, 1998). Those authors showed that dehydration of tolerant pollen and seeds has the potential to cause certain amphiphilic molecules to migrate into membranes, with migration back into the cytomatrix upon rehydration. The amphiphiles were suggested to play a role in maintaining core fluidity of membranes in the dry state. However, subsequent investigations could not confirm this phenomenon. Although the amphiphiles may fluidise membranes, correlation with desiccation-tolerance was uncertain (Golovina and Hoekstra, 2002).

3.2.11 Control of acquisition and maintenance of desiccation-tolerance

The phenomenon of the control of the acquisition and maintenance of desiccation-tolerance in 'orthodox seeds' is suggested to be pre-programmed and developmentally regulated and initiated by maternal factors rather than directly via environmental signals and later to be under the control of the embryo (reviewed by Bewley and Black, 1994; Bewley, 1979, 1997). A major point of confusion is that desiccation-tolerance overlaps with other maturation processes and the development of dormancy where it occurs.

Studies on viviparous mutants and those characterized by impairment of the maturation process in *Zea mays* (VP series mutants) and *Arabidopsis thaliana* (*LEC1*, *LEC2*, *FUS3* and *ABI3*) have indicated both ABA-independent and ABA-dependent pathways of gene regulation (e. g. Bray, 1993; Kermode, 1990, 1995; Kermode and Finch-Savage, 2002; Bartels, 2005; Vicente-Carbajosa and Carbonero, 2005).

Current understanding of the control of seed maturation and acquisition of desiccation-tolerance in *A. thaliana* suggests that *LEC1*, *LEC2*, *FUS3* and *ABI3* are the four 'master genes'

involved, with the latter three implicated in desiccation-tolerance (To *et al.*, 2006). Another pivotal factor is ABA which, in the context of seed development, is probably best known for its role in regulating *lea* gene transcription (e. g. Bray, 1993; Kermode, 1990, 1995; Cumings, 1999; Kermode and Finch-Savage, 2002).

Furthermore, recent evidence suggests that a delicate balance between various ROS, as secondary messengers, and antioxidants may be intimately involved with seed maturation and the acquisition of desiccation-tolerance (Berjak *et al.*, 2007). In addition, there is interaction and cross-talk in the operation of the factors involved!

4. Desiccation-sensitivity

Besides producing short-lived seeds, many of the 'recalcitrant-seeded species' are threatened by overexploitation, indiscriminate harvesting and habitat loss (Berjak, 2005). Hence, understanding the phenomenon of 'seed recalcitrance', and consequently developing sound conservation practices for species producing such seeds, is of major scientific and practical importance (Berjak and Pammenter, 2008). Such importance is underscored in Target VIII of the Global Strategy for Plant Conservation of the Convention on Biodiversity which calls for 60% of all threatened species to be in accessible *ex situ* collections by 2010 (Berjak and Pammenter, 2008)!

'Recalcitrant seeds' remain sensitive to drying both during development and after they are shed from the parent plant. However, the range of WCs of the embryonic axes when seeds are shed varies markedly among species - 0.4 to 4.4 g g⁻¹ (Chin and Roberts, 1980 ; Berjak and Pammenter, 2004). Some decline in WC prior to shedding has been recorded for several temperate species (e. g. *Acer pseudoplatanus* [Hong and Ellis, 1990], *Aesculus hippocastum* [Tompsett and Pritchard, 1993] and *Quercus robur* [Finch-savage and Blake, 1994]) and some (sub-)tropical provenance (e. g. *Machilus hunbergii* [Lin and Chen, 1995]) and species (e. g. *Ekerbergia capensis* [Berjak and Pammenter, 2008]), leading to the suggestion that a measure of desiccation-tolerance might be acquired during development (Finch-Savage and Blake, 1994), with no further importation of water (Berjak and Pammenter, 2000).

Nevertheless, even for those seeds that are shed at axis WCs towards the lower end of the range, further dehydration is deleterious, indicating that at least some of the mechanisms necessary for complete desiccation-tolerance are not entrained (Berjak and Pammenter, 2008). In contrast, WCs of axes of recalcitrant seeds of most of (sub-)tropical species which have been investigated lie at the high end of the range (> 1.5 g g⁻¹), and the axes are damaged after only slight drying, particularly if water loss is slow (Berjak and Pammenter, 2008). Those authors argue that this observation indicates that few, if any, of the mechanisms putatively affording 'orthodox seeds' tolerance to desiccation are operational.

The degree of 'recalcitrance' may be difficult to quantify (Pammenter *et al.*, 2002a). Nonetheless, seeds of *Avicennia marina* (Farrant *et al.*, 1993a, b) and *Hopea* species (e. g. Chin and Roberts, 1980 ; Sunilkumar and Sudhakara, 1998) are considered highly 'recalcitrant'. In this regard, studies on *Zizania* spp. indicated that, despite differences in WCs at which desiccation damage occurred in embryos at different developmental status, all equated to a common water activity value of 0.90 (Vertucci and Farrant, 1995) ! These observations may be related to a common spectrum of metabolic events that are impaired, which has been

suggested to occur in particular water potential ranges, as 'recalcitrant seeds' are dried (Vertucci and Farrant, 1995).

Until relatively recently, seed screening for non-orthodox behaviour was based initially on water content of ostensibly mature seeds at shedding, followed by ascertaining viability retention upon sequential removal of increasing proportions of tissue water (Hong and Ellis, 1996; International Plant Genetic Resources Institute [IPGRI]/DFSC, 2004). However, because frequently only small numbers of recalcitrant seeds are able to be harvested at any one time, Pritchard *et al.* (2004a) developed the 100-seed test which proved to be a reliable indicator of the desiccation responses of seeds of eight tropical palm species. Berjak and Pammenter (2008), nonetheless, recommend modifications of the IPGRI/DFSC protocol (2004) whenever seed numbers allow. The IPGRI/DFSC protocol includes an assessment of viability retention in storage at a range of temperatures. This approach is time-consuming, but gives a reliable indication of whether seeds are 'orthodox', 'recalcitrant' or fall somewhere between 'orthodoxy' and 'recalcitrance'.

Nevertheless, a variety of studies (reviewed by Daws *et al.*, 2006b) have indicated that there could be a correlation among 'recalcitrance', seed characteristics and variables characterising individual habitats. Acting on these indications, and analysing several of the parameters across 104 tropical species from 37 families deriving from Panama, Daws *et al.* (2006b) developed a reliably predictive model based on the just two of the traits: seed mass and seed coat/coverings ratio ([SCR] i. e. the ratio of seed coverings mass to mass of the whole seed). Desiccation-sensitivity was found to be significantly related to relatively low SRCs, typified by large seed size coupled with thin coverings. The predictive value of the model was convincingly shown when it was further applied to seeds of 28 African species and ten species from Europe, showing in all cases that the prediction was in agreement with published data on responses of the seeds to drying (Berjak and Pammenter, 2008).

The original categorisation of seeds according to their post-harvest storage responses, which embodies the idea of two distinctive groupings - 'orthodox' and 'recalcitrant', was pioneered by Roberts (1973) in a landmark paper! A further category - intermediate - was later introduced (Ellis *et al.*, 1990a). Intermediate storage behaviour implies that the seeds are shed at relatively high WCs, but will withstand considerable dehydration, although not to the extent tolerated by 'orthodox seeds', especially at low temperatures.

Although categorising seeds into the three distinct categories is useful, it is noteworthy that desiccation-sensitivity can be further subcategorised into three subcategories - highly, moderately and minimally - 'recalcitrant' (Farrant *et al.*, 1988). Berjak and co-workers and many now favour an open-ended continuum of seed behaviour, subtended by extreme 'orthodoxy' at the one end and the highest degree of 'recalcitrance' at the other (Berjak and Pammenter, 1994, 1997a, b, 2000, 2001, 2004, 2008; Pammenter and Berjak, 1999; Sun, 1999; Kermode and Finch-Savage, 2002, Berjak, 2005, 2006).

The continuum concept accommodates the marked variability occurring both between and within species and is supported by evidence indicating that provenance has a significant effect on seed development and the degree of drying tolerated for individual species (Daws *et al.*, 2004a,b 2006a). In terms of the continuum concept, it is considered pertinent that even 'orthodox seeds' are not equally desiccation-tolerant!

4.1 Occurrence

Chin and Roberts (1980) published the first list of species recorded as producing 'recalcitrant seeds', and collated what was then known about their post-harvest behaviour. The species those authors listed produce seeds important in agroforestry (e. g. species of *Quercus* and *Shorea*), as crops for seed or fruit consumption (e. g. *Castanea* species and *Artocarpus heterophyllus*), or commodity production (e. g. *Elais guineensis*, *Havea brasiliensis* and *Theobroma cacao*).

Since then, the seed biology of a range of lesser known and generally (sub-)tropical tree species has been studied, revealing many more produce 'recalcitrant or otherwise non-orthodox seeds' (reviewed by Berjak and Pammenter, 2004; Sacandé *et al.*, 2004; Flynn *et al.*, 2006). In a single year, a screening programme focusing on southern African species revealed that seeds of at least 17 herbaceous geophytic amaryllids are 'recalcitrant' - which is unusual in indicating a familial trait - while seeds of a further 13 unrelated trees species have also proved 'recalcitrant' (Erdey *et al.*, 2007)!

While the majority of the species producing 'recalcitrant seeds' are endemic to the humid (sub-)tropics, such seeds are also produced by a small spectrum of mainly trees of temperate provenance, while certain dryland species (Danthu *et al.*, 2000; Gaméné *et al.*, 2004; Pritchard *et al.*, 2004b) have also been identified as being 'recalcitrant seeded'. Studies have also identified seeds of some cycads (e. g. *Encephalartos* spp.) to be 'recalcitrant' (Woodenberg *et al.*, 2007), while Daws *et al.* (2007) reported a considerable incidence of desiccation-sensitive seeds among palms.

4.2 Variability

There are marked differences in the degree of drying that 'recalcitrant seeds' will tolerate, although the lowest WC survived depends on other parameteres, especially the rate at which water is lost (see below) (reviewed by Berjak and Pammenter, 2008). Comparisons of published data on individual species are often not helpful, because of the differing conditions under which dehydration was carried out.

However, a similar pattern of ultrastructural events terminating in cell lysis was recorded as occurring at markedly different WCs when 'recalcitrant seeds' of three unrelated species - a gymnosperm: *Araucaria angustifolia*, a dicotyledonous vine: *Landolphia kirkii* and a herbaceous monocot: *Scadoxus membranaceous* - were dried under identical conditions (Farrant *et al.*, 1989). Significantly, the rate at which the seeds of the three species lost water was inversely related to the WC at which viability was lost!

4.2.1 Genera

Differences in the critical/lethal/lowest 'safe' WC which 'recalcitrant seeds' will withstand are not confined to disparate genera (reviewed by Berjak and Pammenter, 2008). They have also been noted for different species of individual genera.

A thought-provoking finding is that seeds of different species of a single genus may be differently categorised (reviewed by Berjak and Pammenter, 2008). For example, species of *Acer* and *Coffea* are variously categorised (Hong and Ellis, 1990; Eira *et al.*, 1999).

4.2.2 Provenance

Interestingly, recent data indicate that seeds of *A. hippocastum* and *A. pseudoplatanus* from different provenances differ in their response to dehydration (Daws *et al.*, 2004b; 2006a, respectively).

4.2.3 Developmental status

While developing seeds of *Machilus thunbergii* lost viability within thirty days when dried at 73% RH and 25 °C, those that were mature were able to tolerate a 19% loss of water before germinability declined (Li and Chen, 1995). Differing degrees of desiccation sensitivity have been similarly correlated with developmental status for *Landolphia kirkii* and *Camellia sinensis* (Pammenter *et al.*, 1991; Berjak *et al.*, 1992; 1993).

It appears that the least desiccation-sensitive stage generally occurs when the metabolic rate is the lowest which usually but not invariably coincides with shedding (reviewed by Berjak and Pammenter, 2008). However, desiccation sensitivity increases markedly as germinative metabolism progresses (Farrant *et al.*, 1986; Berjak *et al.*, 1989).

4.2.4 Tissues

Zygotic embryonic axes and storage tissues seldom have if ever have the same WC (e. g. *Acer hippocastum* [Tompsett and Pritchard, 1993]). There is also an uneven water distribution between component tissues in *Araucaria hunsteinii* (Pritchard *et al.*, 1995).

Frequently, axes are at higher WCs, and more desiccation-sensitive than cotyledons (reviewed by Berjak and Pammenter, 2008). However, cotyledons have been reported to be more sensitive to dehydration than axes in *Castanea sativa* (Leprince *et al.*, 1999).

4.2.5 Season

A further contribution to the variability among seeds of individual species is that their characteristics differ both intra- and interseasonally (reviewed by Berjak and Pammenter, 2008). Intraseasonal variation includes differing WCs of the component tissues of ostensibly mature seeds depending on the time of harvest (reviewed by Berjak and Pammenter, 1997a,b).

An additional feature that has been consistently been observed is the poor quality of seeds produced late in the season, which are often severely fungally infected. In this regards, an enhanced rate of deterioration upon dehydration has been reported for late-harvested seeds of *Machilus kanoi* (Chien and Lin, 1997). It has also been observed that late-season fruits of *Avicennia marina* and *Syzygium cordatum* have a tendency either to abort or not abscise.

It is probable that the poor quality of late-season seeds may be explained in terms of the cumulative heat sum during development (Daws *et al.*, 2004b). Those authors monitored *Acer hippocastum* seed development along a latitudinal gradient and reported that the greater the cumulative heat sum, the more robust, further developed and less desiccation sensitive were the seeds.

Berjak and Pammenter (2008) argue that a similar interpretation for poor seed quality can be applied to fruits and seeds produced in the latter part of the season in non-equatorial zones.

Temperatures decline as the summer wanes and, accompanied by shortening day-lengths, results in a sub-optimal heat sum to late-developing fruits. This phenomenon is proposed to influence fruit and seed development negatively, resulting in their poor quality, which includes lowered resistance to fungal establishment.

Interseasonal variation among seeds of the same species may be similarly rationalised (Berjak and Pammenter, 2008). For example, *Camellia sinensis* seeds harvested showed axial WCs as disparate as 2.0 ± 0.3 to 4.4 ± 2.4 g g⁻¹ (Berjak *et al.*, 1996).

4.2.6 Individual seeds

There are usually marked differences in axial WCs among individual seeds (reviewed by Berjak and Pammenter, 1997a,b)! These differences persist even when seeds are harvested simultaneously.

4.3 Experimental conditions

A number of experimental parameters determine survival during drying, cooling, warming or thawing and imbibition or rehydration of seed tissues. They include drying rate and temperature, cooling rate and imbibition or rehydration rate and temperature.

Disparate opinions have been expressed about the effect of drying rate on the critical/lethal/lowest WC tolerated by 'recalcitrant seeds without compromising viability' (reviewed by Berjak and Pammenter, 2008). However, it is apparent that the actual rates described as rapid or slow can pertain to very different time scales.

For example, rapid desiccation can be achieved in a matter of as little as fifteen minutes for some species and as much as twenty-four hours for others when dehydrating excised axes by 'flash drying' (e. g. Ntuli and Pammenter, 2009). Both these examples are rapid relative to axes within whole seeds which require a matter of days to attain similarly low WCs (e. g. Pammenter *et al.*, 1998).

Zygotic embryonic axes generally but not invariably constitute a very small proportion of the total mass or volume of a 'recalcitrant seed'. When excised, they can be dried very rapidly in a laminar air-flow or using the technique of 'flash-drying' (reviewed by Pammenter *et al.*, 2002b; Ntuli, 2011c).

Rapidly-dried axes and, occasionally, seeds will survive to far lower WCs than can be attained on slow dehydration (reviewed by Berjak and Pammenter, 2008). This phenomenon has been shown for a variety of species. Fast desiccation facilitates axis viability retention well into hydration level III and, occasionally, just into level II (Vertucci and Farrant, 1995), at which extreme, generally lethal damage is associated with slow water loss.

It is not that 'flash-drying' renders 'recalcitrant seeds' desiccation-tolerant (reviewed by Berjak and Pammenter, 2008). On the contrary, they will rapidly lose viability at ambient or refrigerator temperatures if allowed to remain at the low WCs attained. 'Flash-drying' achieves the rapid passage through the intermediate WC ranges at which aqueous-based metabolism-linked - metabolic damage - occurs.

'Recalcitrant seeds' are metabolically active and initiate germination around shedding (reviewed by Berjak and Pammenter, 2008). As a result, their developmental status is

becoming more advanced and their desiccation sensitivity is increasing. Thus, desiccation-sensitivity could be increasing as drying is proceeding if germination is occurring at the same time frame as drying so reducing the water loss tolerated (Berjak *et al.*, 1984, 1989; Farrant *et al.*, 1986).

The drying rate is markedly affected by the nature of the coverings, size and developmental status (reviewed by Berjak and Pammenter, 2008). There is a lower WC limit below which 'recalcitrant seeds' will not survive. This WC is generally at or near the level at which all the remaining water is structure associated. Desiccation damage *sensu stricto* ensues when structure-associated or non-freezable water. A major difference between orthodox and recalcitrant seeds' is that the former can lose a considerable proportion of this water.

4.4 Storage

Seed storage is imperative, not only to provide good-quality planting material and feedstock from season to season in agriculture, as well as interseasonal food reserves, for food security, but also as base and active collections in the long term conserving of genetic resources for biodiversity conservation (reviewed by Berjak and Pammenter, 2008). As long as 'orthodox seeds' are of high quality after harvest, the period for which they can be stored without deterioration is predictable under defined conditions of low temperature and RH that will maintain low WC.

Storage longevity of 'orthodox seeds' increases logarithmically with decreasing WC (Ellis and Roberts, 1980) although there appear to be limits of drying beyond which no further advantage is gained (Ellis *et al.*, 1990b) and, in fact, if exceeded, may be damaging (e.g. Walters, 1998; Walters and Engels, 1998; reviewed by Berjak, 2006). However, Berjak and Pammenter (2008) noted that there is no unanimity about this issue (Hong *et al.*, 2005). Nonetheless, 'orthodox seeds' have finite lifespans - years, decades or centuries - even under ideal conditions (Walters *et al.*, 2005b).

In contrast, 'recalcitrant seeds' are characterised by post-harvest lifespans of the order of days to months, or for temperate species, a year or two, as long as such seeds will tolerate low but not sub-zero temperatures (e. g. Chin and Roberts, 1980). The inexorable progress of germinative metabolism - which occurs with no requirement for additional water - is one of the major factors hampering short- to medium-term storage of 'recalcitrant seeds'. The developmental status of 'recalcitrant seeds' changes rapidly after they are shed because they not only hydrated but metabolically active.

Enzymic antioxidants have been found to be inadequate in counteracting oxidative stress during storage (Tommasi *et al.*, 2006) in 'recalcitrant seeds' of *Ginkgo biloba* (Liang and Sun, 2002). The viability of the seeds at 25 °C declined from 80 to 46% between three and six months, accompanied by a decline in WC from c. 2.0 to 1.0 g g⁻¹. The seeds stored at 4 °C lost viability precipitously between six and nine months in storage during which the reduction in WC was insignificant.

It is suggested that the viability at 25°C was the direct result of metabolic damage (Pammenter and Berjak, 1999; Walters *et al.*, 2001, 2002) (see above). Generation of free radicals/ROS and accumulation of thiobarbituric acid-reactive substances (TBARS), along with the decreasing ability of antioxidants to modulate the situation, is consistent with the

water stress-induced damage in both cases. It is proposed that death occurred at 4 °C because metabolism progressed albeit slowly to the stage at which exogenous water supply was needed (e. g. Berjak *et al.*, 1989; Pammenter *et al.* 1994; Ntuli, 2011d). Working with 'recalcitrant' *Acer saccharinum* seeds, Ratajczak and Pukacka (2006) concluded from changes in enzymes of the ascorbate-glutathione cycle and levels of ascorbate and glutathione that viability of stored hydrated seeds could be maintained only when a vigorous antioxidant system was operational.

4.4.1 Wet storage

The only way in which vigour and viability of 'recalcitrant seeds' can be maintained is to keep them under conditions not permitting water loss at the lowest temperature they will withstand and to eliminate or, at least, minimise seed-associated mycoflora (reviewed by Berjak and Pammenter, 2008). Nevertheless, storage of whole seeds is strictly short- to medium-term. This limitation is due to the fact that 'recalcitrant seeds' are metabolically active, and will progress from development into germination at shedding.

'Recalcitrant seeds' of some species are shed consireably before development is complete (reviewed by Berjak and Pammenter, 2008). For example, *Trichilia dregeana* seeds can be stored for several months at 16 °C (Goveia *et al.*, 2004).

Storage longevity may be further optimised in the case of 'recalcitrant seeds' that are not chilling sensitive which would be expected for temperate species (reviewed by Berjak and Pammenter, 2008). Chilling sensitivity may be provenance related, and there appears to be distinct genetic differences among plants from different provenances (Bharuth *et al.*, 2007).

It has been sporadically suggested that lowering 'recalcitrant seed' WC to levels permitting basal metabolism but precluding germination in storage might extend their longevity (reviewed by Berjak and Pammenter, 2008). This means has proved to be deleterious to both life span and quality of seeds for a range of species (Corbineau and Côme, 1986a, b, 1988; Drew *et al.*, 2000 ; reviewed by Eggers *et al.*, 2007).

Not only did storage life span decline in the 'sub-imbibed condition' relative to that of seeds stored at the shedding WCs, but fungal proliferation was exacerbated. In this respect, it is noteworthy that mild desiccation stress paradoxically stimulates germination of recalcitrant seeds before the damaging effects set in (reviewed by Eggers *et al.*, 2007).

Seeds will have been stimulated to entrain germinative metabolism sooner, when they are placed into storage after loss of a small proportion of the water originally present (reviewed by Eggers *et al.*, 2007). Hence, they become increasingly desiccation sensitive more rapidly than if not dried. This situation results in a greater water stress and thus seed debilitation (Pammenter *et al.*, 1994), and favours more rapid fungal proliferation from seed-associated inoculum (Calistru *et al.*, 2000; Anguelova-Merhar *et al.*, 2003; Dos Santos *et al.*, 2006).

In this regard, 'recalcitrant seeds' appear able to elaborate antifungal enzymes and other compounds (Calistru *et al.*, 2000; Anguelova-Merhar *et al.*, 2003; Dos Santos *et al.*, 2006). These defences become decreasingly effective during storage.

Fungicide treatment has been shown to be highly effective in extending storage life span of 'recalcitrant seeds' in hydrated storage (e. g. Sunilkumar and Sudharata, 1998 ; Calistru *et al.*,

2000). However, application of non-penetrative fungicides will be effective only in situations where the inoculum is primarily located on the seed surfaces !

4.4.2 Cryostorage

However effectively the storage life span of 'recalcitrant seeds' can be extended, it remains a short- to medium-term option (reviewed by Berjak and Pammenter, 2008). This situation is because of the fact that germination at the shedding WC will virtually inevitably occur!

Seedling slow growth offers an alternative to wet storage of seeds (Chin, 1996). This means of long-term conservation is less than ideal. Hence cryostorage - generally in liquid nitrogen at 196 °C or, less ideally, at below -80 ° - presently appears to offer the only option for long-term storage (reviewed by Berjak and Pammenter, 2008)!

It would be ideal if whole seeds could be cryopreserved. This event is generally not possible because 'recalcitrant seeds' of most species are large, and at high WCs when shed. As discussed above, large seeds cannot be dried rapidly, and slow dehydration to WCs commensurate with efficient cooling or freezing is lethal.

WC must be reduced to a level obviating lethal ice crystallisation during cooling for survival at cryogenic temperatures. Successful cryopreservation of small 'non-orthodox seeds' has been achieved in cases where desiccation could be achieved rapidly (e. g. *Azadiachta indica* [Berjak and Dumet, 1996], *Warburgia salutaris* [Kioko *et al.*, 1999, 2003] and *Wasubia japonica* [Potts and Lumpkin, 2000]).

If whole seeds are optimally warmed or thawed and imbibed or rehydrated after retrieval from cryostorage, seedlings should, theoretically, be able to be generated in a greenhouse without an intervening *in vitro* stage. However, 'recalcitrant seeds' are far too large in the great majority of cases necessitating the use of the excised embryonic axes as explants for cryopresearvation.

5. Concluding remarks, future perspectives and prospects

Although a number of phenomena and mechanisms, as discussed above, have been implicated in the acquisition and maintenance of desiccation-tolerance, it seems likely that the picture is not yet complete according to Berjak and Pammenter (2008). This situation makes unequivocal identification of the differences underlying 'recalcitrant seed' behavior presently unattainable.

Similarly, it is not yet possible to present a coherent view integrating the control of the acquisition of desiccation-tolerance. In view of its complexity and our presently fragmentary understanding of the events at the control level, and also of the expression of the many phenomena characterising the acquisition and retention of desiccation-tolerance, it is perhaps not surprising that we have a long way to go before comprehending the basis of the 'recalcitrant condition' (Berjak and Pammenter, 2008).

The use of axes complicated the cryopreservation procedure. They may be injured on excision (e. g. Goveia *et al.*, 2004). Potentially injurious treatments are also required to eliminate seed-associated microorganism inoculum (Berjak *et al.*, 1999). In addition, the extent of 'flash-drying' and cooling rates must be determined (e. g. Wesley-Smith *et al.*,

2001a, b, 2004a, b). Furthermore, the desirability of using cryoprotectants needs to be ascertained. Moreover, the *in vitro* technologies ensuring excised axes will establish vigorous seedlings must be developed. Additionally, thawing and especially rehydration must be optimised (e. g. Berjak *et al.*, 1999; Berjak and Mycock, 2004). Further, the means for efficient dissemination of explants retrieved from cryostorage must be established (e. g. Peran *et al.*, 2006). A further aspect that could be profitably pursued is to induce a measure of axis desiccation and chilling tolerance prior to cryopreservation (e. g. Beardmore and Whittle, 2005).

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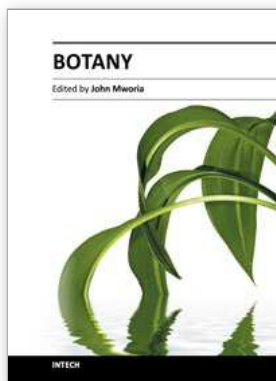
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This book is devoted to botany and covers topical issues in this diverse area of study. The contributions are designed for researchers, graduate students and professionals. The book also presents reviews of current issues in plant-environment interactions making it useful to environmental scientists as well. The book is organized in three sections. The first section includes contributions on responses to flood stress, tolerance to drought and desiccation, phytotoxicity to Chromium and Lead; the second has aspects of economic botany including a review of Smut disease in sugarcane and properties of plant extract used Tassaboount date juice; the last covers topical issues on morphogenesis and genetics on cotton fiber special cell, secretory glands *Asphodelus aestivus* flower ,pollen tube growth in *Leucocjum aestivum* , morphological studies of *Ardisia crenata* complex, and hybrid lethality in the Genus *Nicotiana*.

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