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INVITED REVIEW

Unifying perspectives of some mechanisms basic to desiccation tolerance across life forms

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Abstract

Desiccation-tolerant organisms, or life-cycle stages of particular taxa, occur among animals, higher and lower plants, terrestrial micro-algae, lichens and bacteria. Recent investigations have revealed that a number of mechanisms conferring desiccation tolerance appear to be common to the diversity of life forms able to survive extreme dehydration. In particular, parallel processes involving late embryogenic abundant (LEA) or LEA-like proteins, accumulation of sugars, aspects of active oxygen species (AOS) and non-enzymic and enzymic antioxidants have been the recent focus of attention, some across a diversity of organisms. The present contribution considers advances made from the study of these processes, particularly in enhancing current understanding of the composition of, and protection afforded by, the glassy state in desiccated organisms. Strong evidence that proteins, particularly LEAs, are implicated in glass formation is reviewed, and behaviour of such proteins upon dehydration is discussed in this context. The question of the ability of cells to survive complete water removal is considered in the context that it is unlikely, and that the basis of the deterioration of very dry seeds results from abstraction of water necessary to maintain the integrity of the intracellular glassy state. Finally, the revelation that desiccated seeds deteriorate with time, even under extremely good genebanking conditions, is discussed.

Keywords: active oxygen species, antioxidants, desiccation tolerance, intracellular glasses, LEA, localized reactivity, seed survival, sugars

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Introduction

Desiccation tolerance, while a rare property of living organisms generally, is a remarkable survival mechanism that characterizes life-cycle stages of a surprising number of taxa. Although the phenomenon is presently recognized in species of only five phyla among animals, desiccation tolerance has been identified in one or more life-cycle stages in all the major plant taxa, and is a property *inter alia* found in bacteria, terrestrial micro-algae and lichens (Alpert, 2005). Emerging evidence is that there may be considerable commonality among the mechanisms facilitating desiccation tolerance across the spectrum of organisms that exhibit this trait, irrespective of their phylogeny. The present review seeks to extend the seed biologist's horizons beyond the realm of orthodox propagules of spermatophytes, by a consideration of the commonality of some of the characteristics of desiccation-tolerant resurrection plants, animals, lichens and micro-organisms. This is, however, not a wide-ranging review of all the features considered in the context of desiccation tolerance, but focuses particularly on the implication of proteins (generally late embryogenic abundant proteins, LEAs) with sugars in the intracellular glassy state; free radicals, particularly active oxygen species, and antioxidants; and the revelation that even under what are considered to be ideal conditions, stored seeds are not immortal. For an overview of life in the dry state, the reader is referred to Black and Pritchard (2002), and to papers emanating from a recent symposium, 'Drying without dying: The comparative mechanisms and evolution in animals, plants and microbes' published in the journal, *Integrative and Comparative Biology.*

Desiccation tolerance is not synonymous with drought tolerance

As stated so cogently by Alpert (2005), desiccation tolerance of a whole organism – which is defined as the ability of a living structure to survive drying to equilibrium with low (<50%) relative humidity (RH) – is just *one mechanism* of drought tolerance. That author correctly differentiates desiccation tolerance as a phenomenon based on low intracellular water concentrations, from drought tolerance as meaning survival of low environmental water availability, while maintaining high internal water concentrations. 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 circumstances. Seeds would not be described as drought tolerant; they come to equilibrium (albeit slowly, in some cases) with ambient humidity. If they are desiccation tolerant they will survive, if not, they will die.

Desiccation tolerance is generally understood to pertain to organisms that will survive dehydration to an overall water concentration equal to, or less than, 0.1 g per g dry mass ($g\,g^{-1}$), i.e. water potentials (Ψ) ≤ -100 MPa (Vertucci and Farrant, 1995; Walters *et al.*, 2005b). Although the terms anhydrobiotic and anhydrobiosis are used synonymously with desiccation tolerance in the literature to describe 'tolerance to complete desiccation' (Alpert, 2005 and references therein), that terminology is not used in this review, as it implies complete absence of intracellular water, which is not the case at water concentrations of $\sim 0.1\,g\,g^{-1}$. Intracellular water concentrations $\leq 0.1\,g\,g^{-1}$ are considered to represent levels at which macromolecules can no longer be surrounded by a water monolayer, thereby effectively precluding enzymatic activity and, thus, all metabolism (Billi and Potts, 2002). However, unique water properties pertain at such low intracellular concentrations, and this is not the same thing as complete dryness. Water, in fact, should be viewed not only as an intracellular medium, but also as a structural component of macromolecules, e.g. 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 that a residual water layer remains in close association with proteins, small solutes – which must include inorganic ions – are likely to perturb the residual water, where their localized effects could contribute to macromolecule denaturation. This may well impose one constraint on biological material being able to survive completely anhydrous conditions, thus imposing a limitation on absolute desiccation tolerance.

The trait of desiccation tolerance in seeds is the outcome of the interaction of a spectrum of phenomena and properties (Pammenter and Berjak, 1999) that are under genetic control, and are best described as intrinsic cell characteristics (Walters *et al.*, 2005b). However, the degree of expression of these characteristics – and, indeed, whether all are present or only some are actively expressed – must underlie the differences in response to dehydration between highly desiccation-tolerant, less tolerant and variously desiccation-sensitive organisms, or life-cycle stages of species (Pammenter and Berjak, 1999). It must be stressed 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.

Do orthodox seeds have traits in common with other desiccation-tolerant organisms?

Resurrection plants

These plants, so far known to be constituted by some 330 species, have been described from nine pteridophyte and ten angiosperm families (Proctor and Pence, 2002). The vegetative tissues of all resurrection plants are characterized by desiccation tolerance. The plants concerned are able to remain viable despite considerable dehydration, resuming metabolic activity when water once again becomes available. Desiccation tolerance in angiospermous resurrection plants, like orthodox seeds, is based on a spectrum of relatively complex protection mechanisms that accompany dehydration (Illing *et al.*, 2005).

The occurrence and molecular biology of late embryogenic abundant (LEA) proteins came into prominence from studies on developing cotton seeds in the 1980s (Galau *et al.*, 1986; Hughes and Galau, 1987). These proteins are synthesized and accumulate coincident with the acquisition of desiccation tolerance during orthodox seed development, as do sucrose and certain oligosaccharides (Steadman *et al.*, 1996; reviewed by Buitink *et al.*, 2002), and particular antioxidant enzymes become prominent (Bailly, 2004). In their recent study, Illing *et al.* (2005) compared physiological and molecular data relevant to the acquisition of desiccation tolerance in developing seeds with that obtained from vegetative tissue of abiotically stressed desiccation-sensitive plants, and resurrection plants subjected to extreme dehydration. Those authors also analysed available data for gene expression of many LEA proteins and a large spectrum of antioxidant enzymes in both the developing,

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orthodox seeds, and in abiotically stressed, desiccation-sensitive, vegetative tissue of *Arabidopsis thaliana*. For *A. thaliana*, no particular class of LEAs was uniquely expressed in the stressed vegetative tissues, in contrast to the range of these proteins that become apparent during seed development. In particular, a spectrum of LEA genes that are significantly expressed in developing seeds [LEA 1: At2g40170, At3g51810; LEA 6: At3g22490, At3g22500, At5g27980; LEA 9: At2g41280; LEA 10: At1g04560; present on AtGenexpress Genechip (Illing *et al.*, 2005)] are not expressed in desiccation-sensitive plant tissue subjected to abiotic stress (Illing *et al.*, 2005). In contrast, the expression of at least 16 different LEA genes occurs in the leaves of the xerotolerant resurrection plant, *Xerophyta humilis*, during dehydration (Collett *et al.*, 2004). These 16 genes came from a survey of only 424 cDNAs, making the former a significantly high number (J.M. Farrant, Department of Molecular and Cell Biology, University of Cape Town, South Africa, personal communication).

In terms of the antioxidant enzymes, Illing *et al.* (2005) concluded that most constituted 'housekeeping' protectants that were nevertheless responsive in stressed *A. thaliana* tissues. Those authors suggested, however, that only if mechanisms are in place to protect enzymes, such as ascorbate peroxidase, glutathione reductase and superoxide dismutase, will they be operative during dehydration of *tolerant* tissues. A notable exception to such 'housekeeping' antioxidant enzymes is a 1-cys-peroxiredoxin associated with desiccation tolerance in seeds (Aalen, 1999), which was shown to be expressed abundantly in tissues of the resurrection plants, *X. humilis* and *X. viscosa*. The necessity for a highly effective antioxidant system as one of the fundamental properties facilitating desiccation tolerance was highlighted by Pammenter and Berjak (1999), and has been emphasized recently by Kranner and Birtić (2005). The latter authors ascribe the eventual death of tolerant organisms to failure of components of the antioxidant system during long-term desiccation. Regarding the accumulation of sucrose upon dehydration, Illing *et al.* (2005) reported that this sugar accumulated only in the tissues of the desiccation-tolerant *Eragrostis nindensis*, and not in the sensitive *Eragrostis* species, *E. teff* and *E. curvula*. These observations indicate a further commonality between desiccation-tolerant seeds, in general, and vegetative tissues of resurrection plants. From their analyses, Illing *et al.* (2005) suggest that certain of the mechanisms are likely to have evolved in seeds, where they are developmentally regulated, and the same mechanisms appear to be up-regulated in response to environmental cues in the vegetative tissues of resurrection plants.

Lichens

Lichens represent a symbiosis between a fungus (the mycobiont) and a green alga or cyanobacterium (the photobiont). The remarkable outcome of this symbiosis is that neither of the partners remains constrained to the cryptic habitats that would be obligatory for either one alone (Kranner *et al.*, 2005). This facilitates the formation and existence of the characteristic above-ground lichen thallus, which, in this habitat, may be exposed to high levels of irradiation and desiccating conditions. From their work on the lichen, *Cladonia vulcani*, Kranner *et al.* (2005) have shown that, although when separated both the mycobiont and the photobiont can survive in the desiccated state for differing periods (the fungus being particularly short lived), both the photoprotective and antioxidant mechanisms in the thallus are 'more effective by orders of magnitude' than they are in either of the isolated partners. Furthermore, as a result of these uprated properties, the lichen is highly desiccation-tolerant, which is a prerequisite for its occurring above ground. Some details of the mechanisms involved will be discussed below (see 'Active oxygen species and antioxidants').

Desiccation-tolerant animals

Although desiccation tolerance appears to be far more restricted across the spectrum of animals than of plants (Alpert, 2005), the phenomenon has been documented for nematodes (Browne *et al.*, 2002) and bdelloid rotifers (Lapinski and Tunnacliffe, 2003; Caprioli *et al.*, 2004), for example, and, classically, for the encysted embryos of the brine shrimp, *Artemia* spp. (see Clegg, 2005 and references therein). Solomon *et al.* (2000) reported that a heat-stable, water-stress-related protein (showing homology to a cold-responsive plant dehydrin linked to drought stress) was accumulated by entomopathogenic nematodes held at 97% RH for 3 d. Browne *et al.* (2002) have identified a gene coding for a protein identified as a Group 3 LEA in the nematode, *Aphelenchus avenae*, which was upregulated when the worms were exposed to 90% RH for 24 h. In parallel with the synthesis of this protein, Browne *et al.* (2002) reported that *A. avenae* accumulates trehalose in response to dehydration, leading to their suggestion that the LEA–trehalose combination might act synergistically in the formation of a glass, as is currently favoured for LEA–sucrose-based glasses in plant cells (see below). However, a caveat needs to be introduced at this point, as most of the nematodes in which LEA genes have been cloned do not appear to survive RHs $<90\%$, while desiccation-tolerant organisms are those that survive in equilibrium with RHs $\leq 50\%$; hence, such

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nematodes may show a degree of drought tolerance, but are not desiccation tolerant (Alpert, 2005; and see above).

In vitro, protein–trehalose synergism has been demonstrated for the heat-shock protein and molecular chaperone, p26, one of such proteins occurring in the extremely desiccation-tolerant encysted embryos of *Artemia* (Viner and Clegg, 2001; Clegg, 2005). Lapinski and Tunnacliffe (2003) mention the specific recognition of a dehydration-regulated protein in bdelloid rotifers by cross-reaction with an antiserum against a nematode LEA protein. Interestingly, however, neither of two species of bdelloid rotifers accumulated trehalose – nor other simple sugars, aside from glucose (Lapinski and Tunnacliffe, 2003) – and Caprioli *et al.* (2004) reported similar observations for an additional species of bdelloid rotifer. The implications of the lack of trehalose in these desiccation-tolerant organisms will be considered later.

Micro-organisms

Billi and Potts (2002) point out that prokaryotes in soils – which might periodically become very dry – need to be able to protect against the consequences of dehydration, and that survival of bacteria in the dry state is also important in health issues. They cite data that tubercle bacilli survive for 120 d in dust, a phenomenon presumably requiring desiccation tolerance. In the stationary phase under salt-stress conditions, *Bacillus subtilis* is characterized by considerable accumulation of a general stress-related protein, GsiB (Stacy and Aalen, 1998). Those authors have demonstrated a similarity between GsiB and LEA Group 1 proteins (although they are not homologues), suggesting related functionality among the bacterial and plant proteins in stress-related responses. *Deinococcus radiodurans* is a non-spore-forming, desiccation-tolerant bacterium that expresses two proteins, one of marked similarity to LEA 76 (a Group 3 LEA), and another sharing features with PCC13-62 (from *Craterostigma plantagineum*) (Battista *et al.*, 2001). When the *D. radiodurans* genes (*DR1172* and *DRB0118*) coding for these two proteins were inactivated, the bacteria became markedly sensitized to desiccation, leading those authors to conclude that both the proteins, like their homologues in plants, are implicated in desiccation tolerance.

From the survey to date for LEAs or LEA-like proteins in desiccation-tolerant organisms outside the plant kingdom, one is tempted to predict that this, and possibly other, features of maturing orthodox seeds may emerge as common to any organism that can remain viable in the desiccated condition. However, to substantiate this proposition, one must assess the mechanism by which LEAs contribute to desiccation tolerance, which, in turn, requires consideration of the intracellular condition in a dehydrated organism.

The intracellular glassy state

Perusal of the recent literature (some of which is cited below) leaves no doubt that attention has been sharply focused on the fundamentals of LEAs, di- and oligosaccharides and the glassy state in retention of viability in dry, desiccation-tolerant seeds.

A marked increase in cytomatrical (cytoplasmic) viscosity has been described for orthodox pea (*Pisum sativum*) seeds at tissue water concentrations below $\sim 0.3\,g\,g^{-1}$, which is indicative of glass formation (Buitink and Leprince, 2004). As described by those authors, dehydration below this level effectively causes super-saturation of intracellular solutions, which increases intermolecular cohesive forces and restricts mobility within the cytomatrix. Although dehydration is the principal factor resulting in glass formation during maturation drying of orthodox seeds, cooling below the glass transition temperature, T_g, will result in vitrification of any liquid, as long as the crystalline phase is avoided; hence, glasses may be defined as super-cooled liquids – or super-saturated solutions – of extremely high viscosity (Burke, 1986). The crystalline phase is solid and has a regular structure, whereas a glass, which does not have a regular structure, is described as an amorphous solid (Walters, 1998). As described by Walters (1998), the molecules of a glass are interconnected, resulting in a matrix that imposes restricted mobility compared with a liquid. However, in common with liquids, glasses lack specific molecular orientation, resulting in free volume ('holes') within the matrix. This, in turn, allows diffusion of small molecules (Walters, 1998) or other chemical species, e.g. free radicals (Hendry, 1993; Benson and Bremner, 2004), through the matrix. If sufficient water is present, the amorphous matrix has the looser characteristics of a rubber, which has a higher free volume and lower viscosity, increasing diffusivity (Walters, 1998).

The original realization that the intracellular solution within orthodox seeds at low water concentrations assumed the glassy state (e.g. Koster and Leopold, 1988; Williams and Leopold, 1989; Koster, 1991) stimulated a surge of interest, and many investigations on glasses and glass transitions followed in the 1990s, as noted by Walters (1998). The excitement that was generated came from the realization that the existence of the glassy state could explain the basis of seed survival in the desiccated state and also, because the glassy state is essentially metastable, could account for the fact that seed post-harvest (storage) lifespan is not infinite. However, the

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original conceptualization was that glass formation in seeds occurred as a consequence of their sucrose and oligosaccharide content, and other possible contributors to the intracellular glasses were largely overlooked. As commented by Walters (1998): 'The glassy state is a property of the entire aqueous phase and not the product of a few solutes.' This does not mean that sucrose and oligosaccharides, such as raffinose and stachyose, do not contribute to the glassy matrix, but that intracellular glasses in dry seeds are based on matrices that necessarily must include other cytomatrical constituent molecules. In short, intracellular glasses in seeds cannot merely be sugar-glasses.

LEAs with sugars as the basis of intracellular glasses

One of the first observations implicating an interaction between LEA proteins and sugars came from the work of Walters and co-workers in 1997. Those authors showed that only ~50% of the sugars from the heat-soluble protein–sugar fraction, extracted from mature wheat embryos, could be removed by exhaustive dialysis, with the remainder appearing to be tightly protein-associated. Although the nature of a synergistic relationship between the heat-soluble LEA proteins and sugars in desiccation tolerance was then not clear, Walters *et al.* (1997) suggested the possibility that their interaction might be important in resolving the role of LEAs in the phenomenon. From work examining seed quality of *Brassica campestris* (*rapa*), Sinniah *et al.* (1998) showed that the content of a 58 kDa heat-stable protein correlated well with potential longevity, and that the accumulation of certain oligosaccharides and the protein(s) was equally likely to be important in seed quality.

Later work by Buitink *et al.* (2000) indicated that there were no differences between T_g or intracellular molecular mobility after osmo-priming, compared with the original (non-primed) condition. These observations are significant in terms of intracellular glass composition, because, as shown by those authors, priming decreases the oligosaccharide content, while the sucrose content increases. Despite these changes in the primed seeds, the work of Buitink *et al.* (2000) showed that the intrinsic stability of the intracellular glassy state did not change. Nevertheless, the seed storage lifespan of the primed seeds was drastically reduced compared with the non-primed seeds stored at both 63 and 75% RH, despite the fact that water concentration was similar in both under the same storage conditions (Buitink *et al.*, 2000). Obviously, therefore, factors other than sugar composition and relative concentrations, and the physical properties of the intracellular glasses *per se*, affect the seed storage lifespan. Consequently, Buitink *et al.* (2000) tentatively suggested that oligosaccharides may not play a role in the longevity of desiccated, orthodox seeds. However, in a subsequent review, Buitink and Leprince (2004) express the opinion that, considering the abundance of non-reducing sugars in seeds, it is inevitable that they must participate in the glassy state. Those authors suggest that the sugars may contribute to filling free volume between large molecules, thereby increasing the density of the glass. While this might well be the case, it presents intracellular glasses in dehydrated tissues in a rather different way, compared with the original opinion that basically they are sugar-glasses (e.g. Koster and Leopold, 1988; Williams and Leopold, 1989; Koster, 1991).

Should this shift the focus away from sugars in relation to intracellular glasses? The answer, enigmatically, appears to be both no and yes.

Working with desiccation-tolerant pollen of *Typha latifolia*, Wolkers *et al.* (2001) characterized a heat-soluble protein as having sequence homology with a D76 LEA (LE76_brana, from *Brassica napus* seeds) and a D-7 LEA (LE7_GOSHI, from upland cotton). The isolated *T. latifolia* LEA – suggested by those authors to be a D-7 LEA of the LEA Group III family – had an unordered, random-coil conformation in solution, as has been demonstrated for other LEAs (e.g. Goyal *et al.*, 2003; Wise and Tunnacliffe, 2004). However, the conformation of the protein changed considerably upon drying, and the conformations adopted after rapid or slow drying also differed (Wolkers *et al.*, 2001). When the *T. latifolia* LEA was dried rapidly, the protein assumed a largely α-helical conformation, whereas slow dehydration resulted in both α-helices and extended intermolecular β-sheets. However, when dehydration of that LEA protein was carried out in the presence of sucrose, only the α-helical conformation occurred, whether dehydration was slow or rapid (Wolkers *et al.*, 2001). Those results indicate that there are major, albeit somewhat different, dehydration-related conformational changes in the D-7 LEA, depending to some extent on whether sucrose is present or not. However, the most interesting of the results obtained by Wolkers *et al.* (2001) emanated from a comparison of pure sucrose glasses with glasses containing both sucrose and the LEA protein at concentrations of 0.5 and 0.8 mg LEA mg^{-1} sucrose. The presence of the protein increased both the T_g of the glasses ($0.8 > 0.5$ mg LEA mg^{-1} sucrose), as well as the average strength of hydrogen bonding. These results led Wolkers *et al.* (2001) to suggest that the protein acted synergistically with the sucrose in the formation of the glassy matrix, thus attributing a function to both LEA protein and the disaccharide in conferring long-term stability of intracellular glasses in desiccation-tolerant organisms in the dry state.

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In a review of various factors involved concomitantly in desiccation tolerance, Oliver *et al.* (2001) postulate that it is the *de novo* synthesis of LEAs, rather than oligosaccharides, that confers the remarkable intracellular stability of dry, orthodox seeds. Nevertheless, in view of the considerable accumulated evidence of high sucrose concentrations with appropriate concentrations of other oligosaccharides and/or galatosyl cyclitols (e.g. Steadman *et al.*, 1996; Obendorf, 1997), one is led to the conclusion that the most stable of the intracellular glasses might result from the synergistic association between sucrose/oligosaccharides and proteins – perhaps predominantly LEAs. Alternatively, the significance of the accumulation of sucrose at the expense of monosaccharides, which accompanies maturation drying in orthodox seeds, might reside primarily in causing respiratory substrates to become increasingly unavailable. This is not a new idea, as Rogerson and Matthews (1977) correlated the fall in respiratory rate with a decline in respiratory substrate in developing pea seeds.

Characterization of a protein synthesized in response to dehydration stress by the nematode, *Aphelenchus avenae*, has resulted in its being described as AavLEA1, a Group 3 LEA-like protein (Goyal *et al.*, 2003). This protein, like the D-7 LEA isolated from *T. latifolia* pollen (Wolkers *et al.*, 2001), has been described as natively unfolded, but having the propensity to develop a significant α-helical component upon dehydration (Goyal *et al.*, 2003). What is perhaps highly significant is that those authors speculate that upon dehydration, AavLEA1 could form coiled coils similar in morphology to cytoskeletal intermediate filaments (IFs), a concept taken further and well illustrated by Wise and Tunnacliffe (2004). As components of the cytoskeleton in animal cells, IFs are flexible structures that impart both strength and resilience, and further, the lamins comprising the nucleoskeleton in animal cells and the intranuclear filaments in plant cells are also IFs (Osborne and Weber, 1987; Mínguez and de la Espina, 1993). While Goyal *et al.* (2003) dehydrated the AavLEA1 protein in the absence of oligosaccharides, those authors speculated that LEA protein filaments could act synergistically with sugar glasses 'in a manner analogous to steel-reinforced concrete, where the filaments might increase the tensile strength of the amorphous carbohydrate matrix'. This is a highly attractive idea that provides a functional role for both LEAs and sucrose in seeds – or trehalose in desiccation-tolerant animal cells – in providing a relatively stable, dehydrated intracellular matrix.

In the case of LEAs, Wise and Tunnacliffe (2004) express the opinion that the function of these proteins has been largely obscure, mainly because of their natively unfolded structure in solution (the hydrated state). They are also critical of conventional approaches, such as the BLAST algorithm, which demonstrate low relatedness between LEAs and other polypeptides. This, those authors maintain, may be because LEAs contain regions of low sequence complexity that are routinely masked out during searches for sequence similarity. Therefore, Wise and Tunnacliffe (2004) have developed a speculative computational approach, the Protein or Oligonucleotide Probability Profile (POPP), which facilitates analyses based on similarities in peptide composition of proteins, rather than on sequence similarities. This analytical tool enables the interrogation of a database of POPPs for proteins of known function, in order to query proteins of unknown function, but similar peptide profiles. As a consequence, Wise and Tunnacliffe (2004) have tentatively suggested a revised system for LEA classification. The findings (above) about the similarities among IFs and the LEAs from *T. latifolia* and the nematode, *A. avenae*, are described as fitting in well with predictions made by POPP analysis.

From the information presented above, it is apparent that views about the intracellular amorphous state in desiccation-tolerant organisms have moved from the concept of sugar glasses to that of glasses in which proteins – and especially LEAs – play a dominant role. However, one must not lose sight of the fact that there is a multiplicity of other cytomatrical components that will be caught up in the intracellular glass. Buitink and Leprince (2004) draw attention to the situation that, while intracellular glasses in dehydrated, desiccation-tolerant organisms are probably based on sugar–protein mixtures, many other components, including amino acids, ions and salts, must also somehow be involved, as would cell wall components in plant tissues. Additionally, particles, such as ribosomal subunits, must also be components of the glassy matrix. One must also consider that intra-organellar contents are also likely to form glasses at low water concentrations in desiccation-tolerant organisms, and these would have a different compositional basis compared with those of the cytomatrix (Buitink and Leprince, 2004). Furthermore, localized pools of higher water concentration were suggested, and have been demonstrated, to exist in dehydrated cells (Berjak *et al.*, 1986; Bruni and Leopold, 1992; Rinne *et al.*, 1999; Leubner-Metzger, 2005). The interior of organelles, such as mitochondria and plastids, as well as the *mileau* of the chromatin, might represent such loci, suggesting that 'intracellular glass' as such, is likely to be of variable local density. (In this respect, bulk seed, or axis, water concentration, or Ψ, must mask such internal variation.) Angell (1995) has provided microscopical evidence that, on manipulation of an originally homogeneous inorganic system, two glass phases can coexist: a lower density droplet phase was

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interpreted as having nucleated from the surrounding, denser phase.

One might, in fact, further conjecture that there are intracellular 'pockets' of sufficient residual water to obviate localized glass formation. In desiccated rye seeds, Elder *et al.* (1987) recorded the progressive action of endo- and exonucleases, surely arguing for the existence of sufficient residual water in the *mileau* of the chromatin to facilitate the activity of these enzymes. From their work on birch buds, Rinne *et al.* (1999) were able to conjecture that enzyme activity was maintained in dehydrin-associated local pools of water, occurring in the otherwise-dehydrated cells. Furthermore, the work of Leubner-Metzger (2005) on the living cells of the inner testa of air-dry tobacco seeds has demonstrated convincingly that not only does localized β-1,3-glucanase activity occur, but so does translation, following low-level transcription of the β-1,3-glucanase gene. That author proposes that these are key after-ripening events alleviating coat-imposed dormancy. Thus, the conjecture and evidence support the concept of a non-homogeneous intracellular glass of variable density, with pockets that are sufficiently fluid to permit some chemical reactions in dehydrated, desiccation-tolerant tissues. The implication of this view is that dehydrated desiccation-tolerant tissues are not at thermodynamic equilibrium. This suggested state of dis-equilibrium is maintained, not by the expenditure of energy, but simply by the rate at which processes leading to equilibrium occur. Perhaps the achievement of a state of equilibrium is coincident with death.

Despite the accumulation of sucrose during dehydration in plant cells, and trehalose in animal cells and in yeast cells entering the stationary phase, it has become apparent that desiccation tolerance is possible in the absence of simple sugars. Lapinski and Tunnacliffe (2003), working with clonal populations of two species of bdelloid rotifers, have demonstrated that these organisms are well able to withstand desiccation, but do not contain trehalose or any equivalent disaccharide, and Caprioli *et al.* (2004), investigating another bdelloid rotifer species, reached the same conclusion. However, Lapinski and Tunnacliffe (2003) do mention the occurrence of a protein that accumulates with dehydration of the rotifers, and which appears to be related to nematode LEA protein. As it is unlikely in the extreme that intracellular glasses are not formed in the dehydrated rotifers [water concentration 0.06–0.10 $g\,g^{-1}$ according to Lapinski and Tunnacliffe (2003)], it appears that our horizons must be broadened when considering the major determinants of desiccation tolerance.

Oksanen and Zografi (1993) determined water vapour isotherms for amorphous solid polyvinylpyrrolidone (PVP) and the change in glass transition temperature in relation to water content. From their results, those authors showed that even at low water contents, the water is relatively mobile in the polymer glass, and they hypothesized that water in glasses might be represented by two populations with different modes or time-scales of motion. The hypothesis of Oksanen and Zografi (1993) is supported by studies on the model protein, crambin, by Teeter *et al.* (2001). From their work on crystals of crambin – the water dynamics of which match those of certain enzymes – those authors demonstrated a glass transition to occur around 200 K. Crambin has four water cavities, with much of the water having multiple sub-states, and ~30% of the crambin residues have multiple conformational states, which are structurally coupled to the water sub-states (Teeter *et al.*, 2001). Those authors assert that the coupling is the basis of the temperature-related glass transition, not only of crambin, but of all proteins. While consideration of glass transitions in desiccation-tolerant organisms is based on decreasing water concentrations irrespective of the temperature, the 'message' from the work on crambin is that a protein will undergo a glass transition in the absence of extraneous substances such as sugars. This may have significance with respect to the reports by Wolkers *et al.* (2001) and Goyal *et al.* (2003), showing conformational changes upon dehydration in LEA/LEA-like proteins of plant and animal origin, respectively, even in the absence of sugars.

Sugars, however, appear to be ubiquitous in desiccated seeds [and apparently, resurrection plants (Illing *et al.*, 2005)], and a most interesting interpretation of their functional significance, where not associated with LEAs, has emanated from the work of Bryant *et al.* (2001). They consider that the presence of small solutes, concentrated in the water as dehydration proceeds, would provide a buffer between adjacent membranes, reducing physical stresses, and the associated increase in fluid-to-gel transition temperature (T_m) of the lipid (relative to the higher T_m that would prevail were sugars absent). Furthermore, were sugar-glasses to be formed in these locations, the mechanical resistance of the glass would enhance the protection provided by the small solutes. Bryant *et al.* (2001) suggest that large solutes (perhaps the LEAs) may well be excluded from narrow intermembrane spaces, and so would not provide protection against an increase in T_m. However, this interpretation does not detract from the suggested synergism between sugars and LEAs that would presumably prevail in cytomatrical locations outside of narrow intermembrane spaces.

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Active (reactive) oxygen species and antioxidants

One of the basic tenets of the efficacy of the intracellular glassy state in maintaining viability of desiccated organisms is that molecular and free radical mobility – and thus interaction – is curtailed. Nevertheless, during the process of desiccation, intracellular structures are highly vulnerable, as conditions for radical generation are enhanced (Fig. 1; Vertucci and Farrant, 1995; Pammenter and Berjak, 1999; Kranner and Birtić, 2005; Walters *et al.*, 2005b).

A free radical is a highly reactive chemical species containing one or more unpaired electrons, which is able to exist independently (e.g. Hendry, 1993). The availability or non-availability of di-oxygen (O_2) is a matter of life or death for aerobic organisms. Yet oxygen itself has two unpaired electrons of parallel spin in the outermost orbital, making it a radical (Hendry, 1993). However, according to Benson and Bremner (2004), oxygen is not a free radical as such, and its normal ground state is the triplet state, $^3O_2$. Oxygen acts as an oxidizing agent by accepting additional electrons one at a time, thus making it far less reactive than would be the case for a true free radical (Hendry, 1993; Benson and Bremner, 2004). Nevertheless, oxygen can lose, or, more commonly, gain electrons to form a spectrum of free radicals (FRs) (Hendry, 1993). These collectively, along with H_2O_2 (the non-radical, hydrogen peroxide), are termed active or reactive oxygen species (AOS or ROS, respectively).

The most frequently discussed AOS in the context of intracellular phenomena, are the superoxide radical ($O_2^{\bullet-}$), H_2O_2, the hydroxyl radical ($^{\bullet}OH$) and singlet oxygen ($^1O_2$). While superoxide itself is relatively short lived, it may dismutate to form H_2O_2 which, if not reduced to water, can be involved in a major route of $^{\bullet}OH$ formation by the Fenton reaction, $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + HO^-$ (Hendry, 1993; Benson and Bremner, 2004). The major source of Fe^{2+} for this

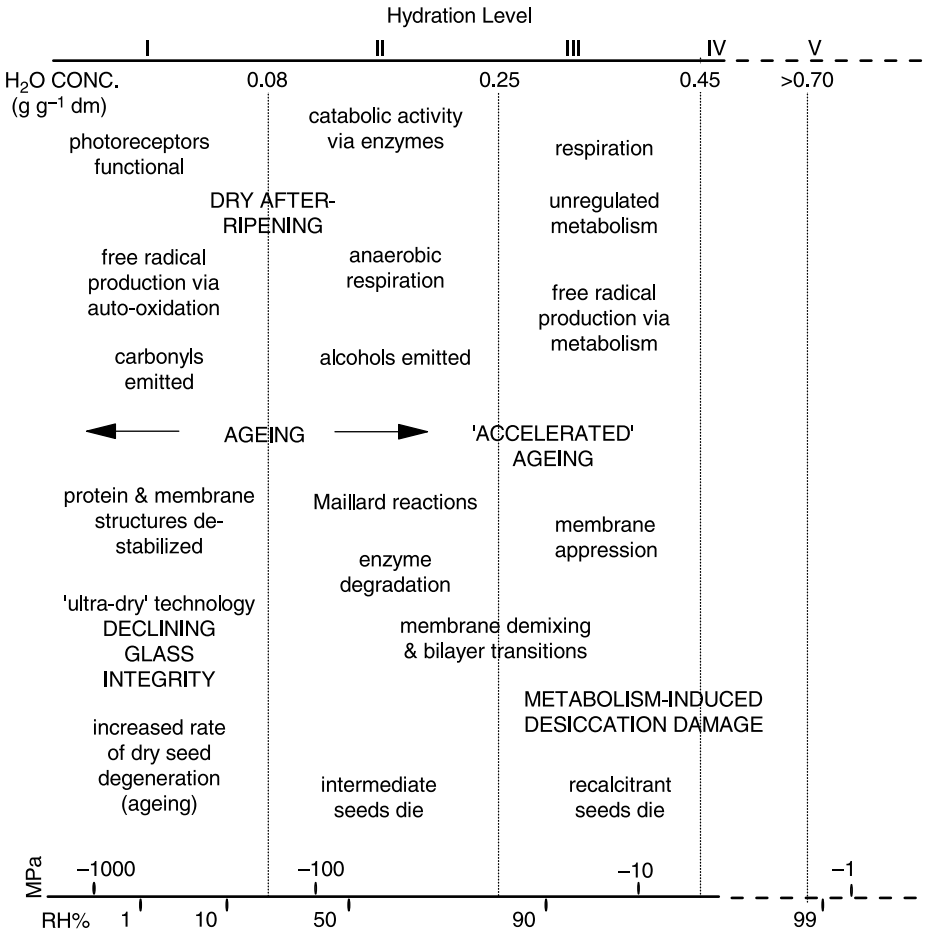

Figure 1. A scheme of events and processes in seeds in the hydration levels III to I. Approximate water concentrations (dry mass basis) are given at the top of the figure, and the equivalent water potentials (ψ) and equilibrium relative humidities (%) at the bottom. (Modified from Vertucci and Farrant, 1995; Walters *et al.*, 2005b.).

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reaction is thought to be the reduction of Fe^{3+} by O_2^- (Hendry, 1993). The hydroxyl radical is considered to be the most damaging AOS in biological tissues, reacting with nucleic acids, lipids and, indirectly, with proteins (Benson and Bremner, 2004). Although singlet oxygen production can occur in non-photosynthetic tissue, $^1O_2$ generation is of particular concern in desiccating chlorophyll-containing tissues in the light when water deficit restricts photosynthesis (Kranner and Birtić, 2005). This AOS can react directly with lipids, thus initiating peroxidation (Halliwell, 1987).

This brief excursion into AOS stresses that in order to reap the benefits of being aerobic but still survive, organisms must be equipped with an array of effective antioxidants, which are comprised both of enzymes and non-enzymic free-radical scavengers. Control of levels of AOS is of prime importance in homeostasis, as these chemical species are not only potentially highly destructive, but also have a critical role in intracellular signalling (Finkel and Holbrook, 2000; Laloi *et al.*, 2004). While strict control is taken for granted in hydrated cells, possession and effective operation of a suite of antioxidants is of prime importance during dehydration of orthodox seeds and desiccation-tolerant vegetative tissues, and again as soon as water uptake commences by the desiccated cells (Pammenter and Berjak, 1999; Kranner *et al.*, 2002; Kranner and Birtić, 2005). Additionally, non-enzymic antioxidants are likely to confer protection during the desiccated state in seeds (Bailly, 2004). The same situation must also prevail in the cells of any organism or structure capable of surviving extreme desiccation. In his 2004 review on AOS and antioxidants in seeds, Bailly provides a thorough update of the topic, as well as stressing that AOS are implicated in a variety of signalling functions. That author makes the point that most studies on AOS and antioxidants have been focused on the final stages of seed desiccation, but that the subject needs to be explored at all stages of seed development – and during germination. The work by Aalen (1999) and Stacy *et al.* (1999) makes a good start to this extension.

Some aspects of the antioxidant situation in dehydrated resurrection plants were considered above, among which, notably, Illing *et al.* (2005) discussed the abundant expression of 1-cys-peroxiredoxin. Peroxiredoxins are thiol-requiring antioxidants having one or two conserved cysteine residues, and no tightly bound metal ions (Aalen, 1999). They function to reduce H_2O_2 and $^{\bullet}OH$. Although the family of peroxiredoxins is wide-ranging across organisms generally, prior to the studies of Illing *et al.* (2005) the expression of 1-cys-peroxiredoxin genes was thought to be unique to seeds, taking place, in barley, only in the embryo and aleurone, i.e. in those seed tissues that survive desiccation (Stacy *et al.*, 1999). Those authors have localized 1-cys-peroxiredoxin to the nuclei of imbibed, dormant barley embryo and aleurone cells, where it is held to afford antioxidant protection to DNA. Additionally, 1-cys-peroxiredoxin is implied to afford protection by preventing free-radical attack during desiccation. If the observations made above about localized water pools occurring *inter alia* in the mileau of the chromatin are correct, then it is possible that 1-cys-peroxiredoxin can, and does, function to protect the genome against AOS in the desiccated seed. Nevertheless, for this to be an effective mechanism in the long term, regeneration of the cysteinyl residue of the catalytic centre of 1-cys-peroxiredoxin should also be possible. This is normally achieved by intramolecular or intermolecular thiol-disulphide reactions, and ultimately by electron donors such as thioredoxins and glutaredoxins (Dietz, 2003). If such interaction is established, then the proposal that 1-cys-peroxiredoxin affords long-term protection to the genome in desiccated seeds would be substantiated.

What is the situation early during seed development, i.e. during histodiffentiation, and later during reserve importation (seed filling) and maturation drying? Histodifferentiation is a phase of high metabolic activity, when mitochondrial respiration – and, in some cases, photosynthetic electron transport – takes place. Both these processes are sources of AOS, and it is implicit that the antioxidant system must be efficiently operational. Ascorbate peroxidase (APX) is involved in the conversion of H_2O_2 to water, which is of vital importance, considering that the peroxide can be a source of $^{\bullet}OH$ formation. The enzymes, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (which comprise the intracellularly widely distributed ascorbate–glutathione cycle) regenerate the antioxidants, ascorbic acid, reduced glutathione and α-tocopherol (Foyer and Halliwell, 1976; Bailly, 2004). From work on developing seeds, Bailly (2004) also records that the activity of the major antioxidant enzymes changes with development. High activity of superoxide dismutase (SOD, responsible for the conversion of O_2^- to H_2O_2) and APX were found to characterize the desiccation-sensitive stage in bean seeds, when the activities of catalase (CAT, also involved in $H_2O_2 \rightarrow H_2O$) and GR were low. In contrast, the desiccation-tolerant condition was characterized by high activity of CAT and GR, and low SOD and APX activity. de Tullio and Arrigoni (2003) are of the opinion that neither ascorbate nor APX are essential for the desiccation phase, and furthermore, that the presence of ascorbate could pose a hazard at that stage because, in association with transition metals, it could act as a pro-oxidant, leading to $^{\bullet}OH$ formation. As discussed by Bailly (2004), a trend of increasing CAT activity during seed

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desiccation seems to be emerging, occurring in sunflower and other species, and leading to the conjecture that transcription of the catalase gene might be finely regulated by water loss.

Kranner and Birtić (2005), in a comparison of orthodox seeds, lichens and resurrection plants, have concluded that the antioxidant activity of glutathione is critical for survival of desiccation. During maturation drying of *Pisum sativum* seeds, glutathione (GSH) is converted to glutathione disulphide (GSSG) (Kranner and Grill, 1996), particularly in the water concentration range that is held to enhance free-radical production accompanying unregulated metabolism (hydration level III, Fig. 1; Walters *et al.*, 2005b). Ongoing dehydration limits the re-reduction of GSSG to GSH, although GSSG may play a role in binding to, and protecting, protein SH-groups (Kranner and Grill, 1996; Kranner and Birtić, 2005). It is, however, imperative that GSSG is reduced to GSH when the seeds are imbibed (reviewed by Kranner and Birtić, 2005). Those authors have equated a substantial increase in glutathione redox potential ($E_{GSSH:2GSH}$) accompanying continuing desiccation stress, with sustained oxidative stress – which is one of the triggers of programmed cell death. In reviewing the evidence from work on many plant and fungal cells, Kranner and Birtić (2005) have indicated that viability is lost when $E_{GSSH:2GSH}$ values are in the range -180 to -160 mV; any plant material with $E_{GSSH:2GSH}$ values more positive than -160 mV will be dying or dead. For the resurrection plant, *Myrothamnus flabellifolia*, those authors have shown that the $E_{GSSH:2GSH}$ remains below -160 mV for material that maintained viability after being held in the desiccated state for 4 months, but after 8 months viability was lost, coinciding with $E_{GSSH:2GSH}$ values more positive than -160 mV. Similarly, a precipitous loss of viability in ageing pea seeds was accompanied by $E_{GSSH:2GSH}$ values more positive than -180 mV (Kranner and Birtić, 2005). Those authors suggest that $E_{GSSH:2GSH}$ affords a reliable marker for viability of material that has been desiccated.

When the water concentration of orthodox seeds declines from ~ 0.45 to below $0.25\,g\,g^{-1}$ (Ψ from -3 to below -11 MPa), unregulated metabolism prevails, with the propensity for metabolically linked free-radical production (Fig. 1; Walters *et al.*, 2005b). Thus, it is particularly necessary that the components of the appropriate antioxidant system are fully expressed and active in this water concentration (Ψ) range.

In the desiccated condition – and especially if they are stored – seeds at water concentrations $< 0.1\,g\,g^{-1}$ remain vulnerable to free-radical production by autoxidation (Fig. 1; Walters *et al.*, 2005b). At such low water concentrations ($\Psi < -100$ MPa) and considering the prevalence of the glassy state, conditions are supposed not to be conducive to enzymic antioxidant operation, and non-enzymic mechanisms are suggested to prevail (Bailly, 2004). However, as the same arguments about restricted molecular mobility must apply to both sorts of antioxidants, one must conjecture that the appropriate scavengers are located where they are required, and that localized conditions permit their reactivity. This argument was made above for 1-cys-peroxiredoxin in nuclei, and may be appropriate also for a spectrum of non-enzymic antioxidants, if localized water pools (Berjak *et al.*, 1986; Bruni and Leopold, 1992; Rinne *et al.*, 1999; Leubner-Metzger, 2005) do exist within organelles. However, should such a situation exist, one must question whether other localized antioxidant enzyme activity could be possible, given that the appropriate enzymes are present. Considering the observations of Elder *et al.* (1987) and Leubner-Metzger (2005), and of Rinne *et al.* (1999) implicating enzyme activity in specific intracellular loci of dry seeds and buds, respectively, the possibility of localized antioxidant enzyme activity, as well as of scavengers, should be seriously considered. However, as pointed out by Bailly (2004), this is easier said than done, as conventional enzyme assays *in vitro* are carried out in aqueous media. This argues that microscopical immunocytochemical (ICC) methodology should be widely used to locate specific molecules in freeze-substituted specimens, thus avoiding the uncertainty arising from aqueous-based analytical approaches. As an indication of the benefits, Stacy *et al.* (1999) localized 1-cys-peroxiredoxin to nuclei by means of ICC and confocal fluorescence microscopy.

If not able to be controlled, free radicals generated while seeds are in the desiccated condition will accumulate, especially during prolonged storage under poor conditions. Thus, it is essential that an appropriate battery of antioxidants be mobilized as soon as the seeds begin imbibing water. The work of several investigators and groups of investigators has revealed that H_2O_2 is produced early during imbibition in a variety of seeds, when non-enzymic antioxidants come into play (see Bailly, 2004 and references therein). However, increased activity of antioxidant enzymes, particularly GR and CAT, have been recorded as occurring prior to radicle protrusion in sunflower seeds (Bailly *et al.*, 2002).

While much progress has been made in the area of AOS and antioxidants in seeds – not forgetting the positive aspects of AOS reviewed in 2004 by both Bailly and Laloi *et al.* – clearly there are still gaps that require to be filled before an overall appreciation of the fine details can be obtained.

This brief overview of AOS and antioxidants in relation to desiccation tolerance would not be complete without a consideration of the intriguing findings on the lichen, *Cladonia vulcani*, by Kranner *et al.* (2005), which may be generally pertinent to all

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lichens. Those authors investigated certain biochemical parameters in the lichen in relation to responses to desiccation and irradiation, compared with the abilities of the isolated fungal and algal partners. They found that although the isolated partners could both survive in the desiccated state for 3 weeks, only the green alga recovered upon re-hydration after 9 weeks in the dry condition under low light intensity, although the recovery was slow – in line with the loss of chlorophyll, β-carotene and α-tocopherol that had occurred during desiccation. After 9 weeks in the desiccated state, glutathione was completely depleted in the fungus, which apparently relies entirely on the GSH–GSSG antioxidant system. In contrast, the intact lichen survived the prolonged desiccation period, despite a 25-fold higher light intensity (lethal to the isolated alga), with only minor damage (Kranner *et al.*, 2005).

Those authors showed that in the lichenized condition, the photo-protective and antioxidant systems of the alga were both upregulated relative to photo-assimilation – properties that were lost to the alga in the separated state. The fungal partner was deduced to benefit from the association in terms of antioxidant defence, showing only an essentially ineffective glutathione-based system if separated. From that work, Kranner *et al.* (2005) hypothesized that the resilience of the lichen to desiccation is the consequence of mutual stimulation by both partners of the photo-protective and antioxidant capacities, which facilitates not only enhanced desiccation-tolerance, but also rapid and complete recovery upon re-hydration.

How dry *is* dry?

There are a few biological specimens that tolerate overall water concentrations below $0.01\,g\,g^{-1}$ without incurring any obvious damage; encysted embryos of *Artemia* constitute one example (Clegg, 1986), while spores of *Aspergillus niger* showed no reduction in viability when stored at 1% RH (Walters *et al.*, 2005b). Another such example is afforded by the seeds of *Welwitschia mirabilis*, the unique gymnosperm of the Namib Desert, which not only survived after drying from an original $0.077\,g\,g^{-1}$ to $0.007\,g\,g^{-1}$ (Whitaker *et al.*, 2004), but showed no reduction in viability when stored at this low water concentration in sealed foil containers for 2 years at 5.5 or $-20\,°C$ (experiment currently ongoing). However, in all these cases, the structures are heavily encapsulated, and it is feasible that the living tissues were actually at water concentrations considerably higher than the surrounding, non-living structures. Clegg (2005) reported that decapsulation followed by dehydration adversely affected survival of *Artemia franciscana* gastrulae, although the effects of the more rapid dehydration rate could not be discounted. Intriguingly, both the *A. franciscana* cysts (Clegg, 2005) and *W. mirabilis* seeds (at the original water concentration) proved to be extremely thermotolerant, as did the spores of *A. niger* var. *phoenicus* associated with the seeds (Whitaker *et al.*, 2004).

From the results of ultra-dry seed storage experiments, it seems unlikely that viability could be retained in the complete absence of intracellular water (Walters and Engels, 1998). In fact, whether all the water is able to be removed from biological material is a moot point. Even lyophilized viable cells have a measurable water content (e.g. $0.035\,g\,g^{-1}$ for yeast), and on immediate removal from vacuum packaging the water content of freeze-dried coffee granules was found to be $0.04\,g\,g^{-1}$ (author's unpublished data).

Current shock: cryostored desiccated seeds may not be immortal!

While finite mortality of desiccation-tolerant organisms or structures should be presumed [but see, 'Longevity in the desiccated state – even desiccation tolerant organisms do not live forever' (Kranner and Birtić, 2005)], cryostorage, theoretically, is tacitly understood to offer the promise of an almost infinite viability extension. However, it appears that, even when stored under near-ideal genebanking conditions, the viability of desiccated seeds declines (Walters *et al.*, 2005a). In a study of some 42,000 accessions representing 276 species stored for significant periods in the USDA National Plant Germplasm System (NPGS), those authors found that seeds fell into three longevity categories – short, medium and long – typified by P_{50} values [the storage time after which germination (viability) declined to 50%] of <25, 30–70 and 80–500 years, respectively. No correlations were found among the characteristic P_{50} values and content of sucrose or oligosaccharides (Walters *et al.*, 2005a), concurring with the views of Buitink and Leprince (2004) that the stability of the intracellular glassy state is not primarily dependent on these compounds.

Nevertheless, from the extensive investigations of intracellular molecular mobility in desiccation-tolerant plant material by Buitink and co-workers (reviewed by Buitink and Leprince, 2004), it is apparent that the properties of intracellular glasses do change at very low water concentrations. The observations reported by those authors support the earlier data that identified temperature-dependent critical water contents below which the deterioration rate of dry seeds increases (Walters, 1998). However, there is not universal agreement about the rate increase. Ultra-dry seeds of five species, hermetically

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stored at −20 °C for 10 years, did not show increased viability loss, relative to those stored at 'conventional' water concentrations (Hong *et al.*, 2005). Seeds of the desert species, *Welwitschia mirabilis*, may also not be adversely affected in the ultra-dry condition (Whitaker *et al.*, 2004). Nevertheless, on the basis that relaxation (movement) within the glassy matrix may occur with time, Walters (1998) concluded that the nature and kinetics of chemical deteriorative processes might also be expected to change, which could underlie the kinetics of seed ageing. Later work by Walters *et al.* (2004) led to the conclusion that, even at temperatures lower than −130°C, ageing reactions are enabled because molecules remain sufficiently mobile. This, without doubt, has serious implications for cryostorage of desiccated material, especially if previously dried below the critical water content appropriate to the storage temperature. In this regard, Benson and Bremner (2004) draw attention to the possible persistence of free radicals that might occur within the glassy matrix, despite the significant extension of lifespan afforded by cryostorage.

The nature of deteriorative reactions in seeds at water concentrations, or Ψ, below the critical level does change (Vertucci and Farrant, 1995; Walters *et al.*, 2005b). Those authors have defined five hydration levels, of which two are relevant to the desiccated state, namely level II (water concentration 0.25–0.08 g g^{-1}; Ψ, −11 to −150 MPa) and level I (<0.08 g g^{-1}; Ψ, <−150 to −1000 MPa; Fig. 1). While phenomena in level II include catabolic events via enzymes (q.v. Elder *et al.*, 1987), anaerobic respiration and enzyme degradation, those characteristic of level I (noting that photoreceptors may be functional and hence be a source of free radicals) include free-radical production via auto-oxidation, evolution of carbonyls, destabilization of protein and membrane structure and, consequentially, increased seed ageing rates that have been recorded for ultra-dry, stored seeds (Fig. 1; Walters *et al.*, 2005b).

On the basis that intracellular glasses are far more complex than those formed by solutions of inorganic salts or, obviously, by pure water, one must query what might occur below critical water concentrations to account for increased molecular mobility, the change in the nature of deleterious events that occur in hydration level I and the increased rate of deterioration that may characterize most ultra-dry seeds. The answer may be forthcoming in the context of the nature of the glassy state, and how it would change as water concentration declines below the critical level (Fig. 2).

While water is acknowledged as a plasticizer, in that above certain concentrations it loosens the structure of the glassy matrix (Walters, 1998), below T_g it probably plays a critical role in the integrity of the glassy matrix by hydrogen bonding between other components – principally proteins such as LEAs, but also sugars, which are, almost without exception, present in high concentrations. On withdrawal of water beyond the critical level, two effects are

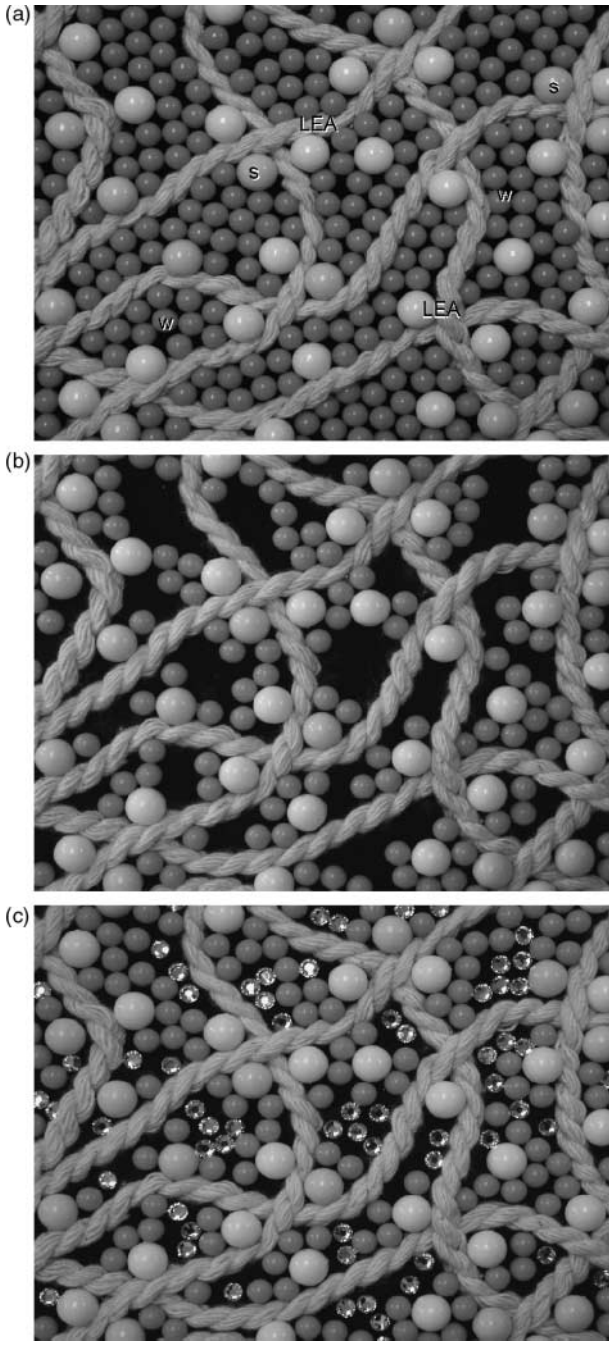

Figure 2. Representation of: (a) a late embryogenic abundant protein (LEA)-based glass in the dry range (w, water; s, sugar); (b) after abstraction of water to the ultra-dry range, leaving interstices where water [w in (a)] has been removed; and (c) migration of free radicals *inter alia*, through the interstices of the more porous glass.

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envisaged: the structural continuity of the glass will be perturbed, and, as suggested by Walters (1998), structural integrity of macromolecules may be compromised. The opinion of Teeter *et al.* (2001) that protein–water coupling underlies the temperature-related glass transition of proteins is relevant in this regard. A further suggestion by Walters (1998) is that macromolecule surfaces may be rendered more vulnerable to attack by, for example, free radicals, and also that metal ions may become exposed to similar attack.

Withdrawal of water below the critical level to maintain glass integrity could result in increased porosity, with the potential for greater molecular – or free radical – mobility (Fig. 2). In this regard, free radicals or other toxic species trapped in a relatively stable glassy matrix above the critical hydration level could become considerably more mobile, once glass integrity is compromised at lower water concentrations. Hence, the rate of deleterious reactions might well increase while the desiccated state prevails, the consequences of which would be cumulative with time. Alternatively, or perhaps additionally, the damaging consequences could be realized once seeds are removed from either cold- or cryo-storage. As noted by Benson and Bremner (2004), Levitt (1962) originally hypothesized that four potentially injurious phases exist in freezing (frost) injury: the moment of freezing; in the frozen state; the moment of thawing; and during the post-thaw period. Despite the desiccated state of orthodox seeds, the same principles apply to those subjected to cryo-storage. Additionally, if free radicals do persist in the glassy state (Benson and Bremner, 2004), the increase in water in the seed during post-storage imbibition is also potentially hazardous.

A considerable amount of recent progress has been made towards the understanding of desiccation tolerance, and about the statics and dynamics of the behaviour of organisms – especially seeds – in the desiccated state. The major challenge is translating this progress into best practices for plant germplasm conservation via the desiccated seed life-cycle stage.

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References

Aalen, R.B. (1999) Peroxiredoxin antioxidants in seed physiology. *Seed Science Research* **9**, 285–295.

Alpert, P. (2005) The limits and frontiers of desiccation-tolerant life. *Integrative and Comparative Biology* **45**, 685–695.

Angell, C.A. (1995) The old problems of glass and glass transition, and the many new twists. *Proceedings of the National Academy of Sciences, USA* **92**, 6675–6682.

Bailly, C. (2004) Active oxygen species and antioxidants in seed biology. *Seed Science Research* **14**, 93–107.

Bailly, C., Bogatek-Leszczynska, R., Côme, D. and Corbineau, F. (2002) Changes in activities of antioxidant enzymes and lipoxygenase during growth of sunflower seedlings and seeds of different vigour. *Seed Science Research* **12**, 47–55.

Battista, J.R., Park, M.-J. and McLemore, A.E. (2001) Inactivation of two homologues of proteins presumed to be involved in the desiccation tolerance of plants sensitizes *Deinococcus radiodurans* R1 to desiccation. *Cryobiology* **43**, 133–139.

Benson, E.E. and Bremner, D. (2004) Oxidative stress in the frozen plant: A free radical point of view. pp. 205–241 *in* Fuller, B.J.; Lane, N.; Benson, E.E. (Eds) *Life in the frozen state*. Boca Raton, CRC Press.

Berjak, P., Dini, M. and Gevers, H.O. (1986) Deteriorative changes in embryos of long-stored, uninfected maize caryopses. *South African Journal of Botany* **52**, 109–116.

Billi, D. and Potts, M. (2002) Life and death of dried prokaryotes. *Research in Microbiology* **153**, 7–12.

Black, M. and Pritchard, H.W. (2002) *Desiccation and survival in plants. Drying without dying*. Wallingford, CABI Publishing.

Browne, J., Tunnacliffe, A. and Burnell, A. (2002) Anhydrobiosis: plant desiccation gene found in a nematode. *Nature* **416**, 38.

Bruni, F. and Leopold, A.C. (1992) Pools of water in anhydrobiotic organisms. A thermally stimulated depolarization current study. *Biophysical Journal* **63**, 663–672.

Bryant, G., Koster, K.L. and Wolfe, J. (2001) Membrane behaviour in seeds and other systems at low water content: the various effects of solutes. *Seed Science Research* **11**, 17–25.

Buitink, J. and Leprince, O. (2004) Glass formation in plant anyhdrobiotes: survival in the dry state. *Cryobiology* **48**, 215–228.

Buitink, J., Hemminga, M.A. and Hoekstra, F.A. (2000) Is there a role for oligosaccharides in seed longevity? An assessment of intracellular glass stability. *Plant Physiology* **122**, 1217–1224.

Buitink, J., Hoekstra, F.A. and Leprince, O. (2002) Biochemistry and biophysics of tolerance systems. pp. 293–318 *in* Black, M.; Pritchard, H.W. (Eds) *Desiccation and survival in plants: Drying without dying*. Wallingford, CABI Publishing.

Burke, M.J. (1986) The glassy state and survival of anhydrous biological systems. pp. 358–363 *in*

https://doi.org/10.1079/SSR2005236 Published online by Cambridge University Press

Leopold, A.C. (Ed.) *Membranes, metabolism and dry organisms*. Ithaca, New York, Comstock Publishing Associates.

Caprioli, M., Katholm, A.K., Melone, G., Ramløv, H., Ricci, C. and Santo, N. (2004) Trehalose in desiccated rotifers: a comparison between a bdelloid and a monogonont species. *Comparative Biochemistry and Physiology A – Molecular and Integrative Physiology* **139**, 527–532.

Clegg, J.S. (1986) The physical properties and metabolic status of *Artemia* cysts at low water contents: the 'water replacement hypothesis'. pp. 169–187 *in* Leopold, A.C. (Ed.) *Membranes, metabolism and dry organisms*. Ithaca, New York, Comstock Publishing Associates.

Clegg, J.S. (2005) Desiccation tolerance in encysted embryos of the animal extremophile. *Artemia. Integrative and Comparative Biology* **45**, 715–724.

Collett, H., Shen, A., Gardner, M., Farrant, J.M., Denby, K.J. and Illing, N. (2004) Towards transcript profiling of desiccation tolerance in *Xerophyta humilis*: Construction of a normalized 11k *X. humilis* cDNA set and microarray expression analysis of 424 cDNAs in response to dehydration. *Physiologia Plantarum* **122**, 39–53.

de Tullio, M.C. and Arrigoni, O. (2003) The ascorbic acid system in seeds: to protect and to serve. *Seed Science Research* **13**, 249–260.

Dietz, K.-J. (2003) Plant peroxiredoxins. *Annual Review of Plant Biology* **54**, 93–107.

Elder, R.H., Dell'Aquila, A., Mezzina, M., Sarasin, A. and Osborne, D.J. (1987) DNA ligase in repair and replication in the embryos of rye. *Secale cereale*. *Mutation Research* **181**, 61–71.

Finkel, T. and Holbrook, N.J. (2000) Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239–247.

Foyer, C. and Halliwell, B. (1976) The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta* **133**, 21–25.

Galau, G.A., Hughes, D.W. and Dure, L. (1986) Abscisic acid induction of cloned cotton late embryogenesis-abundant (lea) mRNAs. *Plant Molecular Biology* **7**, 155–170.

Goyal, K., Tisi, L., Basran, A., Browne, J., Burnell, A., Zurdo, J. and Tunnacliffe, A. (2003) Transition from natively unfolded to folded state induced by desiccation in an anhydrobiotic nematode protein. *Journal of Biological Chemistry* **278**, 12977–12984.

Halliwell, B. (1987) Oxidative damage, lipid peroxidation and antioxidant protection in chloroplasts. *Chemistry and Physics of Lipids* **44**, 327–340.

Hendry, G.A.F. (1993) Oxygen and free radical processes in seed longevity. *Seed Science Research* **3**, 141–153.

Hong, T.D., Ellis, R.H., Astley, D., Pinnegar, A.E., Groot, S.P.C. and Kraak, H.L. (2005) Survival and vigour of ultra-dry seeds after ten years of hermetic storage. *Seed Science and Technology* **33**, 449–460.

Hughes, D.W. and Galau, G.A. (1987) Translational efficiency of Lea mRNAs in cotton embryos: minor changes during embryogenesis and germination. *Plant Molecular Biology* **9**, 301–313.

Illing, N., Denby, K.J., Collett, H., Shen, A. and Farrant, J.M. (2005) The signature of seeds in resurrection plants: a molecular and physiological comparison of desiccation tolerance in seeds and vegetative tissues. *Integrative and Comparative Biology* **45**, 771–787.

Koster, K.L. (1991) Glass formation and desiccation tolerance in seeds. *Plant Physiology* **96**, 302–304.

Koster, K.L. and Leopold, A.C. (1988) Sugars and desiccation tolerance in seeds. *Plant Physiology* **88**, 829–832.

Kranner, I. and Birtić, S. (2005) A modulating role for antioxidants in desiccation tolerance. *Integrative and Comparative Biology* **45**, 734–740.

Kranner, I. and Grill, D. (1996) Significance of thiol-disulphide exchange in resting stages of plant development. *Botanica Acta* **109**, 8–14.

Kranner, I., Beckett, R.P., Wornik, S., Zorn, M. and Pfeifhofer, H.W. (2002) Revival of a resurrection plant correlates with its antioxidant status. *Plant Journal* **31**, 13–24.

Kranner, I., Cram, W.J., Zorn, M., Wornik, S., Yoshimura, I., Stabenheiner, E. and Pfeifhofer, H.W. (2005) Antioxidants and photoprotection in a lichen as compared with its isolated symbiotic partners. *Proceedings of the National Academy of Sciences, USA* **102**, 3141–3146.

Laloi, C., Apel, K. and Danon, A. (2004) Reactive oxygen signalling: the latest news. *Current Opinion in Plant Biology* **7**, 323–328.

Lapinski, J. and Tunnacliffe, A. (2003) Anhydrobiosis without trehalose in bdelloid rotifers. *FEBS Letters* **553**, 387–390.

Leubner-Metzger, G. (2005) β-1,3-glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening. *Plant Journal* **41**, 133–145.

Levitt, J. (1962) A sulfhydryl-disulfide hypothesis of frost injury and resistance in plants. *Journal of Theoretical Biology* **3**, 355–391.

Mínguez, A. and de la Espina, S.M.D. (1993) Immunological characterization of lamins in the nuclear matrix of onion cells. *Journal of Cell Science* **106**, 431–439.

Obendorf, R.L. (1997) Oligosaccharides and galactosyl cyclitols in seed desiccation tolerance. *Seed Science Research* **7**, 63–74.

Oksanen, C.A. and Zografi, G. (1993) Molecular mobility in mixtures of absorbed water and solid poly(vinylpyrrolidone). *Pharmaceutical Research* **10**, 791–799.

Oliver, A.E., Leprince, O., Wolkers, W.F., Hincha, D.K., Heyer, A.G. and Crowe, J.H. (2001) Non-disaccharide-based mechanisms of protection during drying. *Cryobiology* **43**, 151–167.

Osborne, M. and Weber, K. (1987) Cytoplasmic intermediate filament proteins and nuclear lamins A, B and C share the IFA epitope. *Experimental Cell Research* **170**, 195–203.

Pammenter, N.W. and Berjak, P. (1999) A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms. *Seed Science Research* **9**, 13–37.

Parsegian, V.A. (2002) Protein–water interactions. *International Review of Cytology* **215**, 1–31.

Proctor, M.C.F. and Pence, V.C. (2002) Vegetative tissues: Bryophytes, vascular resurrection plants and vegetative propagules. pp. 207–237 *in* Black, M.; Pritchard, H.W. (Eds) *Desiccation and survival in plants: Drying without dying*. Wallingford, CABI Publishing.

Rinne, P.L.H., Kaikuranta, P.L.M., van der Plas, L.H.W. and van der Schoot, C. (1999) Dehydrins in cold-acclimated

apices of birch (*Betula pubescens* Ehrh.): production, localization and potential role in rescuing enzyme function during dehydration. *Planta* **209**, 377–388.

Rogerson, N.E. and Matthews, S. (1977) Respiratory and carbohydrate changes in developing pea (*Pisum sativum*) seeds in relation to their ability to withstand desiccation. *Journal of Experimental Botany* **28**, 304–313.

Sinniah, U.R., Ellis, R.H. and John, P. (1998) Irrigation and seed quality development in rapid-cycling brassica: soluble carbohydrates and heat-stable proteins. *Annals of Botany* **82**, 647–655.

Solomon, A., Salomon, R., Paperna, I. and Glazer, I. (2000) Desiccation stress of entomopathogenic nematodes induces the accumulation of a novel heat-stable protein. *Parasitology* **121**, 409–416.

Stacy, R.A.P. and Aalen, R.B. (1998) Identification of a sequence homology between the internal hydrophilic repeated motifs of Group 1 late-embryogenesis-abundant proteins in plants and hydrophilic repeats of the general stress protein GsiB of *Bacillus subtilis*. *Planta* **206**, 476–478.

Stacy, R.A.P., Nordeng, T.W., Culiáñez-Maciá, F.A. and Aalen, R.B. (1999) The dormancy-related peroxiredoxin antioxidant PER1, is located to the nucleus in barley embryo and aleurone. *Plant Journal* **19**, 1–8.

Steadman, K.J., Pritchard, H.W. and Dey, P.M. (1996) Tissue-specific soluble sugars in seeds as indicators of storage category. *Annals of Botany* **77**, 667–674.

Teeter, M.M., Yamano, A., Stec, B. and Mohanty, U. (2001) On the nature of a glassy state of matter in a hydrated protein: Relation to protein function. *Proceedings of the National Academy of Sciences, USA* **98**, 11242–11247.

Timasheff, S.N. (1982) Preferential interactions in protein–water co-solvent systems. pp. 70–72 *in* Franks, F.; Mathias, S.F. (Eds) *Biophysics of water*. Chichester, Wiley.

Vertucci, C.W. and Farrant, J.M. (1995) Acquisition and loss of desiccation tolerance. pp. 237–271 *in* Kigel, J.; Galili, G. (Eds) *Seed development and germination*. New York, Marcel Dekker.

Viner, R.I. and Clegg, J.S. (2001) Influence of trehalose on the molecular chaperone activity of p26, a small heat shock/α-crystallin protein. *Cell Stress and Chaperones* **6**, 126–135.

Walters, C. (1998) Understanding the mechanisms and kinetics of seed aging. *Seed Science Research* **7**, 223–244.

Walters, C. and Engels, J. (1998) The effects of storing seeds under extremely dry conditions. *Seed Science Research* **8**, 3–8.

Walters, C., Ried, J.L. and Walker-Simmons, M.K. (1997) Heat-soluble proteins extracted from wheat embryos have tightly bound sugars and unusual hydration properties. *Seed Science Research* **7**, 125–134.

Walters, C., Wheeler, L. and Stanwood, P.C. (2004) Longevity of cryogenically stored seeds. *Cryobiology* **48**, 229–244.

Walters, C., Wheeler, L.M. and Grotenhuis, J.M. (2005a) Longevity of seeds stored in a genebank: Species characteristics. *Seed Science Research* **15**, 1–20.

Walters, C., Hill, L.M. and Wheeler, L.M. (2005b) Dying while dry: kinetics and mechanisms of deterioration in desiccated organisms. *Integrative and Comparative Biology* **45**, 751–758.

Whitaker, C., Berjak, P., Kolberg, H. and Pammenter, W. (2004) Responses to various manipulations, and storage potential, of seeds of the unique desert gymnosperm, *Welwitschia mirabilis* Hook. fil. *South African Journal of Botany* **70**, 622–630.

Williams, R.J. and Leopold, A.C. (1989) The glassy state in corn embryos. *Plant Physiology* **89**, 977–981.

Wise, M.J. and Tunnacliffe, A. (2004) POPP the question: what *do* LEA proteins do? *Trends in Plant Science* **9**, 13–17.

Wolkers, W.F., McCready, S., Brandt, W.F., Lindsey, G.G. and Hoekstra, F.A. (2001) Isolation and characterization of a D-7 LEA protein from pollen that stabilizes glasses *in vitro*. *Biochimica et Biophysica Acta* **1544**, 196–206.

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