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Viability loss and free radical processes during desiccation of recalcitrant *Avicennia marina* seeds

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Abstract

At shedding, the moisture content (MC) of *Avicennia marina* (Forssk.) Vierh. propagules was 65% (fresh mass basis), and there was no significant difference in the MC of four tissues (hypocotyl, cotyledons, plumule and root primordia). Viability declined as the propagules were dried below 60% MC, so that only 40% of seeds were capable of germination at 54% MC. At 47% MC all the seeds had died. The four tissues dried at the same constant rate of 0.02 g water g dwt^{-1} h^{-1} throughout this range of MCs. There was no significant depletion of the free-radical-quenching mechanisms measured. In each tissue an organic free-radical was detected by electron paramagnetic resonance (EPR). In the plumule the amplitude of the signal increased by a further 50% at MCs where viability was lost, but there was no increase in the other tissues. There was a concurrent increase in the amount of tocopherol and the activity of superoxide dismutase in the plumule. Lipid peroxidation, assessed by the amount of thiobarbituric acid-reactive substances, also increased in advance of viability loss, suggesting that propagules were experiencing oxidative stress. However, lipid peroxidation decreased at 54–57% MC, where most seeds lost viability. The results presented are consistent with a propagule reacting to oxidative stress, but overtaken by more catastrophic physical damage.

Keywords: *Avicennia marina*, recalcitrant seeds, free radicals, tocopherol, superoxide dismutase, peroxidase

Introduction

The seeds *of Avicennia marina* are both recalcitrant and chilling sensitive (Farrant *et al*., 1993) with a life span in moist storage of 2–3 weeks (Pammenter *et al*., 1994). Ultrastructural observations (Farrant *et al*., 1988, 1992) and respiration measurements (Farrant *et al*., 1997; Greggains, 1998) show that the seeds are metabolically active at shedding and progress rapidly towards germination (Farrant *et al*., 1993). This may contribute to their intolerance to water loss that places them at the sensitive extreme of the distribution of seed desiccation tolerance across recalcitrant species (Farrant *et al*., 1988).

Oxygen radicals are formed during normal oxidative metabolism and respiratory electron transport in seeds, but these reactions are tightly controlled. During desiccation, however, these controls may become impaired (Leprince *et al*., 1999), and this can lead to an increased generation of reactive oxygen species (ROS) and associated lipid peroxidation, membrane damage and eventual cell death (Hendry, 1993; Leprince *et al*., 1993). Evidence for such metabolically induced damage was found during drying of orthodox seeds at the onset of germination, a developmental stage with high respiratory activity (Leprince *et al*., 1990; Puntarulo, 1994). Similarly, recalcitrant seeds can have high respiration rates at shedding (Espindola *et al*., 1994; Leprince *et al*., 1999), and the damage that results from drying has also been linked to oxidative stress (reviewed by Vertucci and Farrant, 1995; Smith and Berjak, 1995; Pammenter and Berjak, 1999). Increased lipid peroxidation and reduction of antioxidant defences are associated with viability loss during drying of recalcitrant seeds (Hendry *et al*., 1992; Finch-Savage *et al*., 1993, 1996; Chaitanya and Naithani, 1994; Li and Sun, 1999).

Oxidative stress resulting from unregulated metabolism is thought to occur when seeds are dried to hydration level 3 of the five hydration levels summarized in Vertucci and Farrant (1995). This hydration level covers the water potential range between –3 and –11 MPa (20–30% moisture content, fresh weight basis), at which critical moisture contents for viability loss are reached during drying

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of relatively desiccation-tolerant recalcitrant seeds, such as those from temperate regions (e.g. *Quercus robur*; Finch-Savage, 1992). However, oxidative stress may also have a role in viability loss at higher moisture contents, since drying *Theobroma cacao* seeds below a critical moisture content of approximately 50% was also associated with increasing lipid peroxidation and a decrease in enzymic protection (Li and Sun, 1999). *A. marina* seeds lose viability above 50% moisture content, and it is not known whether oxidative stress is a factor in viability loss at such high moisture contents. In the present work we monitored the presence of a stable free-radical, lipid peroxidation and enzymatic and non-enzymatic protection mechanisms against oxidative attack during desiccation-induced viability loss in *A. marina*.

Materials and methods

Freshly shed fruits of *Avicennia marina* (Forssk.) Vierh. collected in 1997 from Beachwood Nature Reserve, Durban, South Africa were transported to the UK without loss of moisture. On arrival, 4 days after shedding, the fruits were immersed in water (25°C) for 10 minutes to stimulate pericarp shedding, and undamaged seeds were then placed directly into drying experiments. The seeds were dried to the required moisture contents in a monolayer with air moving through them. The heated drying cabinet was connected to a dehumidifier to provide air at 26 ± 1°C and 25% relative humidity. Six replicate samples were used for moisture content determinations and biochemical assays, with the exception of tocopherol and lipid peroxidation where only three were available. Samples of the plumule, hypocotyl, distal tip of the hypocotyl containing the root primordia (henceforth called root primordia) and the cotyledons (segment of the outer side of the inner cotyledon) were combined from five seeds for each replicate.

Moisture content determinations and germination

Gravimetric moisture contents were determined, on five replicates of tissue from five seeds, after oven drying for 17 h at 103°C and reported on a fresh mass basis (ISTA, 1996). Germination was recorded on three replicates of 10 seeds that were half buried in wet sand with the distal region of the hypocotyl pointing downwards and kept at 25°C in 16/8 h light/dark photoperiod (irradiance 10–15 μmol m^{-2} s^{-1}). Water levels were maintained by placing the seed trays (with holes in the bottom), covered with transparent polystyrene lids, in trays of water. Germination was considered to have occurred when roots first appeared. A note was made of fungal infection during germination, and seedlings were scored for normal development (ISTA, 1996) after approximately 30 days. In normal seedlings, the root primordia typically gave rise to several roots, and a pair of primary leaves were produced within 30 days. Reduced numbers of roots, root thickening and absence of primary leaves were considered abnormal. If left significantly longer than 30 days, the damaged roots and plumule could be replaced in some abnormal seedlings.

Electron paramagnetic resonance (EPR)

Six replicate samples of fresh and dried tissue, each from five seeds, were frozen in liquid nitrogen immediately after treatment. All samples were measured on the same occasion at 150 K; the low temperature was achieved and maintained using a Bruker ER 4111VT variable-temperature unit. EPR spectra were obtained using a Bruker ER-200D EPR (X-band) spectrometer, as described by Leprince *et al.* (1990). The samples of tissue were packed in a consistent manner in 3-mm diameter quartz tubes, care being taken to position the sample reproducibly in the spectrometer cavity. The microwave frequency was set around 9.5 GHz with either 20 dB or 30 dB of nominal power. The modulation frequency was 100 kHz and the modulation amplitude was set at 1 gauss. Other parameters, including receiver gain, were adjusted to obtain the most resolved spectra.

Biochemical assays

Samples were taken from the combined tissue of five seeds and either 100 mg (cotyledon, root primordia and hypocotyl) or 50 mg (plumule) of tissue was used for each assay. Superoxide dismutase (SOD) and guaiacol peroxidase (GPOD) activity was assayed twice on each of six replicate samples. Lipid peroxidation products and tocopherol were assayed on three replicate samples.

Samples were homogenized in ice-cold 50 mM potassium phosphate buffer, pH 7.0, and centrifuged for 2 min at 13,000 *g*. The supernatant was either used immediately or frozen in liquid nitrogen and subsequently stored at –80°C until required. Total protein was determined as in Bradford (1976) and quantified against freshly prepared bovine serum albumin (BSA) standards. SOD activity was determined spectrophotometrically at 30°C by monitoring the reduction in the rate of reaction of superoxide (generated from xanthine using xanthine oxidase) and nitroblue tetrazolium (NBT) at 560 nm (Giannopolitis and Reis, 1977). The rate of reduction of NBT to its blue reduced form was linearly related to the amount of SOD added and to the volume of extract added. GPOD activity was assessed at 30°C using the method of Chance and Maehly (1955), where

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the oxidation of guaiacol to tetraguaiacol on addition of hydrogen peroxide was monitored at 470 nm.

Lipid peroxidation products were determined by the amounts of thiobarbituric acid reactive substances (TBARS) in freshly prepared extracts (see above) equated to malondialdehyde (MDA) as in Heath and Packer (1968) and quantified using 1,1,3,3-tetraethoxypropane as a standard. Butylated hydroxytoluene (2% w/v) was included routinely in the reaction mixture to eliminate artefactual peroxidative damage to the samples during processing, as described by Hendry *et al.* (1992). Tocopherol was extracted by grinding seed tissue in ice-cold 2:1 hexane:aqueous ascorbate (10% w/v) (Kunert and Ederer, 1985). After centrifuging at 13,000 *g* for 30 s, the supernatant was removed and passed through a 5 µm filter (Millipore, Massachusetts, USA). Immediately prior to measurements, the supernatant was evaporated to dryness under nitrogen and the tocopherol was redissolved in methanol. Analytical separation of α- and γ-tocopherol was achieved using the reverse-phase high-performance liquid chromatography (HPLC) method modified from McMurray and Blanchflower (1979), but using 100% methanol in the mobile phase as described by Hendry *et al.* (1993). Detection was by fluorescence-excitation (296–330 nm) and estimated against tocopherol standards (Sigma–Aldrich, Poole, UK).

Results and discussion

At shedding, the moisture content of the cotyledons and hypocotyl was 65%, root primordia 67%, plumule 63% and the whole seed 65%. All the tissues dried at the same constant rate of 0.02 g water g dwt^{-1} h^{-1} throughout the range of moisture contents where viability was lost. Thus, moisture content of tissues did not deviate by more than 2% from that of the whole seed. For consistency, all data are plotted against whole-seed moisture content for reference to viability loss. Viability declined as the seeds dried below 60% moisture content, so that only 40% of seeds were capable of germination at 54% moisture content (Fig. 1). All seeds died by 47% moisture content. Over this range of moisture contents, drying was not associated with any significant change in dry weight or total protein content in the tissues (data not shown).

The resilience of *A. marina* propagules to fungally mediated degradation declines with time (Calistru *et al.*, 2000), and seeds usually begin to die within 10 d after harvest, even when stored moist (Pammenter *et al.*, 1994). The propagules are damaged severely by only slight dehydration, and fungi could exacerbate this damage. However, this was unlikely to be a complicating factor in the present work as seeds put to dry on arrival all died in the first 50 hours of drying. In contrast, no viability loss occurred in parallel storage experiments during the first 5 days of moist-storage after arrival in the UK (data not presented). Indeed, more than 50% of the propagules remained viable after 20 days in moist storage (data not shown). Compared with observations made on propagules in other years (e.g. Pammenter *et al.*, 1994), this unusual longevity was attributed to the comparatively low level of fungal growth observed on the propagules during moist storage. As viability declined during drying, the percentage of seeds with fungi increased during the germination test (Fig. 1). However, this enhanced fungal growth may result from an increased nutrient supply due to solute leakage from tissue damaged during drying.

Free radicals and lipid peroxidation

In common with seeds of other recalcitrant species, respiratory metabolism in *A. marina* continues at a high rate following shedding and then during drying to lethal moisture contents (*A. marina*, Farrant *et al.*, 1992, 1997; *Araucaria angustifolia*, Espindola *et al.*, 1994; *Castanea sativa*, Leprince *et al.*, 1999; reviewed by Pammenter and Berjak, 1999). There is a strong general argument in support of a causal role for metabolic disruption in the desiccation sensitivity of

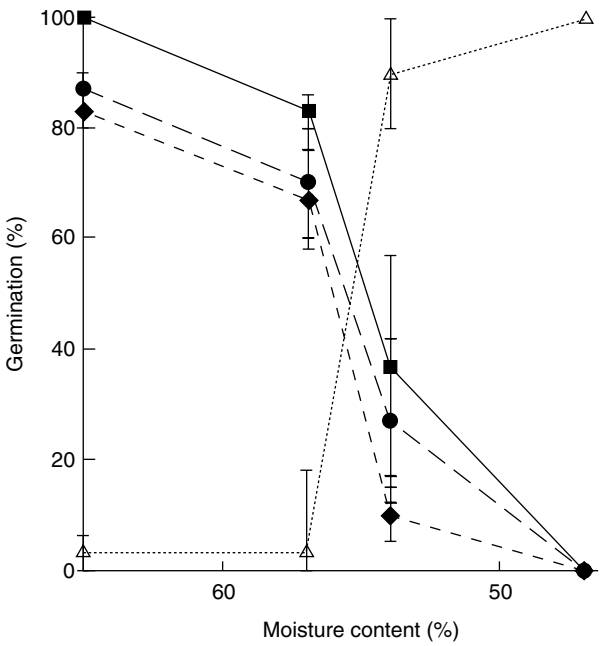

Figure 1. Percentage germination (■), percentage normal roots (●) and seedlings (◆) and percentage of seeds on which fungi proliferated (△) in germination tests of *A. marina* propagules dried to different moisture contents at 26°C. Seeds dried to 60 and 50% moisture content in approximately 13 and 47 hours, respectively.

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recalcitrant seeds (Leprince *et al.*, 1999). If this is the case in *A. marina*, a significant increase in lipid peroxidation and evidence of stable free-radical accumulation in advance of viability loss during drying would be expected.

The plumule, hypocotyl and distal tip containing root primordia are comparatively large in *A. marina* and can be assayed separately. In many other species these tissues must be assayed as a whole embryonic axis, even though their functions are quite distinct. In all four tissues an organic free-radical was detected using EPR, but there was no evidence that it accumulated in the cotyledons, hypocotyl or root primordia as viability was lost during drying (Fig. 2a). However, in the plumule the amplitude of the EPR signal was greater than that in the other tissues, and it increased by a further 50% at moisture contents where viability was lost, after which it subsequently declined. At these damaging moisture contents there was considerable proliferation of fungi during the germination tests (Fig. 1). If this infection was present during drying, free radicals might have been generated in the tissues as part of the plant–pathogen interaction (Goodman, 1994), and these could have contributed to the EPR signal recorded.

In the axes of *Aesculus hippocastanum,* a twofold increase in EPR signal was recorded around the mid-point of viability loss in the seed population during desiccation (Wood *et al.*, 1997). In that species the increase in EPR signal was associated with an increase in lipid peroxidation, as it was in *Q. robur* (Hendry *et al.*, 1992). In *A. marina*, the extent of lipid peroxidation, measured as the amount of thiobarbituric acid reactive substances (TBARS; Fig. 2b), was greater in the cotyledons (196 nmol g^{-1} dwt) at the start of drying than in the hypocotyl or root primordia (52 and 53 nmol g^{-1} dwt, respectively). In the cotyledon and root primordia, there was an initial increase in TBARS during drying when most seeds were still viable, but the amounts in all tissues decreased in the moisture content region where most seeds lost viability and remained relatively constant thereafter. The small size of the plumule made estimation of TBARS impossible in that tissue.

Hendry *et al.* (1992) reported little change in lipid peroxidation in the cotyledons of *Q. robur* during desiccation, but there was a continued increase in the axes even after the point where viability was lost. As with *A. marina*, lipid peroxidation increased during the early stages of moisture loss in *Shorea robusta* (Chaitanya and Naithani, 1994). However, in *S. robusta* accumulation rates slowed down at the point where viability was lost but did not subsequently decline. Axes of jackfruit (*Artocarpus heterophyllus*) and tea (*Camellia sinensis*) showed little change in lipid peroxidation during rapid drying (Chandel *et al.*, 1995). However, the rapid drying of excised axes may have allowed water to be removed fast enough to prevent oxidative damage from occurring.

Defences against free radical damage

There is a range of antioxidant and enzymic mechanisms that normally quench free-radical accumulation in seeds (Hendry, 1993; Leprince *et al.*, 1993; Côme and Corbineau, 1996), but these mechanisms may not be adequate during desiccation stress. For example, in the axes of *Q. robur,* the accumulation of a stable free-radical and associated lipid peroxidation during drying appeared to be linked to a decrease in enzymatic and antioxidant protection (Hendry *et al.*, 1992). Such a decrease in protection also occurred in seeds of other recalcitrant species during drying (Chaitanya and Naithani, 1994;

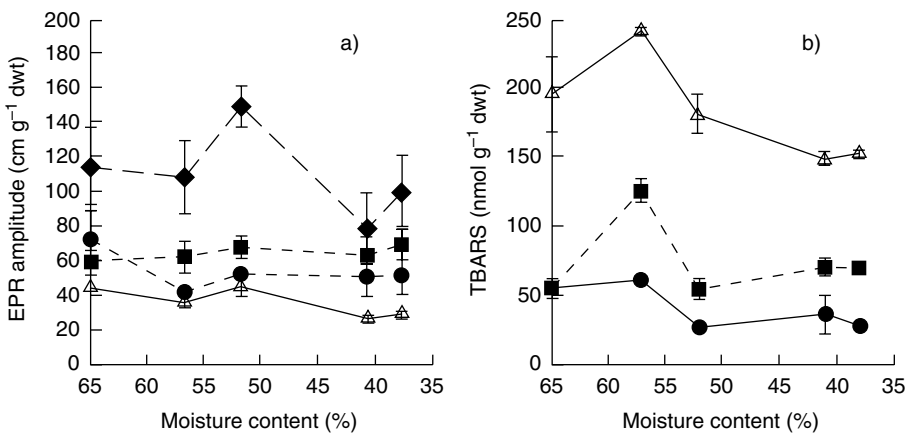

Figure 2. Changes in (a) EPR signal amplitude and (b) lipid peroxidation measured as the amounts of thiobarbituric acid reactive substances (TBARS) during seed drying. (△) Cotyledon; (◆) plumule; (●) hypocotyl; (■) root primordia.

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Li and Sun, 1999), and this may have contributed to their desiccation sensitivity. However, there is a need for caution in the interpretation of measurements associated with damage and with protective mechanisms during viability loss, as it is not possible to determine what contribution is made from the increasing fraction of dead seeds within the population (Hendry, 1993). Even within individual seeds, different tissues can survive different degrees of desiccation (e.g. Leprince *et al*., 1999; Li and Sun, 1999), and so the seed may contain an increasing proportion of dead tissue during desiccation.

In *A. marina* the amounts of the lipid-soluble antioxidant tocopherol and the aqueous-phase free-radical scavenging enzymes SOD and GPOD were measured. The response of all three to drying differed among seed tissues (Fig. 3). The amount of total tocopherol and the activity of SOD in plumules increased (Figs 3a, b) as free-radicals accumulated (Fig. 2a) and seed viability rapidly declined (Fig. 1) below 60% moisture content. The measured amounts and activities then declined. The increase in tocopherol occurred for both α- and γ-tocopherol (data not shown, γ:α ratio 0.08), suggesting that the active α form was increasing as a response to stress and that its precursor, γ-tocopherol, was being synthesized to maintain these levels. In soybean embryonic axes the cellular content of α-tocopherol was similarly adjusted as a response to oxidative stress during imbibition/early stages of germination (Simontacchi *et al*., 1993; Puntarulo, 1994). In contrast, α-tocopherol was reported to decline in axes of recalcitrant *Q. robur* seeds (Hendry *et al*., 1992) as seed viability was lost. In *A. marina* the γ:α tocopherol ratios (0.1) in the hypocotyl and root primordia were similar to that in the plumule, but much lower than that in the cotyledons (γ:α tocopherol ratio approximately 1). The ratios changed little during drying (data not shown). The greater ratio of γ:α tocopherol in the cotyledons may have been due to less stress in the tissue that comprises these large, fleshy organs, and/or the precursor may have been synthesized in preparation for the future photosynthetic role of this tissue.

Hendry *et al*. (1992) reported higher SOD activity in the cotyledons of *Q. robur* compared with the axes, and the activity rose twofold in the cotyledons during desiccation, whereas it declined in the axes. In a similar fashion SOD activity in *S. robusta* seeds peaked sharply before viability was lost and then became undetectable (Chaitanya and Naithani, 1994). However, in *A. marina*, SOD activity did not increase as lipid peroxidation increased in the root primordia at the time of maximum viability loss. In the other tissues of *A. marina* seeds, the activity of SOD and the amount of total tocopherol changed little during drying.

GPOD activity was initially an order of magnitude greater in the root primordia than in other tissues of *A. marina* (Fig. 3c); it then decreased by 50% during drying, with the largest decrease occurring between 57 and 53% moisture content, concurrent with rapid viability loss. The other tissues had very low activity that remained unchanged during drying. In *Q. robur* GPOD activity was also much greater in the axes than the cotyledons (Hendry *et al*., 1992). In *A. marina* the high level of root primordial GPOD activity could be related to germination. For example, germinating wheat grains had a 19-fold increase in GPOD within 72 h of imbibition (Cakmak *et al*., 1993). The subsequent reduction in GPOD activity on drying may therefore be a result of germination arrest rather than damage.

In summary, the viability of *A. marina* propagules was lost at high moisture contents during drying.

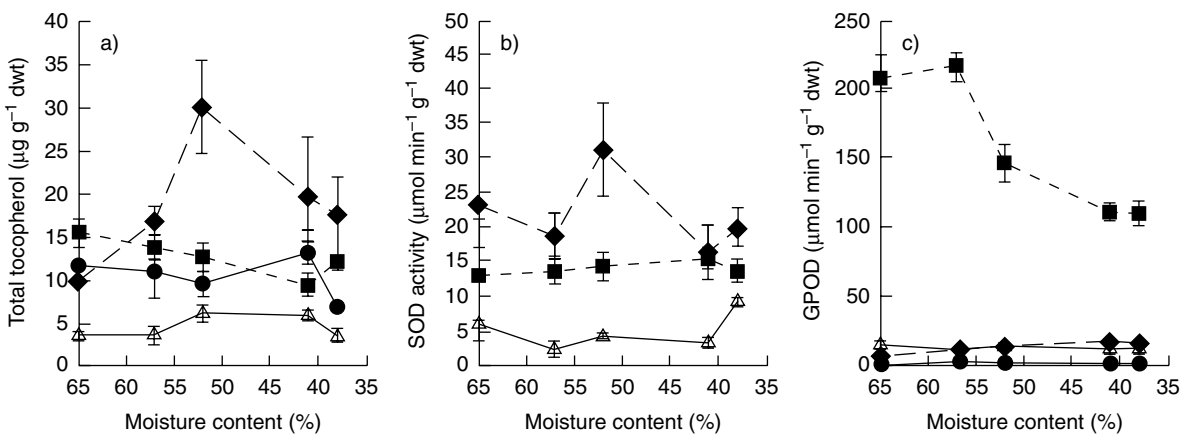

Figure 3. Changes in (a) the amount of total tocopherol and the activities of (b) superoxide dismutase and (c) peroxidase (GPOD). (△) Cotyledon; (◆) plumule; (●) hypocotyl; (■) root primordia.

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Lipid peroxidation increased more than twofold in the root primordia and also increased in the cotyledons as propagules dried to 57% moisture content, suggesting that they were experiencing oxidative stress. These effects were not associated with a significant depletion of the free-radical quenching mechanisms measured. On the contrary, accumulation of the stable free-radical in the plumule was concurrent with an increase in both the amount of tocopherol and the activity of SOD. However, the major reduction in viability occurred in a narrow moisture content range between 57 and 54%. In this range lipid peroxidation decreased. We interpret these results as propagules initially reacting to oxidative stress during drying, but then overtaken by more catastrophic events. At such high moisture contents, the most likely event initiating the degradation of the embryo cells themselves would be mechanical damage resulting from a reduction in cell volume (Pammenter and Berjak, 1999).

A. marina propagules remain susceptible to mechanical damage at the end of development because they are highly vacuolated (Farrant *et al*., 1997) and de-differentiation of subcellular components is limited (Farrant *et al*., 1992). In more tolerant species insoluble reserves accumulate in seeds and these provide mechanical support. This does not occur in *A. marina* propagules, which accumulate large quantities of sugars, including sucrose and stachyose, instead (Farrant *et al*., 1993). These characteristics enable the propagules to germinate rapidly upon shedding and to withstand osmotic stress, a likely adaptation to the moist estuarine environment the species colonizes. Similar to seeds from other tropical wetland species, abscisic acid concentrations do not increase with drying, and dehydrin proteins do not accumulate during development (Farrant *et al*., 1996). In contrast, these proteins accumulate during development in seeds of more desiccation-tolerant species (Farrant *et al*., 1996). DNA integrity can also decrease during viability loss at high moisture contents (Pammenter and Berjak, 1999). In *A. marina*, DNA replication, repair and protein synthesis are markedly compromised early during dehydration (Boubriak *et al*., 2000).

It is likely that a metabolic imbalance will develop during cellular disruption by catastrophic mechanical damage and this will compound oxidative damage. In addition, further damage is possible from that imbalance due to the release of harmful fermentation products, such as ethanol and acetaldehyde, as observed in *Q. robur* (Finch-Savage *et al*., 1993). However, measurements made in the present work suggest that oxidative damage is not the major cause of viability loss during drying at these high moisture contents. There was also no consistent evidence to indicate that oxidative damage was a contributing factor to pre-mortem deterioration during moist storage of *A. marina* (Greggains *et al*., 2000). Events recorded here as viability was lost during desiccation of *A. marina* were different from those in more tolerant recalcitrant seeds that die at lower moisture contents (e.g. *Q. robur*; Hendry *et al*., 1992). This is consistent with the concept of discrete water potential steps in the acquisition of desiccation tolerance (Farrant and Walters, 1998; Walters, 1999). These steps are related to the different desiccation stresses encountered during the course of seed drying. *A. marina* propagules appear unable to survive the likely first step of a reduction in cell volume without irreparable damage.

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