OXFORD JOURNALS
OXFORD UNIVERSITY PRESS

REVIEW

From *Avicennia* to *Zizania*: Seed Recalcitrance in Perspective

PATRICIA BERJAK* and N. W. PAMMENTER

School of Biological & Conservation Sciences, University of KwaZulu-Natal, Durban, South Africa

• *Background* Considered only in terms of tolerance of, or sensitivity to, desiccation (which is an oversimplification), orthodox seeds are those which tolerate dehydration and are storable in this condition, while highly recalcitrant seeds are damaged by loss of only a small proportion of water and are unstorable for practical purposes. Between these extremes, however, there may be a gradation of the responses to dehydration – and also to other factors – suggesting perhaps that seed behaviour might be best considered as constituting a continuum subtended by extreme orthodoxy and the highest degree of recalcitrance. As the characteristics of seeds of an increasing number of species are elucidated, non-orthodox seed behaviour is emerging as considerably more commonplace – and its basis far more complex – than previously suspected.
• *Scope* Whatever the post-harvest responses of seeds of individual species may be, they are the outcome of the properties of pre-shedding development, and a full understanding of the subtleties of various degrees of non-orthodox behaviour must await the identification of, and interaction among, all the factors conferring extreme orthodoxy. Appreciation of the phenomenon of recalcitrance is confounded by intra- and interseasonal variability across species, as well as within individual species. However, recent evidence suggests that provenance is a pivotal factor in determining the degree of recalcitrant behaviour exhibited by seeds of individual species. Non-orthodox – and, in particular, recalcitrant – seed behaviour is not merely a matter of desiccation sensitivity: the primary basis is that the seeds are actively metabolic when they are shed, in contrast to orthodox types which are quiescent. This affects all aspects of the handling and storage of recalcitrant seeds. In the short to medium term, recalcitrant seeds should be stored in as hydrated a condition as when they are shed, and at the lowest temperature not diminishing vigour or viability. Such hydrated storage has attendant problems of fungal proliferation which, unless minimized, will inevitably and significantly affect seed quality. The life span of seeds in hydrated storage even under the best conditions is variable among species, but is curtailed (days to months), and various approaches attempting to extend non-orthodox seed longevity are discussed. Conservation of the genetic resources by means other than seed storage is then briefly considered, with detail on the potential for, and difficulties with, cryostorage highlighted.
• *Conclusions* There appears to be little taxonomic relationship among species exhibiting the phenomenon of seed recalcitrance, suggesting that it is a derived trait, with tolerance having been lost a number of times. Although recalcitrant seededness is best represented in the mesic tropics, particularly among rainforest climax species, it does occur in cooler, drier and markedly seasonal habitats. The selective advantages of the trait are considered.

Key words: Cryostorage, desiccation sensitivity, ecology, evolution, genetic resources, hydrated storage, metabolic activity, mycoflora, recalcitrant seeds.

INTRODUCTION

Seed storage is imperative, not only to provide good-quality planting material from season to season, as well as interseasonal food reserves and feedstock, but also in the long term as base and active collections conserving genetic resources. Given appropriate facilities, storage for all these purposes can readily be achieved – but only if the seeds exhibit orthodox post-harvest physiology (Roberts, 1973). Developing orthodox seeds acquire the ability to tolerate desiccation relatively early, preceding the final developmental phase on the parent plant, i.e. maturation drying, by some time (Bewley and Black, 1994; Vertucci and Farrant, 1995; Kermode and Finch-Savage, 2002). A spectrum of protective mechanisms and processes has been identified, which together confer the property of desiccation tolerance (reviewed by Pammenter and Berjak, 1999; Berjak, 2006), although the modes of their operation and interaction remain largely conjectural (Berjak *et al.*, 2007). As long as orthodox seeds are of high quality after

harvest, the period for which they can be stored without deterioration is predictable under defined conditions of low temperature and relative humidity that will maintain low water (moisture) content. Storage longevity of orthodox seeds increases logarithmically with decreasing water content (Ellis and Roberts, 1980), although there appear to be limits of dehydration below which no further advantage is gained (e.g. Ellis *et al.*, 1990*b*), and, in fact, if exceeded, may be damaging (e.g. Walters, 1998; Walters and Engels, 1998; reviewed by Berjak, 2006) [note, however, that there is not unanimity about this (e.g. Hong *et al.*, 2005)]. Even under ideal conditions, however, orthodox seeds have finite life spans, although these are a matter of many years to decades or centuries, depending on the species (Walters *et al.*, 2005).

Recalcitrant seeds, in contrast, are characterized by post-harvest life spans of the order of days to months, or, for temperate species, perhaps a year or two, as long as such seeds will tolerate low (not sub-zero) temperatures (e.g. Chin and Roberts, 1980). Besides producing short-lived seeds, many of the recalcitrant-seeded species are threatened

* For correspondence: E-mail berjak@ukzn.ac.za

by overexploitation, indiscriminate harvesting and habitat loss (Berjak, 2005). Hence, understanding the phenomenon of seed recalcitrance, and consequently developing sound conservation practices for species producing such seeds, is of major scientific and practical importance. The importance of the conservation of such genetic resources is underscored in Target viii of the Global Strategy for Plant Conservation (of the Convention on Biodiversity) which calls for 60 % of all threatened species to be in accessible *ex situ* collections by 2010.

RECALCITRANT SEEDS ARE ALWAYS DESICCATION SENSITIVE

Recalcitrant seeds remain sensitive to dehydration both during development and after they are shed from the parent plant. However, the range of water concentrations of the embryonic axes when the seeds are shed varies markedly among species [from approx 0.4 g g^{-1} dry mass to extremely high values, e.g. 4.4 g g^{-1} (Chin and Roberts, 1980; Berjak and Pammenter, 2004)]. Some decline in water content prior to shedding has been recorded for seeds of several temperate species, e.g. *Acer pseudoplatanus* (Hong and Ellis, 1990), *Aesculus hippocastanum* (Tompsett and Pritchard, 1993) and *Quercus robur* (Finch-Savage and Blake, 1994), and also some of tropical/sub-tropical provenance, e.g. *Machilus thunbergii* (Lin and Chen, 1995) and *Ekebergia capensis* (our unpublished data), leading to the suggestion that a measure of desiccation tolerance might be acquired during development. However, this apparent decline in water content may result from the continuing accumulation of dry mass which characterizes recalcitrant seed development (Finch-Savage and Blake, 1994), with no further importation of water (Berjak and Pammenter, 2000). Nevertheless, even for those seeds shed at axis water contents towards the lower end of the range, further dehydration is deleterious, indicating that at least some of the mechanisms necessary for complete desiccation tolerance are not entrained. In contrast, water concentrations of axes of recalcitrant seeds of most of the tropical/sub-tropical species which have been investigated lie at the high end of the range (≥ 1.5 g g^{-1}), and the axes are damaged after only slight dehydration – particularly if water loss is slow (see below). This indicates that few, if any, of the mechanisms putatively affording orthodox seeds tolerance to desiccation are operational. Although the degree of recalcitrance may be difficult to quantify (Pammenter *et al.*, 2002*a*), seeds such as those of *Avicennia marina* (Farrant *et al.*, 1993*a, b*) and *Hopea* spp. (e.g. Chin and Roberts, 1980; Sunilkumar and Sudhakara, 1998) are considered to be highly recalcitrant. In this regard, though, studies on *Zizania* spp. indicated that, despite differences in water concentrations at which desiccation damage occurred in embryos at different developmental status, all equated to a common water activity value (a_w) of 0.90 (Vertucci *et al.*, 1995). These observations may be related to a common spectrum of metabolic events that are impaired, which has been suggested to occur in particular water potential ranges, as recalcitrant seeds are dehydrated (Vertucci and Farrant, 1995).

THE WIDESPREAD OCCURRENCE OF SEED RECALCITRANCE

In 1980, Chin and Roberts published the first list of species recorded as producing recalcitrant seeds, and collated what was then known about their post-harvest behaviour. In general, the species those authors listed produce seeds important in agroforestry (e.g. species of *Quercus* and *Shorea*), as crops for seed or fruit consumption (e.g. *Castanea* spp. and *Artocarpus heterophyllus*), or commodity production (e.g. *Elaeis guineensis*, *Hevea brasiliensis* and *Theobroma cacao*). Since then, the seed biology of a range of lesser known, generally tropical/sub-tropical tree species has been studied, revealing many more to produce recalcitrant (or otherwise non-orthodox) seeds (reviewed by Berjak and Pammenter, 2004; Sacandé *et al.*, 2004; Flynn *et al.*, 2006). Over the past year, our screening programme focusing on southern African species has revealed that seeds of at least 17 herbaceous geophytic amaryllids are recalcitrant – which is unusual in indicating a familial trait (see below) – while seeds of a further 13 unrelated tree species have also proved to be recalcitrant (Erdey *et al.*, 2007). While the majority of the species producing recalcitrant seeds are endemic to the humid tropics/sub-tropics, such seeds are also produced by a small spectrum of (mainly) trees of temperate provenance, while certain dryland species (Danthu *et al.*, 2000; Gaméné *et al.*, 2004; Pritchard *et al.*, 2004*b*) have also been identified as being recalcitrant seeded. Current and ongoing studies have also identified seeds of some cycads (exemplified by *Encephalartos* spp.) to be recalcitrant (Woodenberg *et al.*, 2007), while Daws *et al.* (2007) reported a considerable incidence of desiccation-sensitive seeds among the palms.

RECOGNITION AND CATEGORIZATION OF SEED BEHAVIOUR

Until relatively recently, seed screening for non-orthodox behaviour was based initially on water content of ostensibly mature seeds at shedding, followed by ascertaining viability retention following the sequential removal of increasing proportions of tissue water (Hong and Ellis, 1996; IPGRI/DFSC, 2004). However, because frequently only small numbers of recalcitrant seeds are able to be harvested at any one time, Pritchard *et al.* (2004*a*) developed the 100-seed test which proved to be a reliable indicator of the desiccation responses of seeds of eight tropical palm species. The usefulness of the 100-seed test has been confirmed in our laboratory, but, whenever seed numbers allow it, we screen seeds using a modification of the IPGRI/DFSC protocol (2004) which includes an assessment of viability retention in storage at a range of temperatures, at each of the stages of dehydration. This approach is time consuming, but gives a reliable indication of whether seeds are orthodox, recalcitrant or fall somewhere in between orthodoxy and recalcitrance.

However, a variety of studies (reviewed by Daws *et al.*, 2006*a*) have indicated that there could be a correlation among recalcitrance, some seed characteristics and variables characterizing individual habitats. Acting on these

indications, and analysing several of the parameters across 104 tropical forest species from 37 families deriving from Panamá, Daws *et al.* (2006*a*) developed a reliably predictive model based on just two of the traits, seed mass and seed coat/coverings ratio (SCR, i.e. the ratio of seed coverings:mass). Desiccation sensitivity was found to be significantly related to relatively low SCRs, typified by large seed size coupled with thin coverings. The predictive value of the SCR model was convincingly demonstrated when Daws *et al.* (2006*a*) applied it further to seeds of 28 African species and ten species from Europe, showing in all cases that the prediction was in agreement with published data on the responses of the seeds to dehydration.

While the original categorization of seeds according to their post-harvest (storage) responses embodies the idea of two distinct groupings – orthodox and recalcitrant (Roberts, 1973) – a further category, describing post-harvest physiology as being intermediate, was later introduced (Ellis *et al.*, 1990*a*). Intermediate storage behaviour implies that the seeds are shed at relatively high water concentrations, but will withstand considerable dehydration, although not to the extent tolerated by orthodox seeds (Hong and Ellis, 1996). Although categorizing seeds into the three distinct groupings is useful, we favour an open-ended continuum of seed behaviour, subtended by extreme orthodoxy at the one end and the highest degree of recalcitrance at the other (Berjak and Pammenter, 1997, 2004; Pammenter and Berjak, 1999; Sun, 1999; Kermode and Finch-Savage, 2002). The continuum concept accommodates the marked variability occurring both between and within species, and is supported by recent evidence (explored further below) indicating that provenance has a significant effect on seed development and the degree of dehydration tolerated for individual species (Daws *et al.*, 2004*b*, 2006*b*). In terms of the continuum concept, it is considered pertinent that even seeds categorized as orthodox are not equally desiccation tolerant (Walters, 1998).

VARIABILITY AMONG RECALCITRANT SEEDS

Although it is an over-simplification, desiccation sensitivity is generally accepted as the obvious feature identifying seeds of a species as being recalcitrant. Nevertheless, there are marked differences in the degree of dehydration that recalcitrant seeds of individual species will tolerate, although the lowest water content survived depends on other parameters, especially the rate at which water is lost (see below). Comparisons of published data on individual species are often not helpful, because of the differing conditions under which dehydration was carried out. However, when recalcitrant seeds of three unrelated species, a gymnosperm (*Araucaria angustifolia*), a dicotyledonous vine (*Landolphia kirkii*) and an herbaceous monocot (*Scadoxus membranaceous*), were dehydrated under identical conditions, a similar pattern of ultrastructural events terminating in cell lysis was recorded as occurring at markedly different water concentrations (Farrant *et al.*, 1989). What was also a significant observation was that the rate at which the seeds of the three species lost water was inversely

related to the water concentration at which viability was lost. This highlights an important generalization, first noticed in work with *A. marina* (Berjak *et al.*, 1984), i.e. that the more rapidly water can be lost, the lower is the water content reached before intracellular damage becomes limiting. (The important ramifications of this will be elaborated below.)

Differences in the lowest 'safe' water content which recalcitrant seeds will withstand are not confined to disparate genera, but have also been noted for different species of individual genera. For example, *Quercus alba* seeds are more desiccation sensitive than those of *Q. nigra* (Connor and Bonner, 1996), and there are differences between seeds of species of *Baccaurea* (Normah *et al.*, 1997). A thought-provoking finding is that seeds of different species of a single genus may be differently categorized, as exemplified by species of *Acer* and *Coffea* (Hong and Ellis, 1990), and substantiated for *Coffea* spp. by Eira *et al.* (1999) who described *C. liberica* seeds as being the least desiccation tolerant, while those of *C. racemosa* were relatively the most tolerant. Most interesting, though, are the more recent data (explored in more detail below) indicating that seeds of *A. hippocastanum* (Daws *et al.*, 2004*b*) and *A. pseudoplatanus* (Daws *et al.*, 2006*b*) from different provenances differ in their reponse to dehydration. Also relevant in the context of provenance are the findings of Dussert *et al.* (2000) that the relative desiccation sensitivity of seeds of different species of *Coffea* appears to be related to the mean number of dry months typifying each habitat.

A further characteristic of recalcitrant seeds is the fact that they are metabolically active when they are shed. However, the type and intensity of metabolism differ among recalcitrant seeds of different species, depending on the developmental status and water concentration at shedding. To explain this, it must be appreciated that unlike the situation in orthodox seeds, there is no cessation of metabolism [although the rate may slow down as recalcitrant seeds reach maturity (reviewed by Finch-Savage, 1996)]. Instead, developmental events progress, without any outward signs, into those of germination, without an exogenous water supply (Berjak *et al.*, 1989). As discussed by those authors, in some cases germination will ensue in a matter of days after shedding; seeds of some species may be poised for immediate germination; while seeds of yet other species are shed with embryos still having to undergo considerable pre-germination development. These differences have marked effects on the degree of dehydration seeds will tolerate, thereby contributing to unpredictable variability. For example, Lin and Chen (1995), working with *M. thunbergii*, showed that developing seeds lost viability within 30 d when dried at 73 % relative humidity and 25 °C, while those that were mature were able to tolerate a 19 % loss of water (presently recalculated from the data of those authors) before germinability declined. Differing degrees of desiccation sensitivity have been similarly correlated with embryo/seed developmental status for *L. kirkii* and *Camellia sinensis* (Pammenter *et al.*, 1991; Berjak *et al.*, 1992; 1993*a*, *b*). It appears generally that for recalcitrant seeds of most species, the least desiccation-sensitive stage occurs when the metabolic rate is lowest, which

usually (but not invariably) coincides with natural shedding. However, desiccation sensitivity increases markedly as germinative metabolism progresses, macroscopically imperceptibly, to the stage of the onset of mitosis and extensive vacuolation of the embryo cells (Farrant *et al.*, 1986; Berjak *et al.*, 1989). This inexorable progress of germinative metabolism – which occurs with no requirement for additional water – sooner or later (depending on the species) will culminate in radicle protrusion, and is one of the major factors hampering short- to medium-term storage of recalcitrant seeds, as will be discussed later.

Other confounding issues include the fact that axes and storage tissues seldom (if ever) have the same water concentrations, as shown for *A. hippocastanum* (Tompsett and Pritchard, 1993). Working with *Araucaria hunsteinii*, Pritchard *et al.* (1995) reported that in these gymnospermous seeds too, there is uneven water distribution between the component tissues. Most frequently, axes are at higher water contents, and are more desiccation sensitive, than the cotyledons, e.g. as shown for *Q. robur* (Finch-Savage *et al.*, 1992), *M. thunbergii* (Lin and Chen, 1995) and *T. cacao* (Li and Sun, 1999), and for seeds of numerous screened African species [e.g. *Dovyalis caffra* (Erdey and Berjak, 2004); *E. capensis* (Erdey *et al.*, 2004); and *Warburgia salutaris* (Kioko *et al.*, 2004)]. However, to confound the issue, in seeds of *Castanea sativa*, cotyledons have been reported to be more sensitive to dehydration than the axes (Leprince *et al.*, 1999).

A further contribution to the variability among seeds of individual species is that their characteristics differ both intra- and interseasonally. Intraseasonal variation includes differing water contents of the component tissues of ostensibly mature seeds depending on the time of harvest and, even when harvested simultaneously, there are usually marked differences in axis water contents among individual seeds (Berjak and Pammenter, 1997). An additional feature that has been consistently observed for a variety of species is the poor quality of seeds produced late in the season, which are more often than not severely fungally infected. In this regard, an enhanced rate of deterioration upon dehydration has been reported for late-harvested seeds of *Machilus kusanoi* (Chien and Lin, 1997). It has also been observed that late-season fruits of *A. marina* and *Syzygium cordatum* have a tendency either to abort or not to abscise.

It is probable that at least the poor quality of late-season seeds may be explained in terms of the cumulative heat sum during development: Daws *et al.* (2004*b*) monitored *A. hippocastanum* seed development along a latitudinal gradient, and reported that the greater the cumulative heat sum, the more robust, further developed and less desiccation sensitive were the seeds. Although those observations were made along a North–South gradient in Europe over the flowering/fruiting season, a similar interpretation for poor seed quality can be applied to fruits and seeds produced in the latter part of the season in non-equatorial zones. Temperatures decline as the summer wanes and, accompanied by shortening day-lengths, results in a suboptimal heat sum to late-developing fruits. This is proposed to influence fruit and seed development negatively,

resulting in their poor quality, which includes lowered resistance to fungal establishment. (This proposal is based on the assumption that the late-season fruits are derived from flowers produced late in the season, which our casual observation suggests to be the case for *A. marina*.)

Interseasonal variation among seeds of the same species may be similarly rationalized, but in some cases there are remarkable differences. For example, *C. sinensis* seeds harvested in consecutive seasons from the same provenance showed embryonic axis water concentrations as disparate as $2{\cdot}0 \pm 0{\cdot}3$ to $4{\cdot}4 \pm 2{\cdot}4 \mathrm{~g}$ g^{-1} for harvests in different years (Berjak *et al.*, 1996). As mentioned above, recalcitrant seeds generally will entrain germination at the shedding water content, and thus will germinate under storage conditions not allowing dehydration. However, in one particular season, *Q. robur* seeds harvested from the same tree as previously and subsequently had lower than usual water contents, and did not germinate in storage (Finch-Savage *et al.*, 1993; Finch-Savage, 1996), constituting a marked example of interseasonal differences. Similarly, Pritchard *et al.* (1999) have recorded interseasonal differences in germination capacity after a period of dormancy-breaking chilling for seeds of *A. hippocastanum*, which those authors ascribed to mean temperature during seed filling.

While interseasonal differences in heat sum may be a feature of temperate climatic zones, they are less marked in the tropics. Consequently, differing effects of seed dehydration on neotropical rain forest species in Mexico (del Carmen Rodriguez *et al.*, 2000) and interseasonal differences in a variety of traits of *Euterpe edulis* seeds from Brazil (Martins *et al.*, 2000) are rather more difficult to explain. However, in the case of nine species of *Coffea* all originating within tropical Africa, Dussert *et al.* (2000) found no significant correlation between the duration of seed development and the level of desiccation tolerance, but were able to demonstrate a significant inverse correlation between desiccation sensitivity and the mean number of dry months after seed-shed in the various habitats.

Daws *et al.* (2004*a*) have made an interesting observation that could explain some of the intraharvest variability in desiccation sensitivity often observed. They showed that in a collection of *Vitellaria paradoxa* seeds, the fresh mass of which varied from 4 to 10 g, the smaller seeds dried faster than the larger ones, and only the larger seeds survived the drying. This survival was not because of greater tolerance to desiccation, but because the large seeds took longer to dry and so were at a higher water content (above the lethal limit under the conditions used) when compared with the smaller seeds at particular sampling intervals. The apparent variability in sensitivity within a seed lot may be due largely to the variation in seed size within the sample.

FACTORS INVOLVED IN THE ACQUISITION OF DESICCATION TOLERANCE

A suite of mechanisms and processes, under complex genetic control which is still not fully understood (see below), has been implicated in the acquisition and

maintenance of desiccation tolerance in orthodox seeds. The individual components of the suite, and their interactions, have been extensively reviewed (Pammenter and Berjak, 1999; Kermode and Finch-Savage, 2002), and will thus only be outlined here, with information where further data have been forthcoming, or contradictory opinions expressed, particularly in relation to the situation in recalcitrant seeds.

Intracellular physical characteristics

The first set of major components of the suite involve intracellular physical features (Pammenter and Berjak, 1999). These include minimization of vacuolation; protection of the integrity of the DNA; and orderly dismantling of cytoskeletal elements. Both orthodox and recalcitrant seeds (except those of *A. marina*) deal with the potential problem of volume reduction by the accumulation of space-filling insoluble reserves. With respect to the cytoskeleton, Faria *et al.* (2005) used an α-tubulin antibody in an immunocytochemical assay to show that in the dry state of orthodox *Medicago truncutula* seeds, only disassociated tubulin granules were present in the radicle cells, whereas once radicles had protruded by 1 mm, well-established cortical arrays of microtubules could be clearly visualized. In contrast, extensive cortical microtubule arrays were present in embryo cells of fresh mature *Inga vera* seeds, which are recalcitrant (Faria *et al.*, 2004). However, following dehydration from a water content of 1.38 to 0.75 g g^{-1}, microtubules disassociated, giving rise initially to tubulin granules, which disappeared on further dehydration. Upon re-hydration, the damaged cells appeared to have lost the capacity for microtubule reconstitution (Faria *et al.*, 2004). These results support earlier findings indicating the failure of the reconstitution of microfilaments – and hence of a complete cytoskeleton – following damaging degrees of dehydration in embryonic axes of *Q. robur* (Mycock *et al.*, 2000) and *Trichilia dregeana* (Gumede *et al.*, 2003).

Intracellular de-differentiation and metabolic 'switch off'

These constitute the second set of characteristics of the acquisition of desiccation tolerance in orthodox seeds, and comparisons with developing recalcitrant seeds indicated that these phenomena do not occur, although the metabolic rate may be at its lowest at, or shortly before, the seeds are shed (Farrant *et al.*, 1997; reviewed by Pammenter and Berjak, 1999). In embryos of *A. marina*, which are highly recalcitrant, there is only the most transient cessation of DNA replication at shedding, with re-entry into the S phase soon thereafter (Boubriak *et al.*, 2000). Furthermore, those authors found the DNA to be severely damaged after slight dehydration, followed by an inability for its repair after loss of 22 % of the water present at shedding. For *I. vera* embryos, from 6 weeks after flowering to shedding, the 4C DNA content was found to be relatively low and constant in both shoot and root apices, and, in contrast to the findings for *A. marina*, did not change significantly after 13 h of imbibition of mature seeds (Faria *et al.*, 2004). In reviewing previously published information for a range of recalcitrant-seeded species, Faria *et al.* (2004) concluded that the majority of cells appear to be arrested in the G_1 phase of the cell cycle, thus the more vulnerable 4C phase would be avoided when the seeds are shed and at the greatest risk of dehydration.

Reactive oxygen species and antioxidants

Of late there has been a particular focus on free radicals, reactive/active oxygen species (ROS/AOS) and antioxidant systems implicated in the acquisition and maintenance of desiccation tolerance in both seeds and vegetative tissues of resurrection plants. One of the most intriguing aspects of ROS to have emerged recently is their dual role in intracellular signalling as well as intracellular destruction (reviewed by Laloi *et al.*, 2004; Foyer and Noctor, 2005; Suzuki and Mittler, 2006). Formed when high energy state electrons are transferred to molecular oxygen (O_2), ROS include $^1O_2$ (singlet oxygen), H_2O_2 (hydrogen peroxide), $\cdot O_2^-$ (the superoxide radical) and OH· (the hydroxyl radical), which have long been considered as toxic species that can cause oxidative damage to lipids, proteins and nucleic acids (e.g. Halliwell, 1987; Hendry, 1993; Fridovich, 1998; Suzuki and Mittler, 2006). Not surprisingly, the activity of a spectrum of enzymatic and non-enzymatic antioxidants is considered to be of prime importance in quenching ROS activity, i.e. in intracellular protection.

In metabolically active, hydrated plant tissues, however, ROS are now considered to act as second messengers in a diversity of signal transduction cascades (Foyer and Noctor, 2005, and references therein), with H_2O_2 and $\cdot O_2^-$ being singled out because of their implication in many plant developmental and growth processes. Nevertheless, this does not gainsay the vital necessity of their control by the spectrum of antioxidants.

While strict control of ROS is taken for granted in hydrated cells, possession and effective operation of a suite of both enzymic and non-enzymic antioxidants is of prime importance during dehydration of orthodox seeds and desiccation-tolerant vegetative tissues, in the dry state, and again as soon as water uptake by the desiccated cells commences (Pammenter and Berjak, 1999; Kranner *et al.*, 2002; Bailly, 2004; Kranner and Birtić, 2005; Berjak, 2006; Berjak *et al.*, 2007). It is also possible that certain antioxidants may be operative within localized regions of higher water activity within desiccated cells. As an example, 1-cys-peroxiredoxin (Stacey *et al.*, 1999) has been localized to nuclei in imbibed, dormant barley embryos, where it has been suggested to provide antioxidant protection to the DNA. It has been suggested, however, that in the desiccated state there are localized regions with water activity adequate to facilitate molecular mobility ('localized water pools'; Rinne *et al.*, 1999; Leubner-Metzger, 2005). If some such regions occur in the milieu of the chromatin, then it is possible that 1-cys-peroxiredoxin can function to protect the genome against ROS in desiccated seeds, given that the cysteinyl residue (of 1-cys-peroxiredoxin) can be regenerated (Berjak, 2006) ultimately by electron donors such

as thioredoxins and glutaredoxins (Dietz, 2003). In this regard, Rinne *et al.* (1999) conjectured that enzyme activity continues to occur in dehydrin-associated areas of greater water activity in the otherwise dehydrated cells of buds, and Leubner-Metzger (2005) has shown localized β-1,3-glucanase activity in the inner testa to be instrumental in after-ripening in air-dry tobacco seeds. A similar argument may be advanced for the activity of other enzymic, as well as non-enzymic, antioxidants (Bailly, 2004) in localized regions of greater water activity within intracellular glasses in dehydrated seeds (see below).

Unlike the situation in orthodox seeds during the latter stages of development, in recalcitrant seeds metabolism is sustained at measurable levels. When water is lost, and especially when dehydration proceeds slowly, metabolism is considered to become unbalanced. This can result in considerable intracellular damage (termed metabolism-linked damage) and death of seeds/embryos at surprisingly high water contents (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999; Walters *et al.*, 2001, 2002). In recalcitrant seeds, metabolism-linked damage is thought to be intimately associated with the generation of ROS under conditions where the intracellular antioxidant defences are inadequate to quench them.

Recent data for recalcitrant *Araucaria bidwillii* embryos show that there is a transient increase in antioxidant activity upon initial dehydration. However, with further water loss, activity declines, accompanied by an increase in free radicals and thiobarbituric acid-reactive substances (TBARS), the latter indicating increasing lipid peroxidation (Francini *et al.*, 2006). *Ginkgo biloba* seeds are considered to be recalcitrant (Liang and Sun, 2002), and enzymic antioxidants have been found to be inadequate in counteracting oxidative stress during storage (Tommasi *et al.*, 2006). Batches of *G. biloba* seeds were put into storage at 25 and 4 °C at the original water content (approx. $2{\cdot}0$ g g^{-1}) by those authors, who found that the viability of the seeds at the higher temperature declined from 80 to 46 % between 3 and 6 months, accompanied by a decline in water content to about $1{\cdot}0$ g g^{-1}. The seeds stored at 4 °C lost viability precipitously between 6 and 9 months in storage, during which the decline in water content was insignificant (Tommasi *et al.*, 2006). It is suggested that at 25 °C, viability loss was the direct consequence of metabolism-linked desiccation damage (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999; Walters *et al.*, 2001, 2002), while at 4 °C, death occurred because metabolism had progressed, albeit slowly, to the stage at which an exogenous water supply was needed (e.g. Berjak *et al.*, 1989; Pammenter *et al.*, 1994). Generation of free radicals/ROS and accumulation of TBARS, along with the decreasing ability of antioxidants to modulate the situation, is consistent with water stress-induced damage in both cases. Working with recalcitrant *Acer saccharinum* seeds, Ratajczak and Pukacka (2006) concluded, from changes in enzymes of the ascorbate—glutathione cycle, and from levels of ascorbate and glutathione, that viability of stored hydrated seeds could be maintained only when a vigorous antioxidant system was operational.

Late embryogenic accumulating/abundant proteins (LEAs)

Together with sucrose, LEAs have been the focus of much recent attention in the context of the acquisition and retention of desiccation tolerance in orthodox seeds (e.g. Buitink *et al.*, 2002; Kermode and Finch-Savage, 2002; Berjak, 2006; Berjak *et al.*, 2007). These proteins (of which six groups have been identified on the basis of particular peptide motifs) generally lack cysteine residues, are composed predominantly of charged and uncharged polar amino acid residues and, with the exception the Group 5 LEAs, are highly hydrophilic and heat stable (Cuming, 1999). Although that author has indicated that the evidence for LEAs being involved in desiccation tolerance derives from 'correlative and circumstantial evidence rather than by direct experimental demonstration' and this is still the current situation (Berjak *et al.* 2007), the basis of the evidence is convincing: The appearance of LEAs is associated with orthodox seed maturation, as it is with the imposition of a variety of stresses causing water deficits in plant cells (Cuming, 1999). Buitink *et al.* (2006) have demonstrated that 18 genes coding for LEAs and two heat shock proteins (HSPs) were upregulated and identified as being common to the acquisition of desiccation tolerance in *M. truncutula* seeds, and its experimental re-imposition in the seedlings. It has been suggested that because of their hydrophilicity, LEAs of some groups could provide a protective hydration shell around intracellular structures and macromolecules, while others have been hypothesized to sequester ions during dehydration and in the desiccated state. It has also suggested that the lysine-rich K segment of Group 2 LEAs (dehydrins), which has a propensity to form α-helices (Close, 1996), might stabilize hydrophobic domains of other proteins which could become exposed as dehydration proceeds (Close, 1997). Such interactions could counteract inappropriate intermolecular hydrophobic associations (Cuming, 1999), as has been suggested for small HSPs (reviewed by Buitink *et al.*, 2002; Berjak *et al.*, 2007). What is especially significant in terms of desiccation tolerance is that dehydration – particularly in the presence of sucrose – induces at least some LEAs to assume the α-helical conformation (Wolkers *et al.*, 2001), suggested to be the basis of the formation and maintenance of the intracellular glassy state in desiccated cells (Berjak, 2006; Berjak *et al.*, 2007). An additional feature linking LEAs to the phenomenon of desiccation tolerance is that their appearance is concomitant with abscisic acid (ABA) regulation of *lea* gene transcription (reviewed by Bray, 1993; Kermode, 1990, 1995; Cuming, 1999; Kermode and Finch-Savage, 2002; Berjak *et al.*, 2007).

What is the situation pertaining to LEAs in recalcitrant seeds? If LEAs are present in embryo cells of recalcitrant seeds, it must be realized that their various postulated roles in orthodox seeds cannot be more than marginally invoked (at best). This is because slowly dried recalcitrant seeds generally die [as a consequence of metabolism-linked damage (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999; Walters *et al.*, 2001, 2002)] at water contents far above the range at which few, if any, of the mechanisms

of protection suggested for LEAs would be operative. The situation regarding the occurrence of LEAs in recalcitrant seeds is equivocal, as they have been found to occur in a range of species from different habitats, while apparently being absent from others. Group 2 LEAs (dehydrins) have been identified in recalcitrant seeds of some temperate trees (Finch-Savage *et al.*, 1994; Gee *et al.*, 1994), other temperate species and some of tropical/sub-tropical prove-nance (Farrant *et al.*, 1996), and in grasses typified by *Porteresia coarctata*, *Zizania* spp. and *Spartina anglica* (Gee *et al.*, 1994). However, no dehydrin-type LEAs could be demonstrated in seeds of ten tropical wetland species (Farrant *et al.*, 1996). Those investigations were, however, constrained, as analysis was for dehydrin-type, Group 2 LEAs only, and consequently should be extended to the other groups of LEAs unique to seeds. Based on the conjecture about LEA functionality in desiccating/desiccated orthodox seeds, it is difficult to envisage a functional role for such proteins in recalcitrant seeds. However, their presence in recalcitrant embryos of particular species could facilitate more effective survival to lowered water contents (following extremely rapid dehydration by flash drying) necessary to enable the axes to be cryopreserved (see later).

The same might be said about small HSPs, should they occur in recalcitrant embryos. In this regard, Collada *et al.* (1997) showed the abundant presence of a small HSP in cotyledons of recalcitrant *C. sativa*, and concluded that the occurrence could not be linked to desiccation tolerance. Our current investigations suggest that a variety of heat-stable proteins (as yet not identified) are expressed in recalcitrant amaryllid embryos, most of which are amenable to cryopreservation (unpublished data).

Carbohydrates as putative protectants in the desiccated state

It seems invariable that sucrose and certain raffinose series oligosaccharides (or galactosyl cyclitols) accumulate in orthodox seeds during maturation drying (e.g. Leprince *et al.*, 1993; Horowicz and Obendorf, 1994; Blackman *et al.*, 1995; Steadman *et al.*, 1996; Obendorf, 1997; Black *et al.*, 1999), and high sucrose concentrations are also common to desiccated resurrection plant tissues (Berjak *et al.*, 2007, and references therein).

There has been much protracted conjecture, which will not be reiterated here, about the role of sucrose, particularly in the desiccated state of seed tissues (reviewed by Berjak *et al.*, 2007), but the most convincing argument, based on sound evidence, has been promulgated by Bryant *et al.* (2001), Koster and Bryant (2005) and Halperin and Koster (2006). In short, those authors contend that the role of sucrose is dynamic in hindering the close approach of membranes to one another, and hence preventing their lateral proximity. This proximity promotes phase transition of some phospholipids and even the demixing of membrane components which is accompanied by exclusion of integral proteins.

From the relatively few studies carried out, it seems that sucrose with raffinose or stachyose accumulates in the axes and cotyledons of developing recalcitrant seeds. Highly recalcitrant embryos of *A. marina* were found to accumulate substantial amounts of sucrose and stachyose (Farrant *et al.*, 1993*b*), and sucrose accumulation was found to accompany dehydration in the less recalcitrant embryonic axes of *C. sinensis* (Berjak *et al.*, 1989). Similarly, *Q. robur* embryos, which are relatively more desiccation tolerant than those of *A. marina*, accumulate sucrose and raffinose concomitant with the later stage of reserve accumulation (Finch-Savage *et al.*, 1993; Finch-Savage and Blake, 1994), while embryos of *Q. alba* have a high sucrose content (Connor and Sowa, 2003). From a wide-ranging survey of sucrose accumulation among both orthodox and non-orthodox seeds, it appears that a variety of recalcitrant seeds accumulate substantial quantities of sucrose relative to oligosaccharide (Steadman *et al.*, 1996). However, as was pointed out for the LEAs, the sucrose cannot play a part in protecting against desiccation damage as conjectured for orthodox seeds: upon drying in the natural environment, recalcitrant seeds would already have lost viability at water contents well in excess of those at which any benefits could be derived by the contribution of this disaccharide to the intracellular glassy state (see below), or in counteracting lateral contact between membranes, as discussed above. It is probable that in recalcitrant seeds, hydrolysis of sucrose affords a readily available respiratory substrate required to sustain ongoing development which grades imperceptibly into germination, followed by seedling establishment under favourable natural conditions.

The intracellular glassy (vitrified) state

There is considerable evidence for the existence of the intracellular milieu in the glassy or vitrified state in orthodox seeds, once sufficient water has been lost. Although first proposed as being the consequence of a supersaturated sugar solution (Koster and Leopold, 1988; Williams and Leopold, 1989), there has been an increasing realization that there are many other intracellular molecules that must contribute to the glass (e.g. Walters, 1998). It was Koster (1991) who first showed that certain properties of model systems constituted to simulate intracellular sugar mixtures differed from the situation in seeds, while Oliver *et al.* (2001) suggested that the LEAs might underlie the stability of intracellular glasses in the dry state. Existing as unordered random coils in solution, LEAs assume a far more ordered conformation upon dehydration, as demonstrated, for example, by Wolkers *et al.* (2001) for a Group 3 LEA from desiccation-tolerant *Typha* pollen and Boudet *et al.* (2006) for both a Group 1 and Group 5 LEA from *M. truncutula* seeds. In a recent article, Berjak (2006) has proposed that intracellular glasses in dry seeds may be based on coiled LEAs in interaction with sucrose and the residual water, but that in narrow intermembrane spaces, a sugar-based phase might occur, LEAs being excluded on the basis of size, as suggested by Koster and Bryant (2001).

While the relative stability of the intracellular glassy state is held to maintain viability (although not indefinitely) in the desiccated state of orthodox seeds, intracellular glasses just would not normally form in most recalcitrant seeds, as they require water contents ≤ 0.3 g g^{-1}. Water

contents of approx. 0.3 g g^{-1} coincide with a marked increase in cytomatrical (cytoplasmic) viscosity, indicative of glass formation (Buitink and Leprince, 2004), but under the slow drying conditions which would prevail in the natural environment, recalcitrant seeds die at far higher water contents (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999; Walters *et al.*, 2001, 2002). It may, however, be possible that transient intracellular glasses can be formed as a consequence of flash drying (Berjak *et al.*, 1990) excised embryonic axes, which is a procedure intrinsic to the cryopreservation protocol for germplasm conservation of recalcitrant-seeded species (see below).

Other factors that may contribute to desiccation tolerance

Lipid composition. This may contribute to desiccation tolerance in orthodox seeds and its lack in recalcitrant types. For example, Liu *et al.* (2006) showed that the proportion of saturated fatty acids in membrane phospholipids was significantly higher in recalcitrant than in orthodox seeds. When total lipids were evaluated, Nkang *et al.* (2003) found that while agronomically mature seeds of *Telfairia occidentalis* were characterized by predominantly saturated fatty acids, when dried at 28 °C increased accumulation of both mono- and polyunsaturated fatty acids accompanied viability loss. In contrast, when the seeds were dried at 5 °C, high levels of saturated fatty acids were retained and the marked decline in viability was delayed. Interestingly, though, Ajayi *et al.* (2006) reported that *T. occidentalis* seeds lost viability at 6 °C within a relatively short time, suggesting chilling sensitivity, despite the retention of saturated fatty acids during desiccation at 5 °C reported by Nkang *et al.* (2003). Lipid composition has been shown to be associated with deterioration of seeds exhibiting 'intermediate' post-harvest physiology, particularly in terms of their chilling sensitivity. Crane *et al.* (2006) have shown that crystallization of the predominantly saturated storage lipid occurs in *Cuphea carthagenensis* seeds at both high and very low water contents, after maintenance at 5 °C. Those authors showed that rehydration without a preceding melting of crystallized triacylglycerides was lethal. Similarly, Neya *et al.* (2004) showed that hydrating the lipid-rich non-orthodox seeds of *Azadirachta indica* in warm water alleviated effects described as imbibitional stress that occurred when cold water was used. Lipid composition of both membranes and storage bodies thus needs to be extensively surveyed, in relation to desiccation sensitivity and tolerance in seeds.

Repair. Obviously, orthodox seeds must repair any damage accumulated in the dry state, soon after imbibition is initiated, which occurs in the lag phase of water uptake before radicle protrusion occurs (e.g. Osborne, 1983). This demands the unimpaired operation of repair mechanisms and restitution of normal cell structure and function. Simultaneously, the presence and efficient operation of appropriate antioxidants is vital (reviewed by Pammenter and Berjak, 1999). There appear to be few studies on aspects of repair of recalcitrant seeds following sub-optimal short-term storage, or non-lethal dehydration. It was found that when DNA fragmentation was induced by radiation, newly harvested *A. marina* embryos were capable of repair, but that this ability was increasingly compromised if the embryos had first been dehydrated (Boubriak *et al.*, 2000). From Fourier transform infra-red (FTIR) analyses of membrane lipids, Connor and Sowa (2003) showed that after initial dehydration of *Q. alba* acorns, on rehydration a reversible shift occurred between the gel and liquid crystalline phases, but that this ability declined in line with declining seed viability. Those authors also showed that protein secondary structure was irreversibly affected by dehydration.

Endogenous amphiphilic substances. Implication of amphiphilic substances in membrane stability in the dry state was briefly reviewed (Pammenter and Berjak, 1999) as a possible factor in desiccation tolerance. Golovina *et al.* (1998) had shown that dehydration of tolerant pollen and seeds has the potential to cause certain amphiphilic molecules to migrate into the membranes, with migration back into the cytomatrix upon rehydration. The amphiphiles were suggested to play a role in maintaining core fluidity of membranes in the dry state. Subsequent investigations, however, could not confirm this, and indicated that although the amphiphiles may fluidize membrane surfaces, correlation of the phenomenon with desiccation tolerance was uncertain (Golovina and Hoekstra, 2002). Hence it is presently not possible to comment on any significance that intracellular amphiphile distribution may or may not have in recalcitrant seeds subjected to dehydration.

Oleosins. Oleosins in hydrated cells are held to maintain oil bodies as discrete entities, and were suggested to be lacking or to be present in inadequate proportions (to the oil bodies) in lipid-rich recalcitrant seeds (Leprince *et al.*, 1998). In this regard, no oleosins were detected in the highly recalcitrant seeds of the tropical species, *T. cacao*. However, later work involving cloning and characterization of cDNA and peptide sequencing has shown that two oleosins are present in mature *T. cacao* seeds (Guilloteau *et al.*, 2003). Thus perhaps the earlier indication of a lack of oleosins possibly contributing to instability of lipid bodies upon rehydration after dehydration, of *T. cacao* and other oil-rich recalcitrant seeds (Leprince *et al.*, 1998) needs to be re-examined.

Control of the acquisition and maintenance of desiccation tolerance. Although a number of phenomena, as discussed above, have been implicated in the acquisition and maintenance of desiccation tolerance, it seems likely that the picture is not yet complete, making unequivocal identification of the differences underlying recalcitrant seed behaviour presently unattainable. Similarly, it is not yet possible to present a coherent view integrating control of the acquisition of desiccation tolerance. In orthodox seeds the phenomenon is pre-programmed and developmentally regulated, and is suggested to be initiated by maternal factors [rather than directly via environmental signals (Bewley and Black, 1994)], and later to be under control of the embryo. A major point of confusion in attempting

to separate the endogenous factors involved in desiccation tolerance is that its acquisition overlaps with other maturation processes and with the development of dormancy where this occurs. Studies of viviparous mutants and those characterized by impairment of the maturation process in maize (*VP* series mutants) and *Arabidopsis* (*LEC1*, *LEC2*, *FUS3* and *ABI3*) have indicated both ABA-independent and -dependent pathways of gene regulation to be involved (e.g. Bray, 1993; Kermode, 1990, 1995; Kermode and Finch-Savage, 2002; Bartels, 2005; Vincente-Carbajosa and Carbonero, 2005).

Current understanding of the control of seed maturation and acquisition of desiccation tolerance in *Arabidopsis thaliana* suggests that *LEC1*, *LEC2*, *FUS3* and *ABI3* are the four 'master genes' involved, with the latter three being implicated in desiccation tolerance (To *et al.*, 2006). Another pivotal factor is ABA which, in the context of seed development, is probably best known for its role in regulating *lea* gene transcription (e.g. Bray, 1993; Kermode, 1990, 1995; Cuming, 1999; Kermode and Finch-Savage, 2002). Furthermore, recent evidence suggests that a delicate balance between various ROS, as secondary messengers, and antioxidants may be intimately involved with seed maturation and the acquisition of desiccation tolerance. While an attempt has been made to present the most integrated conceptualization possible elsewhere (Berjak *et al.*, 2007), further elaboration on the operation and cross-talk among the various factors involved is beyond the scope of this article.

However, in view of its complexity and our presently fragmentary understanding of events at the control level, and also of expression of the many phenomena characterizing the acquisition and maintenance of desiccation tolerance, it is perhaps not surprising that we have a long way to go before comprehending the basis of the recalcitrant condition.

DAMAGE OF RECALCITRANT SEEDS IN RELATION TO DRYING RATE

Disparate opinions have been expressed about the effect of drying rate on the lowest water content tolerated by recalcitrant seeds without compromising viability. However, it is apparent that the actual rates described as rapid or slow can pertain to very different time scales. For example, when dehydrating excised axes by flash drying, rapid dehydration can be achieved in a matter of 15 min for some species to as much as 3 h for others. Both these examples, however, are rapid relative to axes within whole seeds which require a matter of days to attain similarly low water contents (e.g. Pammenter *et al.*, 1998).

Generally – but not invariably – embryonic axes constitute a very small proportion of the total mass or volume of a recalcitrant seed, and when excised can be dehydrated very rapidly in a laminar air-flow or using the technique of flash drying (Pammenter *et al.*, 2002*b*). Although there are odd exceptions, such rapidly dried axes (and occasionally, seeds, if they can be dehydrated rapidly) will survive to far lower water contents than can be attained on slow dehydration, as has been shown for a variety of species

[e.g. *H. brasiliensis* (Normah *et al.*, 1986); *Q. rubra* (Pritchard, 1991); *E. capensis* (Pammenter *et al.*, 1998); *Aquilaria agallocha* (Kundu and Kachari, 2000); *Wasabia* spp. (Potts and Lumpkin, 2000); *A. heterphyllus* (Wesley-Smith *et al.*, 2001*a*); and *T. occidentalis* (Ajayi *et al.*, 2006)]. Rapid dehydration facilitates axis viability retention well into Hydration Level III, and occasionally just into Level II (Vertucci and Farrant, 1995), at which extreme, generally lethal damage is associated with slow water loss.

It is not that flash drying renders recalcitrant axes desiccation tolerant: on the contrary, they will rapidly lose viability at ambient or refrigerator temperatures if allowed to remain at the low water contents attained. What flash drying does achieve is the rapid passage through the intermediate water content ranges at which aqueous-based metabolism-linked damage occurs, i.e. the time during which unbalanced metabolism occurs and consequent ROS-associated damage accumulates, is strictly curtailed (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999; Walters *et al.*, 2001). The major benefit of the procedure is that it yields explants at water contents suitable for cryostorage, which is currently considered to be the only means by which the genetic resources of recalcitrant-seeded species can be conserved (see below).

When whole seeds are dehydrated, the drying rate is markedly affected by the nature of the seed coverings, seed size and developmental status. In general, the larger the seeds, the more slowly will axis or embryo dehydration proceed. Unless markedly different drying rates can be achieved, the rate of drying will have little effect on response to drying. In the case of the recalcitrant *V. paradoxa* seeds described by Daws *et al.* (2004*a*), the larger seeds (fresh mass 8.93 ± 1.41 g) dried more slowly and, under the conditions used, survived as they had not yet reached a lethal water content. The smaller seeds (fresh mass 5.40 ± 1.02 g), on the other hand, dried more rapidly and so reached the lethal water content sooner and did not survive. Dehydration was not rapid in either case (several days), and so an effect of drying rate would not necessarily be expected. Indeed, a perusal of the data of Daws *et al.* (2004*a*) shows that viability of seeds in both categories was substantially affected at essentially the same hydration level (Vertucci and Farrant, 1995). Viability declined markedly when the seeds dehydrated through the dangerous range of Hydration Level III, where unregulated metabolism accompanied by free radical production is held to proceed while antioxidant defences are inadequate, and virtually no seeds remained viable in Hydration Level II. Loss of viability in Hydration Level III appears to typify recalcitrant seeds across species (Vertucci and Farrant, 1995).

The effect of drying rate on response to drying has a further complication, pertinent particularly to slowly dried whole seeds. Recalcitrant seeds are metabolically active and initiate germination around shedding, and so their developmental status is becoming more advanced and their desiccation sensitivity is increasing. If germination is occurring in the same time frame as drying, desiccation sensitivity could be increasing as drying is proceeding, so

reducing the water loss tolerated (Berjak *et al.*, 1984, 1989; Farrant *et al.*, 1986).

There is, however, a lower water content limit below which recalcitrant axes will not survive; this generally is at or near the level at which all the remaining water is structure associated. Damage ensuing when structure-associated water (also termed non-freezable water, because of its calorimetric properties) is removed is termed desiccation damage *sensu stricto* (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999; Walters *et al.*, 2001). A major difference between desiccation-sensitive and -tolerant seeds is that the latter can lose a considerable proportion of the structure-associated water (Pammenter *et al.*, 1993).

STORAGE OF RECALCITRANT SEEDS/ GERMPLASM

Because recalcitrant seeds are not only hydrated, but also metabolically active, their developmental status changes (more or less rapidly, depending on the species) after they are shed. Hence there are strict guidelines that should be followed to maintain seeds in as near to the same state as at shedding or harvest, before their arrival at the laboratory or seed repository. These have been discussed in detail by Berjak and Pammenter (2004), and will not be reiterated here.

Short- to medium-term storage of recalcitrant seeds

The only way in which vigour and viability of recalcitrant seeds can be maintained is to keep them at the lowest temperature they will withstand, under conditions not permitting water loss, and to eliminate – or at least to minimize – the seed-associated mycoflora. The latter objective is actually difficult to achieve, but the storage parameters can be optimized once preliminary trials have been conducted on a species basis. Nevertheless, storage of whole seeds is strictly a short- to medium-term option. This is because the seeds are metabolically active, and will progress from development to germination at the water content typifying shedding, the only recorded exception being for *Q. robur* in one particular year (Finch-Savage *et al.*, 1993; Finch-Savage, 1996), as discussed above. Recalcitrant seeds of some species, e.g. *T. dregeana*, are shed considerably before development is complete and can be stored for several months at approx. 16 °C under optimized conditions before visible germination in storage is observed (Goveia *et al.*, 2004). In the case of recalcitrant seeds that are not chilling sensitive (which would be expected for temperate species), storage longevity may be further optimized by refrigeration. At the opposite extreme, however, are seeds of *T. cacao*, reputed not to survive below 10 °C (Chin and Roberts, 1980), while *Trichilia emetica* seeds have been found to be lethally damaged at 6 °C (Kioko *et al.*, 2006), as are those of *T. occidentalis* (Ajayi *et al.*, 2006) and *A. marina* (unpublished data). Furthermore, ongoing work on *E. capensis* seeds suggests that chilling sensitivity may be provenance related, and that there appear to be distinct genetic differences among plants from the different provenances

(Bharuth *et al.*, 2007). However, the nature of the chilling injury is still under investigation.

Although it has been sporadically suggested that lowering recalcitrant seed water content to levels permitting basal metabolism but precluding germination in storage might be a means to extend their longevity, this, in fact, has proved to be deleterious to both life span and quality. This has been shown to be the case for seeds of a range of species (Corbineau and Côme, 1986*a*, *b*, 1988; Drew *et al.*, 2000; Eggers *et al.*, 2007). In the cases of *T. dregeana* (Drew *et al.*, 2000; Eggers *et al.*, 2007), *T. emetica*, *S. cordatum* and the gymnosperm, *Podocarpus henkelii* (Eggers *et al.*, 2007), not only did seed storage life span decline in the 'sub-imbibed' condition relative to that of seeds stored at the shedding water contents, but fungal proliferation was exacerbated. The explanation may reside in the fact that, paradoxically, mild dehydration stress actually stimulates germination of recalcitrant seeds (Pammenter *et al.*, 1998; Eggers *et al.*, 2007) before the damaging effects set in. Thus, when the seeds are placed into storage after loss of a small proportion of the water originally present, they will have been stimulated to entrain germinative metabolism sooner, and hence become increasingly desiccation sensitive more rapidly than if not dehydrated (Eggers *et al.*, 2007). This results in a greater water stress and thus seed debilitation (Pammenter *et al.*, 1994), and favours more rapid fungal proliferation from the seed-associated inoculum. In this regard, recalcitrant seeds appear able to elaborate antifungal enzymes and other compounds (Calistru *et al.*, 2000; Anguelova-Merhar *et al.*, 2003; dos Santos *et al.*, 2006), but these defences become decreasingly effective with ongoing duration of storage.

It has become increasingly apparent that the means to optimize short- to medium-term storage of recalcitrant seeds is to maintain the shedding water content and impose the lowest temperature tolerated without chilling damage. Nevertheless, proliferation of microorganisms – particularly fungi – will almost inevitably occur, as recalcitrant seeds are seldom free of inoculum that is often located within the seed tissues. Fungicide treatment has been shown to be highly effective in extending storage life span of recalcitrant seeds in hydrated storage, e.g. for *A. marina* (Calistru *et al.*, 2000) and *Hopea parviflora* (Sunilkumar and Sudhakara, 1998). However, application of non-penetrating fungicides will be effective only in situations where the inoculum is primarily located on the seed surfaces. Thus we are presently experimenting with the application of systemic and surface-penetrating fungicides in terms of both their efficacy and possible deleterious effects on the embryos.

Long-term storage of recalcitrant germplasm

However effectively the storage life span of recalcitrant seeds can be extended, this remains a short- to medium-term option, because of the fact that germination at the shedding water content will virtually inevitably occur. Seedling slow growth does offer an alternative to hydrated storage of seeds (Chin, 1996), but this is less than ideal

as a long-term means of conservation. Hence cryostorage – generally in liquid nitrogen at –196 °C or, less ideally, at some temperature below –80 °C – presently appears to offer the only option for long-term storage.

It would be ideal if whole seeds could be cryopreserved, although this is generally not possible because recalcitrant seeds of most species are large, and at high water contents when shed. As discussed above, large seeds cannot be dried rapidly, and slow dehydration to water contents commensurate with efficient cooling (freezing) is lethal. However, for survival at cryogenic temperatures, water content must be reduced to a level obviating lethal ice crystallization during cooling. While reduction of water content to, or near to, the level where only non-freezable water is present achieves this for orthodox seeds, there are only a few documented cases of non-orthodox seeds transiently surviving such drastic levels of dehydration (reviewed by Berjak and Pammenter, 2004).

Successful cryopreservation of small non-orthodox seeds has been achieved in cases where dehydration could be achieved rapidly, e.g. *Azadirachta indica* (Berjak and Dumet, 1996), *W. salutaris* (Kioko *et al.*, 1999, 2003) and *Wasabia japonica* (Potts and Lumpkin, 2000). If whole seeds are optimally thawed and rehydrated after retrieval from cryostorage, seedlings should, theoretically, be able to be generated in a greenhouse without an intervening *in vitro* stage. However, in the great majority of cases, recalcitrant seeds are far too large, necessitating the use of the excised embryonic axes as explants for cryopreservation. The great advantage offered by excised axes (which generally constitute only an insignificant component by volume or mass of the entire seed) is that they are very small and amenable to rapid dehydration by flash drying (Pammenter *et al.*, 2002*b*). However, use of axes also complicates the cryopreservation procedure significantly, as: (*a*) they may be injured on excision (e.g. Goveia *et al.*, 2004); (*b*) in all cases *a priori* requirements include potentially injurious treatments to eliminate seed-associated microorganism inoculum from the axes themselves (Berjak *et al.*, 1999); (*c*) the extent of flash drying and cooling rates must be determined (e.g. Wesley-Smith *et al.*, 2001*a*, *b*, 2004*a*, *b*); (*d*) the desirability of using cryoprotectants needs to be ascertained (work in progress); (*e*) the *in vitro* technology ensuring that excised axes will establish vigorous seedlings must be developed; (*f*) thawing and especially rehydration must be optimized (Berjak *et al.*, 1999; Berjak and Mycock, 2004); and (*g*) the means for efficient dissemination of explants retrieved from cryostorage must be established (Perán *et al.*, 2006). A further aspect that could be profitably pursued is to induce a measure of axis desiccation and chilling tolerance prior to cryopreservation, as carried out for the temperate species, *A. saccharinum* (Beardmore and Whittle, 2005). However, whether or not this would be successful for highly recalcitrant tropical species is a matter of conjecture.

Exploration of all the complications involved in achieving successful cryopreservation of excised embryonic axes – or of alternative explants (e.g. nodal buds, shoot apices or somatic embryos) where the embryonic axes are not amenable to cryopreservation – is beyond the scope of the present review. However, for the interested reader, the topic has been presented in somewhat more detail by Berjak and Pammenter (2004).

EVOLUTIONARY AND ECOLOGICAL CONSIDERATIONS

Any review of recalcitrant seeds would be incomplete without a consideration of the evolutionary and ecological aspects of their biology. Seed desiccation sensitivity is uncommon: of the approx. 8000 species for which data are available, >90 % can tolerate drying to low water contents (Flynn *et al.*, 2006). Perhaps it is because recalcitrant seeds constitute such a small proportion of the total that seed ecologists and evolutionary biologists have, until recently, almost ignored this group. Seed ecologists study the composition and dynamics of soil seed banks, but rarely consider the water content of the seeds, whilst seed physiologists understand the importance of water content, but have a good knowledge of the seeds of only a few species, mostly crops or weedy invaders. Recently there has been increased interest in the general biology of recalcitrant seeds, and the reviews of Garwood (1989) and Vázquez-Yanes and Orozco-Segovia (1993) were early steps in bridging this divergent approach.

A difficulty faced by biologists interested in evolutionary or ecological aspects of recalcitrant seeds is the paucity of data. Currently the best collection of information is the Seed Information Database of the Royal Botanic Gardens Kew, Millennium Seed Bank (Flynn *et al.*, 2006), but even this contains data on less than 4 % of the world's seed-bearing flora. Consequently, much that is published is accompanied by caveats from the authors concerning sample size, and recommendations that interpretations should be treated with a certain amount of caution. Studies on the evolutionary aspects of seed recalcitrance are particularly prone to this lack of data but, despite this, some thought has been given to the matter.

Seed recalcitrance, although of limited occurrence, is spread widely among seed-bearing plant taxa, and there is no clear phylogenetic pattern (Dickie and Pritchard, 2002). Although originally thought to be absent from the cycads, ginkgos and gnetophytes (Dickie and Pritchard, 2002), recent studies have demonstrated recalcitrance in seeds of two cycads (Woodenberg *et al.*, 2007), and at least two physiology groups (Liang and Sun, 2002; Tomassi *et al.*, 2006) have described seeds of *G. biloba* to be recalcitrant. Reviewing 45 dicotyledonous families, von Teichman and van Wyk (1994) found that seeds that were recalcitrant had certain attributes considered to be ancestral states of the ovule. Based on this, they tentatively concluded that desiccation sensitivity was the ancestral state in seeds. Pammenter and Berjak (2000) concurred with this view, but suggested that tolerance evolved early, and probably a number of times. More recently, Dickie and Pritchard (2002) have analysed a much larger data set and concluded that desiccation tolerance was the ancestral state, similar to the suggestion of Farnsworth (2000). Desiccation tolerance is a complex trait, under the control of several genes, but it requires the loss of only a single gene to reverse this trait,

and so sensitivity could have arisen, independently, a number of times (Dickie and Pritchard, 2002). If this is the case, it has implications in terms of the presence (or lack thereof) of some putative tolerance mechanisms in recalcitrant seeds, and may be related to the considerable interspecies variation that is observed.

Seed desiccation sensitivity would be thought to place constraints on the environments in which reproductive success can occur, and so it is not surprising that most of the early reports on recalcitrance were of seeds native to tropical mesic forests. However, as data have accumulated, it has become apparent that species producing recalcitrant seeds are not confined to tropical forests, with such seeds being produced by species in temperate regions and tropical and sub-tropical drylands. This accumulation of data is now permitting more rigorous ecological analysis, rather than simple records of habitat of the pertinent species. Tweddle *et al.* (2003) analysed a data set pertaining to the seeds of 886 tree and shrub species from 93 families, and showed that the proportion of species producing recalcitrant seeds decreases as the habitat becomes drier, or shows a seasonality with a pronounced dry season. The highest proportion of species with desiccation-sensitive seeds occurs in non-pioneer rainforest trees (where they form seedling, rather than seed banks), although nearly half of these species produce tolerant seeds. There is also a relationship between desiccation sensitivity and the non-dormant state, although this is probably not causal. This study by Tweddle *et al.* (2003) was confined to trees and shrubs, largely because of a lack of data concerning the seeds of tropical herbs. However, as more studies are undertaken, there is an increasing number of reports of recalcitrant seeds being produced by monocots (reviewed by Sershen *et al.*, 2007).

Although recalcitrant-seeded species are more common in aseasonal mesic tropical forests, they do occur in other habitats that are less favourable to germination and seedling establishment. Under these conditions, the regeneration niche appears to be more specialized. Commonly, recalcitrant seeds are shed in the wet season (Farnsworth, 2000). Dussert *et al.* (2000) assessed the desiccation tolerance of nine *Coffea* species and found that the degree of tolerance was related to the number of dry months between dispersal and the start of the following wet season. Pritchard *et al.* (2004*b*) examined seeds produced by some tropical African dryland trees and showed that although the proportion producing recalcitrant seeds was low, the size of the seeds, their rapid germination, and shedding in months of high rainfall contributed to maintenance of high water content and successful germination and seedling establishment. Instead of desiccation tolerance, which presumably has costs, an alternative strategy could be a combination of physical characteristics that reduce the rate of water loss, with rapid germination when water is available. This appears to be the case in some amaryllid and palm species; additionally, some of these herbaceous species reproduce vegetatively, reducing the reliance on seeds for reproduction (Sershen *et al.*, 2007). The low occurrence of recalcitrant-seeded species in arid habitats is not surprising; it is possible that they represent relict populations

consequent on climate change that have developed specialized regeneration strategies, rather than re-acquired desiccation tolerance.

If desiccation sensitivity is a derived trait, there must presumably be some selective advantage to losing tolerance. While it is easy to see that the loss of tolerance in mesic tropical forest species may not be a disadvantage, it is, at first sight, more difficult to identify a selective advantage of sensitivity. However, Pritchard *et al.* (2004*b*) and Daws *et al.* (2005) have noted a number of characteristics of tropical recalcitrant seeds that may confer advantages. These seeds are generally larger than their co-occurring orthodox counterparts, germinate more rapidly and invest less in protection against predators. Large seeds would reduce the rate of water loss and provide substantial reserves for rapid establishment (or survival in a seedling bank); rapid germination would lead to rapid establishment and the ability to acquire transiently available water close to the soil surface; and, as seedlings are less susceptible to predation, a reduction in seed coverings implies a more efficient utilization of resources.

LITERATURE CITED

Ajayi SA, Berjak P, Kioko J, Dulloo ME, Vodouhe RS. 2006. Responses of fluted pumpkin (*Telfairia occidentalis* Hook.f.) seeds to desiccation, chilling and hydrated storage. *South African Journal of Botany* **72**: 544–550.

Anguelova-Merhar VS, Calistru C, Berjak P. 2003. A study of some biochemical and histopathological responses of wet-stored recalcitrant seeds of *Avicennia marina* infected by *Fusarium moniliforme*. *Annals of Botany* **92**: 1–8.

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

Bartels D. 2005. Desiccation tolerance studied in the resurrection plant *Craterostigma plantagineum*. *Integrative and Comparative Biology* **45**: 696–701.

Beardmore T, Whittle C-A. 2005. Induction of tolerance to desiccation and cryopreservation in silver maple (*Acer saccharinum*) embryonic axes. *Tree Physiology* **25**: 965–972.

Berjak P. 2005. Protector of the seeds: seminal reflections from southern Africa. *Science* **307**: 47–49.

Berjak P. 2006. Unifying perspectives of some mechanisms basic to desiccation tolerance across life forms. *Seed Science Research* **16**: 1–15.

Berjak P, Dumet D. 1996. Cryopreservation of seeds and isolated embryonic axes of neem (*Azadirachta indica*). *CryoLetters* **17**: 99–104.

Berjak P, Mycock DJ. 2004. Calcium, with magnesium, is essential for normal seedling development from partially-dehydrated recalcitrant axes: a study on *Trichilia dregeana* Sond. *Seed Science Research* **14**: 217–231.

Berjak P, Pammenter NW. 1997. Progress in the understanding and manipulation of desiccation-sensitive (recalcitrant) seeds. In: Ellis RH, Black M, Murdoch AJ, Hong TD, eds. *Basic and applied aspects of seed biology*. Dordrecht, The Netherlands: Kluwer Academic Publishers, 689–703.

Berjak P, Pammenter NW. 2000. What ultrastructure has told us about recalcitrant seeds. *Revista Brasileira de Fisiologia Vegetal* **12**: 22–55.

Berjak P, Pammenter NW. 2004. Recalcitrant seeds. In: Benech-Arnold RL, Sánchez RA, eds. *Handbook of seed physiology: applications to agriculture*. New York: Haworth Press, 305–345.

Berjak P, Dini M, Pammenter NW. 1984. Possible mechanisms underlying the differing dehydration responses in recalcitrant and orthodox seeds: desiccation-associated subcellular changes in propagules of *Avicennia marina*. *Seed Science and Technology* 12: 365–384.

Berjak P, Farrant JM, Pammenter NW. 1989. The basis of recalcitrant seed behaviour. In: Taylorson RB, ed. *Recent advances in the development and germination of seeds*. New York: Plenum Press, 89–108.

Berjak P, Farrant JM, Mycock DJ, Pammenter NW. 1990. Recalcitrant (homoiohydrous) seeds: the enigma of their desiccation-sensitivity. *Seed Science and Technology* 18: 297–310.

Berjak P, Pammenter NW, Vertucci CW. 1992. Homoiohydrous (recalcitrant) seeds: developmental status, desiccation sensitivity and the state of water in axes of *Landolphia kirkii* Dyer. *Planta* 186: 249–261.

Berjak P, Vertucci CW, Pammenter NW. 1993a. Effects of developmental status and dehydration rate on characteristics of water and desiccation sensitivity in recalcitrant seeds of *Camellia sinensis*. *Seed Science Research* 3: 155–166.

Berjak P, Farrant JM, Pammenter NW, Vertucci CW, Wesley-Smith J. 1993b. Current understanding of desiccation-sensitive (recalcitrant) seeds: Development, states of water and responses to dehydration and freezing. In: Côme D, Corbineau F, eds. *Proceedings of the Fourth International Workshop on Seeds: Basic and applied aspects of seed biology*. Paris: ASFIS, 715–722.

Berjak P, Mycock DJ, Wesley-Smith J, Dumet D, Watt MP. 1996. Strategies for *in vitro* conservation of hydrated germplasm. In: Normah MN, Narimah MK, Clyde MM, eds. *In vitro conservation of plant genetic resources*. Kuala Lumpur, Malaysia: Percetakan Watan Sdn. Bhd, 19–52.

Berjak P, Walker M, Watt MP, Mycock DJ. 1999. Experimental parameters underlying failure or success in plant germplasm conservation: a case study on zygotic axes of *Quercus robur* L. *CryoLetters* 20: 251–262.

Berjak P, Farrant JM, Pammenter NW. 2007. Seed desiccation tolerance mechanisms. In: Jenks M, ed., *Plant desiccation tolerance*. Ames, IO: Blackwell Publishing, in press.

Bewley JD, Black M. 1994. *Seeds. Physiology of development and germination*, 2nd edn. New York: Plenum Press.

Bharuth V, Berjak P, Pammenter NW, Naidoo T. 2007. Responses to chilling of recalcitrant seeds of *Ekebergia capensis* from different provenances. Abstracts from the 5th International Workshop on Desiccation Tolerance and Sensitivity of Seeds and Vegetative Plant Tissues. *South African Journal of Botany* 73: 498.

Black M, Corbineau F, Gee H, Côme D. 1999. Water content, raffinose, and dehydrins in the induction of desiccation tolerance in immature wheat embryos. *Plant Physiology* 120: 463–471.

Blackman SA, Obendorf RL, Leopold AC. 1995. Desiccation tolerance in developing soybean seeds: the role of stress proteins. *Physiologia Plantarum* 93: 630–638.

Boubriak I, Dini M, Berjak P, Osborne DJ. 2000. Desiccation and survival in the recalcitrant seeds of *Avicennia marina*: DNA replication, DNA repair and protein synthesis. *Seed Science Research* 10: 307–315.

Boudet J, Buitink J, Hoekstra FA, Rogniaux H, Larré C, Satour P, Leprince O. 2006. Comparative analysis of the heat stable proteome of radicles of *Medicago truncatula* seeds during germination identifies late embryogenesis abundant proteins associated with desiccation tolerance. *Plant Physiology* 140: 1418–1436.

Bray EA. 1993. Molecular responses to water deficit. *Plant Physiology* 103: 1035–1040.

Bryant G, Koster KL, 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, Leprince O. 2004. Glass formation in plant anydrobiotes: survival in the dry state. *Cryobiology* 48: 215–228.

Buitink J, Hoekstra FA, Leprince O. 2002. Biochemistry and biophysics of tolerance systems. In: Black M, Pritchard HW, eds. *Desiccation and survival in plants. Drying without dying*. Wallingford, Oxon: CABI Publishing, 293–318.

Buitink J, Leger JJ, Guisle I, Vu BL, Wuilleme S, Lamirault G, et al. 2006. Transcriptome profiling uncovers metabolic and regulatory processes occurring during the transition from desiccation-sensitive to desiccation-tolerant stages in *Medicago truncatula* seed. *The Plant Journal* 47: 735–750.

Calistru C, McLean M, Pammenter NW, Berjak P. 2000. The effects of mycofloral infection on the viability and ultrastructure of wet-stored recalcitrant seeds of *Avicennia marina* (Forssk.) Vierh. *Seed Science Research* 10: 341–353.

Chien CT, Lin P. 1997. Effects of harvest date on the storability of desiccation-sensitive seeds of *Machilus kusanoi* Hay. *Seed Science and Technology* 25: 361–371.

Chin HF. 1996. Strategies for conservation of recalcitrant species. In: Normah MN, Narimah MK, Clyde MM, eds. *In vitro conservation of plant genetic resources*. Kuala Lumpur, Malaysia: Percetakan Watan Sdn. Bhd., 203–215.

Chin HF, Roberts EH. 1980. *Recalcitrant crop seeds*. Kuala Lumpur, Malaysia: Tropical Press Sdn. Bhd.

Close TJ. 1996. Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. *Physiologia Plantarum* 97: 795–803.

Close TJ. 1997. Dehydrins: a commonality in the response of plants to dehydration and low temperature. *Physiologia Plantarum* 100: 291–296.

Collada C, Gomez L, Casado R, Aragoncillo C. 1997. Purification and *in vitro* chaperone activity of a class I small heat-shock protein abundant in recalcitrant chestnut seeds. *Plant Physiology* 115: 71–77.

Connor KF, Bonner FT. 1996. Effects of desiccation on temperate recalcitrant seeds: differential scanning calorimetry, gas chromatography, electron microscopy, and moisture studies on *Quercus nigra* and *Quercus alba*. *Canadian Journal of Forest Research* 26: 1813–1821.

Connor KF, Sowa S. 2003. Effects of desiccation on the physiology and biochemistry of *Quercus alba* acorns. *Tree Physiology* 23: 1147–1152.

Corbineau F, Côme D. 1986a. Experiments on the storage of seeds and seedlings of *Symphonia globulifera* L.f. (Guttiferae). *Seed Science and Technology* 14: 585–591.

Corbineau F, Côme D. 1986b. Experiments of germination and storage of the seeds of two dipterocarps: *Shorea roxburghii* and *Hopea odorata*. *The Malaysian Forester* 49: 371–381.

Corbineau F, Côme D. 1988. Storage of recalcitrant seeds of four tropical species. *Seed Science and Technology* 16: 97–103.

Crane J, Kovach D, Gardner C, Walters C. 2006. Triacylglycerol phase and 'intermediate' seed storage physiology: a study of *Cuphea carthagenesis*. *Planta* 223: 1081–1089.

Cuming AC. 1999. LEA proteins. In: Shewry PR, Casey R, eds. *Seed proteins*. Dordrecht, The Netherlands: Kluwer Academic Publishers, 753–779.

Danthu P, Gueye A, Boye A, Bauwens D, Sarr A. 2000. Seed storage behaviour of four Sahelian and Sudanian tree species (*Boscia senegalensis*, *Butyrospermum parkii*, *Cordyla pinnata* and *Saba senegalensis*). *Seed Science Research* 10: 183–187.

Daws MI, Gaméné CS, Glidewell SM, Pritchard HW. 2004a. Seed mass variation masks a single critical water content in recalcitrant seeds. *Seed Science Research* 14: 185–195.

Daws MI, Lydall E, Chmielarz P, Leprince O, Matthews S, Thanos CA, Pritchard HW. 2004b. Developmental heat sum influences recalcitrant seed traits in *Aesculus hippocastanum* across Europe. *New Phytologist* 162: 157–166.

Daws MI, Garwood NC, Pritchard HW. 2005. Traits of recalcitrant seeds in a semi-deciduous tropical forest in Panamá: some ecological implications. *Functional Ecology* 19: 874–885.

Daws MI, Garwood NC, Pritchard HW. 2006a. Prediction of desiccation sensitivity in seeds of woody species: a probabilistic model based on two seed traits in 104 species. *Annals of Botany* 97: 667–674.

Daws MI, Cleland H, Chmielarz P, Gorian F, Leprince O, Mullins CE, et al. 2006b. Variable desiccation tolerance in *Acer pseudoplatanus* seeds in relation to developmental conditions: a case of phenotypic recalcitrance? *Functional Plant Biology* 33: 59–66.

Daws MI, Wood CB, Marks T, Pritchard HW. 2007. Desiccation sensitivity in the Arecaceae: correlates and frequency. Abstracts from the 5th International Workshop on Desiccation Tolerance and Sensitivity of Seeds and Vegetative Plant Tissues. *South African Journal of Botany* 73: 483.

del Carmen Rodrígues M, Orozco-Sergovia A, Sánchez-Coronado ME Vázquez-Yanes C. 2000. Seed germination of six mature neotropical rain forest species in response to dehydration. *Tree Physiology* **20**: 693–699.

Dickie JB, Pritchard HW. 2002. Systematic and evolutionary aspects of desiccation tolerance in seeds. In: Black M, Pritchard HW, eds. *Desiccation and survival in plants. Drying without dying.* Wallingford, Oxon: CABI Publishing, 239–259.

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

Drew PJ, Pammenter NW, Berjak P. 2000. 'Sub-imbibed' storage is not an option for extending longevity of recalcitrant seeds of the tropical species, *Trichilia dregeana. Seed Science Research* **10**: 355–363.

Dussert S, Chabrillange N, Engelmann F, Anthony F, Louarn J, Hamon S. 2000. Relationship between seed desiccation sensitivity, seed water content at maturity and climatic characteristics of native environments on nine *Coffea* species. *Seed Science Research* **10**: 293–300.

Eggers S, Erdey D, Pammenter NW, Berjak P. 2007. Storage and germination responses of recalcitrant seeds subjected to mild dehydration. In: Adkins S, ed. *Seed science research: advances and applications.* Wallingford, Oxon: CABI Publishing, 85–92.

Eira MTS, Walters C, Caldas LS, Fazuoli LC, Sampaio JB, Dias MCLL. 1999. Tolerance of *Coffea* spp. seeds to desiccation and low temperature. *Revista Brasileira de Fisiologia Vegetal* **11**: 97–105.

Ellis RH, Roberts EH. 1980. Improved equations for the prediction of seed longevity. *Annals of Botany* **45**: 13–30.

Ellis RH, Hong TD, Roberts EH. 1990a. An intermediate category of seed storage behaviour? I. Coffee. *Journal of Experimental Botany* **41**: 1167–1174.

Ellis RH, Hong TD, Roberts EH, Tao KL. 1990b. Low-moisture-content limits to relations between seed longevity and moisture. *Annals of Botany* **65**: 493–504.

Erdey D, Berjak P. 2004. Germination of *Dovyalis caffra* (Hook. F. et Harv.) seeds. In: Sacandé M, Jøker D, Dulloo ME, Thomsen KA, eds. *Comparative storage biology of tropical tree seeds.* Rome: IPGRI, 105–107.

Erdey D, Mbatha Z, Berjak P. 2004. Conservation of *Ekebergia capensis* seeds from South Africa. In: Sacandé M, Jøker D, Dulloo ME, Thomsen KA, eds. *Comparative storage biology of tropical tree seeds.* Rome: IPGRI, 108–121.

Erdey D, Sershen , Pammenter NW, Berjak P. 2007. Drying out in Africa: physical and physiological seed characteristics of selected indigenous plant species. Abstracts from the 5th International Workshop on Desiccation Tolerance and Sensitivity of Seeds and Vegetative Plant Tissues. *South African Journal of Botany* **73**: in press.

Faria JMR, van Lammeren AAM, Hilhorst HWM. 2004. Desiccation sensitivity and cell cycle aspects in seeds of *Inga vera* subsp. *Affinis. Seed Science Research* **14**: 165–178.

Faria JMR, Buitink J, van Lammeren AAM, Hilhorst HWM. 2005. Changes in DNA and microtubules during loss and re-establishment of desiccation tolerance in germinating *Medicago truncutula* seeds. *Journal of Experimental Botany* **56**: 2119–2130.

Farnsworth E. 2000. The ecology and physiology of viviparous and recalcitrant seeds. *Annual Review of Ecology and Systematics* **31**: 107–138.

Farrant JM, Pammenter NW, Berjak P. 1986. The increasing desiccation sensitivity of recalcitrant *Avicennia marina* seeds with storage time. *Physiologia Plantarum* **67**: 291–298.

Farrant JM, Pammenter NW, Berjak P. 1989. Germination-associated events and the desiccation sensitivity of recalcitrant seeds – a study on three unrelated species. *Planta* **178**: 189–198.

Farrant JM, Berjak P, Pammenter NW. 1993a. Studies on the development of the desiccation-sensitive (recalcitrant) seeds of *Avicennia marina* (Forsk.) Vierh.: the acquisition of germinability and response to storage and dehydration. *Annals of Botany* **71**: 405–410.

Farrant JM, Pammenter NW, Berjak P. 1993b. Seed development in relation to desiccation tolerance: a comparison between desiccation-sensitive (recalcitrant) seeds of *Avicennia marina* and desiccation-tolerant types. *Seed Science Research* **3**: 1–13.

Farrant JM, Pammenter NW, Berjak P, Farnsworth EJ, Vertucci C.W. 1996. Presence of dehydrin-like proteins and levels of abscisic acid in recalcitrant (desiccation sensitive) seeds may be related to habitat. *Seed Science Research* **6**: 175–182.

Farrant JM, Pammenter NW, Berjak P, Walters C. 1997. Subcellular organization and metabolic activity during the development of seeds that attain different levels of desiccation tolerance. *Seed Science Research* **7**: 135–144.

Finch-Savage WE. 1996. The role of developmental studies in research on recalcitrant and intermediate seeds. In: Ouédraogo A-S, Poulsen K, Stubsgaard F, eds. *Intermediate/recalcitrant tropical forest tree seeds.* Rome: IPGRI, 83–97.

Finch-Savage WE, Blake PS. 1994. Indeterminate development in desiccation-sensitive seeds of *Quercus robur* L. *Seed Science Research* **4**: 127–133.

Finch-Savage WE, Clay HA, Blake PS, Browning G. 1992. Seed development in the recalcitrant species *Quercus robur* L.: water status and endogenous abscisic acid levels. *Journal of Experimental Botany* **43**: 671–679.

Finch-Savage WE, Grange RI, Hendry GAF, Atherton NM. 1993. Embryo water status and loss of viability during desiccation in the recalcitrant species *Quercus robur* L. In: Côme D, Corbineau F, eds. *Basic and applied aspects of seed biology.* Paris: ASFIS, 723–730.

Finch-Savage WE, Pramanik SK, Bewley JD. 1994. The expression of dehydrin proteins in desiccation-sensitive (recalcitrant) seeds of temperate trees. *Planta* **193**: 478–485.

Flynn S, Turner RM, Stuppy WH. 2006. Seed Information Database ([SID] release 7·0, http://www.kew.org/data/sidOctober.

Foyer CH, Noctor G. 2005. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. *Plant, Cell and Environment* **28**: 1056–1071.

Francini A, Galleschi L, Saviozzi F, Pinzino C, Izzo R, Sgherri C, Navari-Izzo F. 2006. Enzymatic and non-enzymatic protective mechanisms in recalcitrant seeds of *Araucaria bidwillii* subjected to desiccation. *Plant Physiology and Biochemistry* **44**: 556–563.

Fridovich I. 1998. Oxygen toxicity: a radical explanation. *Journal of Experimental Biology* **201**: 1203–1209.

Gaméné CS, Pritchard HW, Daws MI. 2004. Effects of desiccation and storage on *Vitellaria paradoxa* seed viability. In: Sacandé M, Jøker D, Dulloo ME, Thomsen KA, eds. *Comparative storage biology of tropical tree seeds.* Rome: IPGRI, 40–56.

Garwood NC. 1989. Tropical soil seed banks: a review. In: Leck MA, Parker VT, Simpson RL, eds. *Ecology of soil seed banks.* San Diego: Academic Press, 149–209.

Gee OH, Probert RJ, Coomber SA. 1994. 'Dehydrin-like' proteins and desiccation tolerance in seeds. *Seed Science Research* **4**: 135–141.

Golovina EA, Hoekstra FA. 2002. Membrane behavior as influenced by partitioning of amphiphiles during drying: a comparative study on anhydrobiotic plant systems. *Comparative Biochemistry and Physiology Part A* **131**: 545–558.

Golovina EA, Hoekstra FA, Hemminga MA. 1998. Drying increases intracellular partitioning of amphiphilic substances into the lipid phase: impact on membrane permeability and significance for desiccation tolerance. *Plant Physiology* **118**: 975–986.

Goveia M, Kioko JI, Berjak P. 2004. Developmental status is a critical factor in the selection of excised recalcitrant axes as explants for cryopreservation. *Seed Science Research* **14**: 241–248.

Guilloteau M, Laloi M, Blais D, Crouzillat D, Mc Carthy J. 2003. Oil bodies in *Theobroma cacao* seeds: cloning and characterization of cDNA encoding the 15·8 and 16·9 kDa oleosins. *Plant Science* **164**: 597–606.

Gumede Z, Merhar V, Berjak P. 2003. Effect of desiccation on the microfilament component of the cytoskeleton in zygotic embryonic axes of *Trichilia dregeana.* In: *Proceedings of the 4th International Workshop on Desiccation Tolerance and Sensitivity of Seeds and Vegetative Plant Tissues.* Blouwaterbaai, South Africa, **22**.

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

Halperin SJ, Koster KL. 2006. Sugar effects on membrane damage during desiccation of pea embryo protoplasts. *Journal of Experimental Botany* **57**: 2303–2311.

Hendry GAF. 1993. Oxygen and free radical processes in seed longevity. *Seed Science Research* **3**: 141–153.

Hong TD, Ellis RH. 1990. A comparison of maturation drying, germination and desiccation tolerance between developing seeds of *Acer pseudoplatanus* L. and *Acer platanoides* L. *New Phytologist* **116**: 589–596.

Hong TD, Ellis RH. 1995. Interspecific variation in seed storage behaviour within two genera: *Coffea* and *Citrus*. *Seed Science and Technology* **23**: 165–181.

Hong TD, Ellis RH. 1996. *A protocol to determine seed storage behaviour*. rnoIPGRI Technical Bulletin No. 1, Engels JMM, Toll J, eds. Rome: International Plant Genetic Resources Institute.

Hong TD, Ellis RH, Astley D, Pinnegar AE, Groot SPC, Kraak HL. 2005. Survival and vigour of ultra-dry seeds after ten years of hermetic storage. *Seed Science and Technology* **33**, 449–460.

Horowicz M, Obendorf RL. 1994. Seed desiccation tolerance and storability; dependence on flatulence-producing oligosaccharides and cyclitols – review and survey. *Seed Science Research* **4**: 385–405.

IPGRI-DFSC. 2004. The desiccation and storage protocol. In: Sacandé M, Jøker D, Dulloo ME, Thomsen KA, eds. *Comparative storage biology of tropical tree seeds*. Rome: IPGRI, 345–351.

Kermode AR. 1990. Regulatory mechanisms involved in the transition from seed development to germination. *Critical Reviews in Plant Sciences* **9**: 155–195.

Kermode AR. 1995. Regulatory mechanisms in the transition from seed development to germination: interactions between the embryo and the seed environment. In: Kigel J, Galili G, eds. *Seed development and germination*. New York: Marcel Dekker, 273–332.

Kermode AR, Finch-Savage WE. 2002. Desiccation sensitivity in orthodox and recalcitrant seeds in relation to development. In: Black M, Pritchard HW, eds. *Desiccation and survival in plants: drying without dying*. Wallingford, Oxon: CABI Publishing, 149–184.

Kioko J, Berjak P, Pritchard H, Daws M. 1999. Studies of post-shedding behaviour and cryopreservation of seeds of *Warburgia salutaris*, a highly endangered medicinal plant indigenous to tropical Africa. In: Marzalina M, Khoo KC, Jayanthi N, Tsan FY, Krishnapillay B, eds. *Recalcitrant seeds*. Kuala Lumpur, Malaysia: Forest Research Institute, 365–371.

Kioko JI, Berjak P, Pammenter NW. 2003. Responses to dehydration and conservation of the non-orthodox seeds of *Warburgia salutaris*. *South African Journal of Botany* **69**: 532–539.

Kioko J, Baxter D, Berjak P. 2004. Tolerance to desiccation and storability of *Warburgia salutaris (ugandensis)* seeds from Kenya. In: Sacandé M, Jøker D, Dulloo ME, Thomsen KA, eds. *Comparative storage biology of tropical tree seeds*. Rome: IPGRI, 131–139.

Kioko JI, Berjak P, Pammenter NW. 2006. Viability and ultrastructural responses of seeds and embryonic axes of *Trichilia emetica* to different dehydration and storage conditions. *South African Journal of Botany* **71**: 167–176.

Koster K. 1991. Glass formation and desiccation tolerance in seeds. *Plant Physiology* **96**: 302–304.

Koster KL, Bryant G. 2005. Dehydration in model membranes and protoplasts: contrasting effects at low, intermediate and high hydrations. In: Chen THH, Uemura M, Fujikawa S, eds. *Cold hardiness in plants: molecular genetics, cell biology and physiology*. Wallingford, UK: CAB International, 219–234.

Koster KL, Leopold AC. 1988. Sugars and desiccation tolerance in seeds. *Plant Physiology* **88**: 829–832.

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

Kranner I, Beckett RP, Wornik S, Zorn M, Pfeifhofer HW. 2002. Revival of a resurrection plant correlates with its antioxidant status. *The Plant Journal* **31**: 13–24.

Kundu M, Kachari J. 2000. Desiccation sensitivity and recalcitrant behavior of seeds of *Aquilaria agallocha* Roxb. *Seed Science and Technology* **28**: 755–760.

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

Leprince O, Hendry GAF, McKersie BD. 1993. The mechanisms of desiccation tolerance in developing seeds. *Seed Science Research* **3**: 231–246.

Leprince O, van Aelst A, Pritchard HW, Murphy DJ. 1998. Oleosins prevent oil-body coalescence during seed imbibition as suggested by a low-temperature scanning electron microscope study of desiccation-tolerant and -sensitive seeds. *Planta* **204**: 109–119.

Leprince O, Buitink J., Hoekstra FA. 1999. Axes and cotyledons of recalcitrant seeds of *Castanea sativa* Mill. exhibit contrasting responses of respiration to drying in relation to desiccation sensitivity. *Journal of Experimental Botany* **50**: 1515–1524.

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

Li CR, Sun WQ. 1999. Desiccation sensitivity and activities of free radical-scavenging enzymes in recalcitrant *Theobroma cacao* seeds. *Seed Science Research* **9**: 209–217.

Liang YH, Sun WQ. 2002. Rate of dehydration and cumulative desiccation stress integrated to modulate desiccation tolerance of Cocoa and Ginkgo embryonic tissues. *Plant Physiology* **128**: 1323–1331.

Lin TP, Chen M-H. 1995. Biochemical characteristics associated with the development of the desiccation-sensitive seeds of *Machilus thunbergii* Sieb. & Zucc. *Annals of Botany* **76**: 381–387.

Liu M-S, Chang C-Y, Lin T-P. 2006. Comparison of phospholipids and their fatty acids in recalcitrant and orthodox seeds. *Seed Science and Technology* **34**: 443–452.

Martins CC, Nakagawa J, Bovi MLA. 2000. Desiccation tolerance of four seedlots from *Euterpe edulis* Mart. *Seed Science and Technology* **28**: 101–113.

Mycock DJ, Berjak P, Finch-Savage WE. 2000. Effects of desiccation on the subcellular matrix of the embryonic axes of *Quercus robur*. In: Black M, Bradford KJ, Vázquez-Ramos J, eds. *Seed biology: advances and applications*. Wallingford, Oxon: CABI Publishing. 197–203.

Neya O, Golovina EA, Nijsse J, Hoekstra FA. 2004. Ageing increases the sensitivity of neem (*Azadirachta indica*) seeds to imbibitional stress. *Seed Science Research* **14**: 205–217.

Nkang A, Omokaro D, Egbe A, Amanke G. 2003. Variations in fatty acid proportions during desiccation of *Telfairia occidentalis* seeds harvested at physiological and agronomic maturity. *African Journal of Biotechnology* **2**: 33–39.

Normah MN, Chin HF, Hor YL. 1986. Desiccation and cryostorage of embryonic axes of *Hevea brasiliensis* Muell.-Arg. *Pertanika* **9**: 299–303.

Normah MN, Ramiya SD, Gintangga M. 1997. Desiccation sensitivity of recalcitrant seeds – a study on tropical fruit species. *Seed Science Research* **7**: 179–183.

Obendorf RL. 1997. Oligosaccharides and galatosyl cyclitols in seed desiccation tolerance. *Seed Science Research* **7**: 61–74.

Oliver AE, Leprince O, Wolkers WF, Hincha DK, Heyer AG, Crowe JH. 2001. Non-disaccharide-based mechanisms of protection during drying. *Cryobiology* **43**: 151–167.

Osborne DJ. 1983. Biochemical control of systems operating in the early hours of germination. *Canadian Journal of Botany* **61**: 3568–3577.

Pammenter NW, Berjak P. 1999. A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms. *Seed Science Research* **9**: 13–37.

Pammenter NW, Berjak P. 2000. Evolutionary and ecological aspects of recalcitrant seed biology. *Seed Science Research* **10**: 301–306.

Pammenter NW, Vertucci CW, Berjak P. 1991. Homeohydrous (recalcitrant) seeds: dehydration, the state of water and viability characteristics in *Landolphia kirkii*. *Plant Physiology* **96**: 1093–1098.

Pammenter NW, Vertucci CW, Berjak P. 1993. Responses to dehydration in relation to non-freezable water in desiccation-sensitive and -tolerant seeds. In: Côme D, Corbineau F, eds. *Proceedings of the 4th International Workshop on Seeds: Basic and applied aspects of seed biology*. Paris: ASFIS, 867–872.

Pammenter NW, Berjak P, Farrant JM, Smith MT, Ross G. 1994. Why do stored hydrated recalcitrant seeds die? *Seed Science Research* **4**: 187–191.

Pammenter NW, Greggains V, Kioko JI, Wesley-Smith J, Berjak P, Finch-Savage WE. 1998. Effects of differential drying rates on viability retention of recalcitrant seeds of *Ekebergia capensis*. *Seed Science Research* **8**: 463–471.

Pammenter NW, Naidoo S, Berjak P. 2002a. Desiccation rate, desiccation response and damage accumulation: can desiccation sensitivity be quantified? In: Nicolás N, Bradford KJ, Côme D, Pritchard HW,

eds. *The biology of seeds – recent advances*. Wallingford, Oxon: CABI Publishing, 319–325.

Pammenter NW, Berjak P, Wesley-Smith J, Vander Willigen C. 2002b. Experimental aspects of drying and recovery. In: Black M Pritchard HW, eds. *Desiccation and survival in plants: drying without dying*. Wallingford, Oxon: CABI Publishing, 93–110.

Perán R, Berjak P, Pammenter NW, Kioko JI. 2006. Cryopreservation, encapsulation and promotion of shoot production of embryonic axes of a recalcitrant species, *Ekebergia capensis* Sparrm. *CryoLetters* **27**: 1–12.

Potts SE, Lumpkin TA. 2000. Cryopreservation of *Wasabia* spp. seeds. *CryoLetters* **18**: 185–190.

Pritchard HW. 1991. Water potential and embryonic axis viability in recalcitrant seeds of *Quercus rubra*. *Annals of Botany* **67**: 43–49.

Pritchard HW, Tompsett PB, Manger K, Smidt WJ. 1995. The effect of moisture content on the low temperature responses of *Araucaria hunsteinii* seed and embryos. *Annals of Botany* **76**: 79–88.

Pritchard HW, Steadman KJ, Nash VJ, Jones C. 1999. Kinetics of dormancy release and the high-temperature germination response in *Aesculus hippocastanum* seeds. *Journal of Experimental Botany* **50**: 1507–1514.

Pritchard HW, Wood CB, Hodges S, Vautier HJ. 2004a. 100-seed test for desiccation tolerance and germination: a case study on 8 tropical palm species. *Seed Science and Technology* **32**: 393–403.

Pritchard HW, Daws MI, Fletcher BJ, Gaméné CS, Msanga HP, Omondi W. 2004b. Ecological correlates of seed desiccation tolerance in tropical African dryland trees. *American Journal of Botany* **91**: 863–870.

Ratajczak E, Pukacka S. 2006. Changes in the ascorbate–glutathione system during storage of recalcitrant seeds of *Acer saccharinum* L. *Acta Societatis Botanicorum Poloniae* **75**: 23–27.

Rinne PLH, Kaikuranta PLM, van der Plas LHW, 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.

Roberts EH. 1973. Predicting the storage life of seeds. *Seed Science and Technology* **1**: 499–514.

Sacandé M, Jøker D, Dulloo ME, Thomsen KA, eds . 2004. *Comparative storage biology of tropical tree seeds*. Rome, IPGRI.

dos Santos ALW, Wiethölter N, El Gueddari NE, Moerschbacher BM. 2006. Protein expression during seed development of *Araucaria angustifolia*: transient accumulation of class IV chitinases and arabinogalactan proteins. *Physiologia Plantarum* **127**: 138–148.

Sershen, Pritchard HW, Pammenter NW, Berjak P, Dytham L Suzuki K, Daws MI. 2007. Ecological aspects of seed desiccation sensitivity in the monocots: a review. *Seed Science Research* in press.

Stacey RAP, Nordeng TW, Culiñáez-Maciá FA, Aalen RB. 1999. The dormancy-related peroxiredoxin antioxidant PER1, is located to the nucleus in barley embryo and aleurone. *The Plant Journal* **19**: 1–8.

Steadman KJ, Pritchard HW, Dey PM. 1996. Tissue-specific soluble sugars in seeds as indicators of storage category. *Annals of Botany* **77**: 667–674.

Sun WQ. 1999. Desiccation sensitivity of recalcitrant seeds and germinated orthodox seeds: can germinated orthodox seeds serve as model systems for studies of recalcitrance? In: Marzalina M, Khoo KC, Jayanthi N, Tsan FY, Krishnapillay B, eds. *Recalcitrant seeds*. Kuala Lumpur, Malaysia: Forest Research Institute, 29–42.

Sunilkumar KK, Sudhakara K. 1998. Effect of temperature, media and fungicides on the storage behavior of *Hopea parviflora* seeds. *Seed Science and Technology* **26**: 781–797.

Suzuki N, Mittler R. 2006. Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. *Physiologia Plantarum* **126**: 45–51.

von Teichman I, van Wyk AE. 1994. Structural aspects and trends in the evolution of recalcitrant seeds in dicotyledons. *Seed Science Research* **4**: 225–239.

To A, Valon C, Savino G, Jocylyn G, Devic M, Giraudat J, Parcy F. 2006. A network of local and redundant gene regulation governs *Arabidopsis* seed maturation. *The Plant Cell* **18**: 1642–1651.

Tommasi F, Paciolla C, de Pinho MC, De Gara L. 2006. Effects of storage temperature on viability, germination and antioxidant metabolism in *Ginkgo biloba* L. seeds. *Plant Physiology and Biochemistry* **44**: 359–368.

Tompsett PB, Pritchard HW. 1993. Water status changes during development in relation to the germination and desiccation tolerance of *Aesculus hippocastanum* L. seeds. *Annals of Botany* **71**: 107–116.

Tweddle JC, Dickie JB, Baskin CC, Baskin JM. 2003. Ecological aspects of seed desiccation sensitivity. *Journal of Ecology* **91**: 294–304.

Vázquez-Yanes C, Orozco-Segovia A. 1993. Patterns of seed longevity and germination in the tropical rainforest. *Annual Review of Ecology and Systematics* **24**: 69–87.

Vertucci CW, Farrant JM. 1995. Acquisition and loss of desiccation tolerance. In: Kigel J, Galili G, eds. *Seed development and germination.* New York: Marcel Dekker Inc., 237–271.

Vertucci CW, Crane J, Porter RA, Oelke EA. 1995. Survival of *Zizania* embryos in relation to water content, temperature and maturity status. *Seed Science Research* **5**: 31–40.

Vicente-Carbajosa J, Carbonero P. 2005. Seed maturation: developing an intrusive phase to accomplish a quiescent state. *International Journal of Developmental Biology* **49**: 645–651.

Walters C. 1998. Understanding the mechanisms and kinetics of seed aging. *Seed Science Research* **8**: 223–244.

Walters C, Engels J. 1998. The effects of storing seeds under extremely dry conditions. *Seed Science Research* **8** (Supplement no. 1): 3–8.

Walters C, Pammenter NW, Berjak P, Crane J. 2001. Desiccation damage, accelerated ageing and respiration in desiccation tolerant and sensitive seeds. *Seed Science Research* **11**: 135–148.

Walters C, Farrant JM, Pammenter NW, Berjak P. 2002. Desiccation stress and damage. In: Black M, Pritchard HW, eds. *Desiccation and survival in plants: drying without dying*. Wallingford, Oxon: CABI Publishing, 263–291.

Walters C, Wheeler L, Grotenhuis JM. 2005. Longevity of seeds stored in a genebank: species characteristics. *Seed Science Research* **15**: 1–20.

Wesley-Smith J, Pammenter NW, Berjak P, Walters C. 2001a. The effects of two drying rates on the desiccation tolerance of embryonic axes of recalcitrant jackfruit (*Artocarpus heterophyllus* Lamk.) seeds. *Annals of Botany* **88**: 653–664.

Wesley-Smith J, Walters C, Pammenter NW, Berjak P. 2001b. Interactions of water content, rapid (non-equilibrium) cooling to –196°C and survival of embryonic axes of *Aesculus hippocastanum* L. seeds. *Cryobiology* **42**: 196–206.

Wesley-Smith J, Walters C, Berjak P, Pammenter NW. 2004a. Non-equilibrium cooling of *Poncirus trifoliata* L. embryonic axes at various water contents. *CryoLetters* **25**: 121–128.

Wesley-Smith J, Walters C, Berjak P, Pammenter NW. 2004b. The influence of water content, cooling and warming rates upon survival of embryonic axes of *Poncirus trifoliata*. *CryoLetters* **25**: 129–138.

Williams RJ, Leopold AC. 1989. The glassy state in corn embryos. *Plant Physiology* **89**: 977–981.

Wolkers WF, McCready S, Brandt WF, Lindsey GG, Hoekstra FA. 2001. Isolation and characterization of a D-7 LEA protein in pollen that stabilizes glasses *in vitro*. *Biochimica et Biophysica Acta* **1544**: 196–206.

Woodenberg W, Erdey D, Pammenter N, Berjak P. 2007. Post-shedding seed behaviour of selected *Encephalartos* species. Abstracts from the 5th International Workshop on Desiccation Tolerance and Sensitivity of Seeds and Vegetative Plant Tissues. *South African Journal of Botany* **73**: 496.