

Chapter **22**

## Understanding and Handling Desiccation-Sensitive Seeds



**Patricia Berjak and Norman W. Pammenter**  
School of Life and Environmental Sciences,  
University of Natal, Durban 4041, South Africa



**Summary**

Recalcitrant seeds are shed in the hydrated state, are desiccation sensitive, and lose viability on hydrated storage. Consequently, they require special attention during collection, transport and short-term storage. A hallmark of recalcitrant seeds is variability in terms of desiccation and storage responses, both between species and within a species, where variation can occur inter- and intra-seasonally, with provenance, and drying method. Thus a 'critical water content' for desiccation damage cannot be unequivocally determined.

Fungal contamination is ubiquitous in recalcitrant seeds, and is a major cause of deterioration; thus surface sterilisation of the seeds immediately on removal from the fruit is critical. Recalcitrant seeds can be stored only in the short-term. They should be stored hydrated (partial drying ['sub-imbibed' storage] is not recommended). Effective sterilisation of the seeds and containers is essential; the use of systemic fungicides to reduce internal contamination is being investigated. Seeds should be stored at the lowest temperature commensurate with their chilling tolerance. Long term storage is possible only using cryopreservation, and some relevant studies are described.

## Understanding the Basis of Seed Behaviour

### 1. Broad Concepts on a Narrow Foundation

Examination of the voluminous seed biology literature reveals that, aside from works dealing with seed structure and dispersal (e.g., Corner, 1976; van der Pijl, 1982; Boesewinkel and Bouman, 1995), most of the amassed knowledge is derived from studies on a limited spectrum of 'orthodox' crop seeds. Orthodoxy (Roberts, 1973), describes seeds that can tolerate drying to low water contents, and the storage life span of which increases with reductions in both moisture content and temperature, in a quantifiable and predictable way. Roberts (1973) simultaneously introduced the term 'recalcitrant' to describe seeds that are shed at high water contents, do not tolerate much dehydration, and so cannot be stored in the desiccated condition and are not amenable to long-term storage. A further class, the 'intermediate' seeds (Ellis and Hong, 1990), are more tolerant of desiccation than recalcitrant seeds, but not to the extent of orthodox seeds, and many lose viability more rapidly at reduced temperatures. (It should be noted that there is considerable intra-species variation in the responses of seeds to desiccation and in their storage behaviour [see below], which has led to a lack of consistency in classification. Post-harvest behaviour of seeds might better be considered as constituting a continuum, subtended by the highest degree of recalcitrance at one end and of orthodoxy at the other, with subtle gradations of non-orthodoxy between the two extremes [Berjak and Pammenter, 1997]).

Despite the passage of almost 30 years since Roberts (1973) coined the terms orthodox and recalcitrant, most standard texts on seeds or plant propagation accord only cursory attention to recalcitrant seeds (e.g., Bewley and Black, 1994; Hartmann *et al.*, 1997). Yet, as the characteristics of an ever-broadening spectrum of seeds of species of tropical and sub-tropical provenance become known, there is increasing documentation of non-orthodox behaviour emerging as a relatively common feature.

## **2. A Wide Spectrum of Unrelated Species Produces Non-orthodox Seeds**

A recently released seed database (Tweddle *et al.*, 2002) lists over 500 species producing recalcitrant seeds, or seeds that are possibly recalcitrant, and a further 145 showing 'intermediate' or possibly intermediate storage behaviour. Although recalcitrant seeds seem to be most common among woody species from the mesic tropics, they are not confined to this group; a number of herbaceous monocots produce recalcitrant seeds, as do many trees of temperate provenance. Some species in dry environments are recorded as producing recalcitrant seeds (e.g., Gamene, 1997; Danthu *et al.*, 2000), while a survey of 87 aquatic species, revealed that only 6.9% produced recalcitrant seeds (Hay *et al.*, 2000). Seed recalcitrance can be seen to be widespread across families with little taxonomic relationship (e.g., von Teichman and van Wyk, 1994; Pammenter and Berjak, 2000). There are few guidelines to assist predictions that a particular species does produce recalcitrant seeds, necessitating carefully controlled trials such as those proposed by Hong and Ellis (1996).

## **3. Variability is a Hallmark of Non-orthodox Seeds**

One of the most important points to appreciate when working with recalcitrant seeds is that they are enormously variable. Conflicting reports concerning responses of recalcitrant seeds of individual species are *not* inaccurate for the particular harvest, or for the conditions under which the particular experiments were conducted. However, individual reports may not be directly comparable because of lack of consistency about the developmental status, the conditions under which the seeds were obtained, and the details of the experimental manipulation – particularly to drying parameters. All these factors have their basis in the fact that recalcitrant seeds are not only hydrated when shed or harvested, but also are in a state of ongoing metabolism. Thus the developmental status of the seeds will inexorably change after they are collected (Berjak *et al.*, 1989).

When one considers recalcitrant seeds across a range of species deriving from different families, marked variations in size, structure, internal morphology, and the nature of the seed coverings are immediately apparent and there is also variation in chemical makeup. All these features contribute

to differences in the responses of seeds to dehydration. There are, however, further, more subtle variations among the recalcitrant group of species that complicate their handling.

For example, differences have been reported in the water content and desiccation sensitivity between axes and cotyledons of *Quercus robur* L. (Finch-Savage, 1992; Finch-Savage *et al.* 1992). Cotyledons of *Theobroma cacao* L. have been reported as being more tolerant to desiccation than axes (Li and Sun, 1999), while Leprince *et al.* (1999) found *Castanea sativa* Mill. axes to be more tolerant than the cotyledons. We have generally found that for recalcitrant seeds from a spectrum of species, the axes often are at a substantially higher water content than are the storage tissues (unpublished data). Also, water may be unevenly distributed within the tissues of embryonic axes themselves. Additionally, there are generally distinct inter- and intra-seasonal differences in the seeds of individual species, which are presently largely inexplicable. For example, the water content of ostensibly mature seeds of individual species varies, depending on the stage in a season at which they are harvested, and, for most species, there is significant seed-to-seed variability in the axis water content within any single harvest (Berjak and Pammenter, 1997). Seeds from late-season fruits are generally of very inferior quality, often showing extremely high fungal infection levels (Chacko and Pillai, 1997; Chien and Lin, 1997; our unpublished observations).

Inter-seasonal variations include marked differences in embryonic axis water content (dry mass basis). For example, a more than two-fold difference has been recorded for those of *Camellia sinensis* (L.) O. Kuntze harvested in different years (Berjak *et al.*, 1996), while, for *Quercus robur* seeds harvested from the same tree, the visible germination that generally occurs in storage was not apparent in a particular season in which the seeds had a lower water content than usual (Finch-Savage *et al.*, 1993; Finch-Savage, 1996). Seeds of *Aesculus hippocastanum* L. showed inter-seasonal differences in sensitivity to dormancy-breaking chilling (Pritchard *et al.*, 1999). For tropical species, intra-seasonal anomalies in the effects of seed dehydration on germination have recently been reported for rain forest species (Rodríguez *et al.*, 2000) as have differences in a variety of traits among seed lots of *Euterpe edulis* Mart. (Martins *et al.*, 2000).

A factor that may underlie many of the inter- and intra-seasonal differences among harvests is that the response of recalcitrant seeds to dehydration is correlated with developmental status, with tolerance increasing with increasing maturity. However, it must be appreciated that it is extremely difficult to identify precisely, the developmental status of seeds that are ostensibly mature. In fact, this is probably possible only if flowers are tagged on opening, so that seed development may be referred to a fixed datum point, viz. days after flowering (DAF) or anthesis (DAA). While this is occasionally possible (e.g., Farrant *et al.*, 1992), in most cases it is entirely impractical.

#### 4. Unqualified 'Critical' Damaging or Lethal Water Contents are Not Valid

Response of recalcitrant seeds, or axes excised from seeds, to drying depends on the rate at which water is lost (Normah *et al.*, 1986; Pammenter *et al.*, 1991; 1998; Pritchard, 1991; Kundu and Kachari, 2000; Potts and Lumpkin, 2000). Generally, seeds or axes that are dried very rapidly can survive transiently to the lowest water contents (although there are exceptions, e.g., *Warburgia salutaris* (Bertol.f.) Chiov. [Kioko *et al.*, 1999]; *Theobroma cacao* [Liang and Sun, 2000]). When dehydration is rapid, insufficient time is allowed for the accumulation of damage that occurs during drying. However, irrespective of the drying rate, there is an absolute lower limit below which recalcitrant seeds will not survive. It appears therefore, that there are at least two types of damage that can occur when desiccation-sensitive seeds are dried. At higher water contents (above the lower limit) aqueous-based degradative processes result from the disturbance of ongoing metabolism (Vertucci and Farrant, 1995; Pammenter *et al.*, 1998; Walters *et al.*, 2001; Sun and Liang, 2001). During slow dehydration, this damage accumulates and leads to viability loss at relatively high water contents. If drying is sufficiently rapid, the tissue passes through the range of water contents in which aqueous based degradative processes occur, before damage can accumulate to lethal levels. Below the lower limit to which these seeds or axes can be dried, direct, rapidly-lethal damage occurs because of the removal of stabilising water from intracellular surfaces. These types of damage have been referred to as 'metabolism-derived damage' and 'desiccation damage *sensu stricto*', respectively (Pammenter *et al.*, 1998; Walters *et al.*, 2001).

Not only drying rate, but the maturation status of the seeds (Berjak *et al.*, 1992; 1993; Vertucci *et al.*, 1994; 1995) is related to the water content at which damage occurs or is lethal. Additionally, partially dehydrated (but still viable) axes lose viability after very short-term 'storage' (Walters *et al.*, 2001). One cannot, therefore, stipulate *unqualified* 'critical water contents' at which viability will be lost without specifying parameters relating both to the seeds or axes and the experimental conditions (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999).

## Handling Desiccation-Sensitive Seed

Newly-collected seeds are commonly spread out to dry by equilibration to ambient RH, before placing them into whatever storage facility is locally used. While this may be carried out in sophisticated facilities where RH and temperature are controlled, outdoors shade drying is commonly used (e.g., at the Kenya Forestry Research Institute [Kioko *et al.*, 1993]). Alternatively, seeds may be dried in the sun, or indoors with the use of hot-air blowers: both these methods elevate internal temperature of the still-wet seeds considerably, and superimpose accelerated ageing conditions upon the inevitable metabolism-derived dehydration damage sustained by desiccation-sensitive seeds. If the seeds are recalcitrant, such practices inevitably result in lethal damage, and it is imperative that carefully controlled drying trials based on a recognised protocol (e.g., IPGRI/Danida, 1999) are carried out for seeds that do not dry down naturally prior to shedding.

As an illustrative case, it was found that seeds of *Warburgia* spp., which consistently lost viability after slow equilibration drying, would survive more rapid dehydration to water contents so low that they might be considered to be 'non-recalcitrant' (Pritchard and Daws, 1997). However, they will not survive in the dehydrated condition for more than 1–2 weeks either at ambient temperature or when cold-stored, indicating that they are certainly not orthodox (our unpublished observations), but they are dry enough to be successfully cryostored (Kioko *et al.*, 1999) if immediately introduced into liquid nitrogen.

### 1. Harvest and Transport

Ideally, hand-harvesting should be done directly from the parent plant, despite the problem that estimation of the maturity status may necessarily be subjective. Hand-harvesting obviates uncertainty about the time elapsed from shedding, and thus uncertainties about post-shedding changes, and also prevents water loss from seeds lying on the ground (possibly in the full sun). If the seeds are particularly short-lived, frequent (perhaps daily) collections should be made throughout the season. Because of intra-seasonal variation it is imperative that records of collections are meticulously kept. A further advantage of hand-harvesting is that significant fungal invasion occurs once the propagules are on the ground. Once harvested, the fruits/seeds should immediately be enclosed in a moisture-retaining container.

Plastic bags serve the purpose for collection and short-term transport, but more complex arrangements are necessary if longer times intervene before the seeds can be processed. Despite the obvious inconvenience, it is best if seeds can be transported within the intact fruits, which should be surface-sterilised (usually with a 1% sodium hypochlorite solution; a 1:3 dilution of commercial household bleach is generally used) to curtail fungal proliferation

within packaging designed to prevent dehydration. The same precautions are absolutely essential for seeds that are removed from fruits prior to transport. Always being mindful of the possibility of chilling injury, seeds should be transported at the lowest temperature as can be managed. In cases where practical difficulties preclude collection and transport of fruits or seeds, *in vitro* collection is a possibility (Pence, 1996; Engelmann, 1997).

When seeds or *in vitro* collections are to be transported over long distances, every effort needs to be made to minimise the time that this takes. While transport will necessarily be overland from collecting sites in remote areas, wherever possible air freight should be used. Again, because of the hydrated, metabolic nature of the seeds, it is essential that they are transported in the 'live animal' hold of the aircraft, which is pressure and temperature controlled. (It is not advisable to label the seeds as 'perishable' as this could lead to them being transported in the refrigerated hold, with disastrous consequences if the seeds are chilling sensitive.) Equally important is the minimisation of delay in delivery of a seed consignment, which depends on the appropriate documentation and permits being provided. With all the necessary precautions in place, we have found that the best guarantee of the swiftest delivery of seeds is by the use of a reliable door-to-door courier service.

## 2. Short- or Medium-term Storage

A successful storage regime must ensure that seeds retain unimpaired (or only slightly impaired) vigour and viability for a useful period, i.e., from harvesting until required for planting. Data collected by King and Roberts (1980) show storage life spans of hydrated recalcitrant seeds varying from days or weeks for some tropical species to, at most, two and a half years for some temperate species. With few exceptions, for those species where viability was recorded, it was not high, and certainly would not satisfy the definition of 'successful storage'. The ultimate cause of the loss of viability of hydrated recalcitrant seeds in storage is not known, although Pammenter *et al.* (1994) suggested that germinative metabolism in storage generated a requirement for extra water, which if not fulfilled, gave rise to a mild but prolonged water stress. Some progress in increasing storage longevity has been made over the past 20 years, with some important principles emerging: maintenance of water content, quality of seeds, microbial contamination and temperature.

An *a priori* requirement for viability retention of recalcitrant seeds is the maintenance of a high tissue water content, which can be achieved in a variety of ways. Perhaps the most common way of storing recalcitrant seeds is in heavy gauge, water-impermeable, polythene bags. However, such bags will also be air-impermeable, and the ongoing metabolism of the stored seeds could lead to anaerobic conditions (Smith, 1995). Periodic opening of the bags is thus recommended, although care should be taken as this action does increase the chances of physical injury and microbial contamination. Buckets with a sealing lid have also been used for experimental storage, and if the bucket is not full,

the head-space is increased relative to plastic bags, reducing the frequency with which the container must be opened for aeration. In our laboratory, seeds are frequently placed on a grid over liquid water in the base of the bucket. While this prevents any dehydration it can create another problem: with the small (but unavoidable) diurnal changes in store-room temperature, condensation can occur on the inner side of the bucket lid, and water can drip onto the stored seeds, increasing their water content and enhancing germination in storage. Even with the use of plastic bags, condensation induced by temperature changes can be a problem. The use of moisture-retaining packing media such as sawdust, peat or sand has been recommended (Kioko *et al.*, 1993), although there are also problems with this procedure. The mixture should be turned frequently to maintain aerobic conditions, increasing the likelihood of mechanical damage and microbial infection. Additionally, careful control of the water content of the packing material is required: if it is too low, the seeds will dehydrate; if it is too high the seeds will imbibe water which will increase the rate of germination in storage. To avoid this problem Bhattacharjee *et al.* (1994) have suggested the use of sand moistened with a polyethylene glycol solution of a water potential of  $-0.5$  MPa for the storage of *Camellia sinensis* seeds (but see below).

As with orthodox seeds, recalcitrant seeds of poor quality will have reduced storage life spans. The longevity of cold-stored poor quality seeds of *Inga vera* Willd. ssp. *affinis* (DC.) T.D. Penn. was reduced by 20%, relative to good quality seeds (Barbedo and Cicero, 2000). More dramatically, *Trichilia dregeana* Harv. and Sond. seeds of poor quality (as a result of presumed heat-stress after shedding), declined in viability from 100% to *c.* 20% when wet-stored over three weeks at 16°C (Drew *et al.*, 2000), while high-quality, uninfected seeds of the same species have retained viability for 8 months under the same conditions (our unpublished observations). Physically damaged seeds should not be stored as such seeds are more susceptible to fungal infection.

In addition to the intrinsic deterioration of recalcitrant seeds in storage, the activity of microbial contaminants, especially the ubiquitous fungi associated with these seeds, can be a major cause of viability loss (Calistru *et al.*, 2000; Sutherland *et al.*, 2002). Recalcitrant seeds must be stored under moist conditions to avoid dehydration and those of chilling-sensitive species require relatively high ambient temperature. These are the very conditions that also encourage the activity of the associated mycoflora, and so surface sterilisation of seeds to be stored is essential, and sterilisation of containers is also advisable. A 1% solution of sodium hypochlorite is generally used. In addition, frequent inspection and aerosol application of a soluble fungicide, together with removal of visibly contaminated seeds is recommended. Using this approach, the storage life span of the very short-lived seeds of *Avicennia marina* (Forssk.) Vierh. was increased nearly four-fold (Calistru *et al.*, 2000). While sodium hypochlorite and fungicide treatment may be effective in minimising fungal proliferation on seed surfaces, if mycelium is located below the pericarp/testa, then such treatment is unlikely to be effective.

Based on the concept that stored hydrated recalcitrant seeds are metabolically active, the use of systemic fungicidal 'cocktails' is currently being investigated in our laboratory.

If intrinsic metabolism is an underlying cause of the short storage life span of recalcitrant seeds, then reducing metabolic rate should extend longevity. The simplest way of doing this is to store the seeds at reduced (but above freezing) temperatures, which has the added advantage of reducing the activity of microbial contaminants. Most recalcitrant seeds from temperate regions, and some tropical ones are chilling tolerant and cold storage is recommended for these. However, the seeds of other tropical species are chilling sensitive (see King and Roberts, 1980) and it is essential to establish the sensitivity of seeds for which chilling response is not known before cold-storage is used.

Other approaches have been used to reduce metabolic rate of stored recalcitrant seeds, although these are probably more useful for experimental purposes, rather than large-scale storage activities. It may not be advisable to reduce metabolic rate by reducing respiration by using an anoxic atmosphere, as Tompsett (1983) has shown the importance of oxygen for retention of viability of seeds of *Araucaria hunsteinii* K. Schum. However, Sowa *et al.* (1991) increased the storage life span of seeds of *Litchi chinensis* Sonnen and *Dimocarpus longan* Lour. using nitrous oxide, an anaesthetic that reduces respiration rates in seeds. Abscisic acid (ABA) has been used as a metabolic inhibitor, and extended the longevity of cold-stored immature seeds of *Inga uraguensis* Harv. and Sond. (Barbedo and Cicero, 2000), although the inclusion of ABA in an encapsulating gel designed to take the place of the ABA-containing pericarp of *Avicennia marina*, had no beneficial effects in addition to those of the gel alone (Pammenter *et al.*, 1997). The efficacy of ABA in extending storage life span is not likely to be universal as this will depend on maturity status and whether or not the seeds are responsive to this growth regulator.

Many recalcitrant seeds show visible germination in hydrated storage, and a slight reduction in water content has been suggested to prevent this phenomenon (e.g., Hong and Ellis, 1996). However, data are accumulating that partial drying and 'sub-imbibed' storage has adverse effects on recalcitrant seeds (King and Roberts, 1982; Corbineau and Côme, 1988; Xia *et al.*, 1992; Tompsett and Pritchard, 1993; Pritchard *et al.*, 1995; Drew *et al.*, 2000). This is consistent with the view that the time for which recalcitrant seeds are held at reduced water contents is as important as the level of stress.

### 3. Long-term (cryo-) Storage

At best, hydrated storage of recalcitrant seeds at above-freezing temperatures is useful only in the short- to medium-term. For the long-term, such as would be useful for a gene bank, cryostorage is the only viable option. This involves storage of material at temperatures between -80 and -196°C. A major problem, however, is that reducing the temperature of hydrated tissue to these levels will

cause the formation of lethal ice crystals. The first step to avoid this would be to attempt to reduce the water content to levels where the ice crystal formation does not occur.

Where possible, intact seeds should be used for germplasm conservation, as it should be merely a matter of thawing the seeds after storage, and planting them out. This has been successful with seeds of *Azadirachta indica* A. Juss. (Berjak and Dumet, 1996), *Camellia sinensis* (Hu *et al.*, 1993), *Wasabia japonica* Matsum. (Potts and Lumpkin, 2000) and *Warburgia salutaris* (Kioko *et al.*, 1999). However, the seeds of these species were all dried to water contents around 0.17 g g<sup>-1</sup> (dry mass basis) prior to cooling, and they are probably intermediate, rather than recalcitrant.

The seeds of most recalcitrant species are far too large to dry sufficiently rapidly to survive to the low water contents required to avoid ice crystal damage. An approach to overcome this problem is to use excised embryonic axes, which are smaller and so can be dried rapidly to the required water contents without losing viability. A difficulty associated with this approach is that, after retrieval from cryostorage, *in vitro* procedures must be used for recovery of axes and plantlet establishment, which imposes stringent sterilisation procedures. A common occurrence is that although the axes are still 'alive', they often do not regenerate plantlets; rather, callus formation and/or degeneration of the shoot meristem result (reviewed by Berjak *et al.*, 1996; 1999).

Partially dehydrating axes prior to cryopreservation imposes a desiccation stress in addition to the low temperature stress. To ameliorate the desiccation stress, Wesley-Smith *et al.* (1992; 2001) used a combination of rapid but less extensive drying with very rapid cooling by plunging the axes directly into sub-cooled liquid nitrogen. Very rapid cooling (of the order of hundreds of degrees per second) minimises the time for which axes are exposed to temperatures that permit ice crystal formation and growth. (There is an upper limit to the water content at which partially hydrated axes can be rapidly cooled, as with an increase in water content there is a reduction in cooling rate). This approach has been successful with axes of *Camellia sinensis* (Wesley-Smith *et al.*, 1992), *Quercus robur* (Berjak *et al.*, 1999), *Aesculus hippocastanum* L. (Wesley-Smith *et al.*, 2001), *Ekebergia capensis* Sparrm. (Wesley-Smith *et al.*, unpublished results) and *Trichilia emetica* Vahl (Kioko *et al.*, unpublished results).

The science of cryopreservation of zygotic axes is still in its infancy and the optimum combination of water content, cooling rate, thawing technique and *in vitro* recovery procedure still have to be established on a species by species basis. There are some species for which success has not yet been achieved, and others for which the zygotic axes are unsuitable (because of size) for the procedures outlined. For these species it might be necessary to attempt cryopreservation of somatic embryos or shoot apices.

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