THE BASIS OF RECALCITRANT SEED BEHAVIOUR

Cell Biology of the Homiohydrous Seed Condition

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INTRODUCTION

The term 'recalcitrance', defined as obstinate disobedience, refers to seeds that undergo no maturation drying as the final phase of development, tolerate very little post-shedding desiccation and are often chilling-sensitive. Such seeds are unstorables by any of the methods used for air-dry orthodox seeds. Since these terms were introduced by Roberts in 1973, much of the widely-disseminated literature has been systematically collated to afford an overview of recalcitrant seeds, particularly those of crop species (Chin and Roberts, 1980). Two major unresolved issues emerged from that overview: there was no explanation of the basis of recalcitrant seed behaviour, and no successful storage regimes had been established. The present contribution deals with progress that has been made towards an understanding of the responses of post-harvest, recalcitrant seeds in terms of their cell biology.

Plants that produce recalcitrant seeds generally occur in habitats conducive to relatively rapid, if not immediate, seedling establishment, such as aquatic or marshy environments and humid forests, usually where there is no low temperature constraint (Roberts and King, 1980). In such environments there can be little selective advantage to maturation drying. The lack of maturation drying and the inability of recalcitrant seeds to tolerate much post-shedding desiccation, might be viewed as an evolutionary 'hangover', on the assumption that the acquisition of these properties occurred subsequent to the development of the seed habit. In this regard it is interesting that many of the extant gymnosperms of tropical/sub-tropical distribution have seeds that are apparently recalcitrant (unpublished observations).

The storage behaviour of recalcitrant seeds is generally documented as a record of their short lifespan, even under fully or almost fully hydrated conditions, the moisture content at which damage occurs, and the lower limit of temperature tolerated (King and Roberts, 1980). However, recalcitrance is not an all-or-none phenomenon; there are varying degrees of recalcitrance. A study of the published data which are available has led to the proposal that
there is a continuum of recalcitrant seed types, which may be grouped as showing a minimum, moderate or high degree of recalcitrance (Farrant, Pammenter and Berjak, 1988a). According to that grouping, seeds in the first category will tolerate a fair amount of water loss and relatively low temperatures, the species concerned being indigenous to temperate and sub-tropical regions. Highly recalcitrant seeds produced by tropical forest and wetland species, will tolerate

![Image](https://example.com/image1)

**Figure 1.** Succinic dehydrogenase activity (---) and protein synthesis rate (—-) in relation to axis water content. *A. marina* seeds stored at 80% RH (---) and 10% RH (—-) (After Farrant et al., 1985).

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*A. marina: newly-shed. v. vacuole, p. plastid, m. mitochondrion*

*A. marina: cell division occurs within four days in wet storage*

*A. marina: 24 h after planting. p. plastid, m. mitochondrion*

*A. marina: rapidly dried to 90% mc (47% wmb)*

*A. marina: slowly dried to 90% mc (47% wmb)*
only a little water loss and are generally markedly temperature-sensitive. Seeds showing a moderate degree of recalcitrant behaviour are tropical in distribution and characterised by the intermediate degree of dehydration and mid-range temperatures tolerated.

Another general characteristic of recalcitrant seeds is the variation in moisture content at shedding, within any particular harvest of a single species. Results of a survey of 21 recalcitrant species, have shown that without exception there is a marked variation in moisture content on a seed-to-seed basis for both embryonic axes and storage tissues (Report of the International Seed Testing Association [ISTA] Seed Moisture Committee, 1989).

SEEDS OF AVICENNIA MARINA - A CASE HISTORY

Avicennia marina is a mangrove species with highly recalcitrant seeds, that lose viability within 16 days. The embryonic axis, which has a moisture content (mc) of 160 - 190% dry mass basis [dmb] (62 - 67% wet/fresh mass basis [wmb/fmb]) has a well developed shoot apex and several defined root primordia when the seeds are shed.

Initial studies on the response of A. marina seeds to relatively slow desiccation (Berjak, Dini and Pammenter, 1984) showed an enhanced state of axis subcellular development despite 15% water loss in the short-term, compared with newly-shed material. Continued dehydration caused deleterious subcellular changes, culminating in seed death in 14 days, after the loss of 35% of the initial water content. Neither the enhanced development nor the deleterious changes resembled the disassembly accompanying maturation drying of orthodox seeds (Bairn and Mercer, 1966; Klein and Pollock, 1968; Hallam, 1972). These results were interpreted as germination initiation of the hydrated seeds, which was curtailed by continued water loss (Berjak et al., 1984).

Subsequent studies on seeds maintained at their original water content (wet stored) showed that material stored for 3 - 4 days had a higher germination index than material stored for one day. If the index (Timson, 1965) was calculated from the start of storage rather than from the day of planting (after storage), the enhancement was eliminated. These results were interpreted as supporting the proposal of germination initiation in the hydrated seeds during storage (Pammenter, Farrant and Berjak, 1984).

Although A. marina seeds are hydrated, the ultrastructural situation upon shedding was shown to be one of relative quiescence (Farrant, Berjak and Pammenter, 1985). Root primordia cells had mitochondria characterised by electron transparent matrices and limited cristae development, little polysome formation or endomembrane development, a lack of stored reserves in the plastids, and only a small degree of vacuolation (Plate 1). The levels of succinic dehydrogenase activity and protein synthesis were low (Fig. 1), in keeping with this appearance of relative quiescence. After a short period in storage, there was evidence of enhanced subcellular activity (Plate 2) and increases in both succinic dehydrogenase activity and protein synthesis occurred (Fig. 1). The subcellular events were similar to those characterising seeds planted out immediately upon shedding (Plate 3), although proceeding more slowly in storage (Farrant et al., 1985).

The progress of germination-associated events in wet-stored
Recalcitrant seeds implies that they should become more desiccation sensitive with increasing storage time. Rapid dehydration to a constant moisture content using PEG 6000 solution, confirmed this (Farrant, Pammenter and Berjak, 1986). A reduction of 18% of the initial water content had no effect on newly-shed seeds, but caused considerable subcellular damage in seeds stored for 3 - 4 days and reduced the rate of subsequent germination (calculated according to Czabator, 1962). Fresh seeds that had been planted out for 24 hours were similarly adversely affected. For seeds stored for 10 days, the same degree of dehydration caused extensive ultrastructural derangement and reduced totality of germination as well. The rate of dehydration was also found to influence desiccation sensitivity (Plates 4 & 5), rapidly dried seeds surviving to lower water contents than those dried slowly (Farrant et al., 1985). The ultrastructure of root primordium cells showed that less germination-associated change had occurred in rapidly dried seeds than in those dried slowly to the same moisture content.

These data are consistent with the hypothesis that germination of recalcitrant seeds is initiated at, or around, shedding and as the germination-associated changes continue in storage the seeds become increasingly desiccation sensitive. Even under wet storage conditions the seeds ultimately lose viability, so to complete the germination process and establish seedlings, additional water is required. It has been suggested that this coincides with the stage of cell division and extensive vacuolation (Farrant, Pammenter and Berjak, 1988a).

Recalcitrant seeds should thus be viewed as developing seedlings rather than quiescent seeds, although the rate of development will vary considerably among species. A model based on the observations on A. marina (Fig. 2a) but suggested also to be applicable to other species, was developed to explain the behaviour of recalcitrant seeds in these terms (Farrant et al., 1986, 1988a). Although the abscissa is a time-dependent axis, it is not linearly so. The origin coincides with natural shedding and the rate of the germination process will vary widely among species.

BROAD APPLICABILITY OF THE MODEL

Reference to the literature shows that there is wide variation among recalcitrant seed species in the moisture content at which viability is lost and the proportion of water loss that can be tolerated, and many species are also temperature sensitive (Chin and Roberts, 1980; Chin, Aziz, Ang and Samsidar, 1981; Corbineau and Côme, 1988). There is broad agreement that viability is best retained by storage of recalcitrant seeds in an hydrated state, at as low a temperature as is consistent with chilling sensitivity on a species basis. However, even under the most favourable conditions, viability will decline within a relatively short period (King and Roberts, 1980, 1982; Corbineau and Côme, 1988; Farrant, Pammenter and Berjak, 1989). Can the observations made on a wide variety of recalcitrant seed species be explained in terms of the proposed model?

Investigations have been carried out in our laboratory on seeds of several species showing differing degrees of recalcitrant behaviour, and all have responded in terms of the generalisations made in the model. An in-depth study was conducted on seeds of three unrelated species, Araucaria angustifolia, Scadoxus membranaceus and Landolphia Kirkii (Farrant et al., 1989). There is considerable
Interspecific difference in initial moisture content and the time taken for newly-shed seeds to germinate.

The level of subcellular organisation increased in the mesotermic tissue of the three species during short-term wet storage, differing only in the timing of the events and the pattern of reserve utilisation/deposition. In all cases mitochondrial differentiation, strong development of the endomembrane system, and assembly of polysomes occurred. Cell division - which is considered the ultimate marker event in germination initiation - occurred during wet storage in S. membranaceus and L. kirkii (Farrant et al., 1989) and has been subsequently observed for A. angustifolia. Shortly after this, although seed moisture content remained at the original level, viability declined. This is in accordance with the model, which suggests that additional water is required at this stage. Seeds of the three species all lost viability under desiccating storage conditions, but there was no correlation between the initial water content and proportional loss which could be tolerated. However, the rate at which water was lost under the same conditions varied and was correlated with the proportion of water loss tolerated. A. angustifolia seeds dried relatively slowly, but were the most desiccation-sensitive; seeds of L. kirkii dried the most rapidly, but tolerated the greatest water loss; those of S. membranaceus were intermediate for both parameters (Farrant et al., 1989).

The analysis of data about recalcitrant seeds is far from straightforward. In the first instance, it is important to stress that recalcitrant seeds may behave very differently depending on when in the season they are harvested, and on whether they have been naturally shed or the fruits picked. Because there is no clear-cut terminal event common to the development of recalcitrant seeds on the parent plant, it is often impossible to determine the absolute state of maturity on hand-harvesting. Thus such seeds may be pre-mature, at least physiologically, which could prolong the initial phase prior to the events of germination proper (Fig. 2). However, hand-harvesting might be the method of choice regarding collection of such seeds for experimental purposes, as it obviates the uncertainty arising about time of shedding of fallen seeds. We have also found considerable differences in both germination rate and wet storage lifespan among batches of seeds of the same species, hand-harvested early, midway or late in the season. Another point concerning data analysis is that, as the seeds of many of these species start to lose water from the time they are shed, 'newly-collected' and 'newly-harvested' often are not synonymous. Coupled with this is the fact that investigations may be carried out half-the-world away from the place of origin of the seeds. Under these circumstances, the history of the seeds from shedding may be difficult to ascertain precisely, and their packaging from collection to despatch may well allow water loss. Additionally, recalcitrant seeds being hydrated and metabolic, apparently cannot tolerate long-distance transport in an aircraft hold which is not adequately pressurised (Lins, pers. comm.). Further, if germination initiation on, or soon after, shedding is the rule for those recalcitrant seeds with well-differentiated (although not necessarily large) axes, then by the time they are available for storage manipulation, such seeds may well not be truly representative of the newly shed condition.

Investigations on seeds of Hevea brasiliensis illustrate these difficulties graphically (Berjak, 1989). Newly-shed seeds germinate within 10 days (Chin, 1980), although the embryonic axis is not fully differentiated on shedding (Berjak, Wesley-Smith and Mycock, 1988; Berjak, 1989). Thus they undergo the germination progression, per se,
Figure 2 relates dissimilar and similar effects of two consistently different drying rates to the state of axis development at shedding and the timing of the germination-related processes.
rapidly (Fig. 2b). Comparison was made between seeds conveyed to the laboratory in closed polythene bags immediately they were shed, and those collected and transported over a number of days from some distance. The situation of axis cells from the latter showed that considerable subcellular deterioration had accompanied the modest water loss that had occurred, compared with the newly-shed condition (Plates 6 & 7). These seeds were able to repair sub-lethal intracellular damage when planted out, but germination was slow and totality was reduced. They were also able to effect a measure of subcellular repair during wet storage but showed a considerably curtailed lifespan, and lost viability at moisture contents significantly above those documented for this species by Chin et al. (1981).

A further difficulty in the interpretation of results from experiments on recalcitrant seeds arises from the difference in moisture content between axis and storage tissues within individual seeds (Report of the ISTA Seed Moisture Committee, 1989). Thus one cannot compare data sets in which moisture content has been reported on a whole seed basis in one instance, and on the embryonic axes in another (e.g. for Araucaria spp.; cf. Tompsett [1984] and Farrand et al. [1989]). This difficulty is exacerbated in cases where the embryonic axis constitutes only an insignificant fraction of the total seed volume, but is at a considerably higher moisture content than the storage tissues (Report of the ISTA Seed Moisture Committee, 1989). Assessment of the effects of moisture loss, and in particular, the effects of differential drying rates, can be complicated by the fact that some seeds may lose a certain proportion of water slowly, after which dehydration proceeds much more quickly under the same conditions (Prichard and Prendergast, 1986). This underlines the necessity for frequent sampling for moisture content determinations and viability assessments.

All 15 species of recalcitrant seeds investigated in our laboratory have shown enhanced germination rates following wet storage, interpreted as the consequence of germination initiation at, or shortly after, shedding. The eight species we have investigated ultrastructurally have all yielded evidence of the ongoing progression of germination under wet storage conditions. However, the time taken for visible germination to be manifested in wet storage (where this stage was reached), and the timing of the key events of the germination progression, varied among the species investigated.
These are considered to be critical issues when assessing the effects of differential drying rates. There are reports in the literature stating that differential drying rates apparently have no effect on the desiccation sensitivity of recalcitrant seeds (Tompsett, 1982, 1984, 1987; Probert and Longley, 1989). However, we are of the opinion that those results can be explained in terms of the rates of drying relative to the rates of germination in storage. A detailed discussion of Figure 2 might clarify the issue, noting that in all cases the origin of the abscissa corresponds to natural seed shedding.

Figure 2a illustrates the situation where the embryonic axis is fully differentiated on seed shedding. This is followed by a short period of organisation and differentiation at the cell level, a time scale of approximately 1 - 3 days being involved. During this period the tissue is at, or near, its relatively most desiccation tolerant. This phase is followed by that of cell division and extensive vacuolation, when desiccation sensitivity increases. Root protrusion and growth follow shortly thereafter. Differential drying rates normally achieved in the laboratory will have a marked effect. The seeds of Avicennia marina exemplify this behaviour.

However, many of the recalcitrant seed species are shed with relatively undifferentiated axes. Before cell division and subsequent growth can occur, axis differentiation must be completed. This phase, and subsequent germination, can be relatively rapid (Fig. 2b), as is the case for Hevea brasiliensis, where the germination of newly-shed seeds occurs within about 10 days (Chin, 1980). However, the period of relatively low desiccation sensitivity is extended compared with the case illustrated in Figure 2a. If the two drying rates are not sufficiently dissimilar, then a differential effect is unlikely to be apparent.

In some species the period of axis differentiation is quite extended and the subsequent germination process might also be slow (Fig. 2c). Scadoxus membranaceus exhibits this type of behaviour (Farrant et al., 1989). The period of low desiccation sensitivity can be of the order of months, and, unless drying rates are of this order, no differential effect will be apparent. Probert and Longley (1989) showed that for the recalcitrant seeds of the temperate aquatic grasses, Zizania palustris and Spartina anglica, enhanced rates and totality of germination (at 16°C) occurred after storage in the imbibed state at 2°C for several months. Although those data indicate a dormancy-breaking effect of low-temperature imbibed storage, this does not preclude some axis development and slow initiation of germination under those conditions.

Finally, recalcitrant seeds may germinate so rapidly that the relatively desiccation tolerant stage is practically obviated (Fig. 2d). In such cases, not even the most rapid drying rate is fast enough to prevent death of most of the seeds on dehydration. Thus there may be no practical difference between the effects of rapid and slower dehydration.

Very few studies on recalcitrant seeds provide data concerning rates of germination or information on changes occurring in storage. Thus it is difficult, if not impossible, to assess many published findings in terms of the model.

An additional problem is that it is often not simple to achieve differential drying rates of seeds. Some recalcitrant seeds are so large that it is not possible to dehydrate them rapidly, no matter how
desiccating the conditions. For the genera Araucaria and Dipterocarpus (Tompsett, 1984 & 1987, resp.), it is the species with the largest seeds that dry the slowest and are the most desiccation sensitive. This is in agreement with results for other species (Farrant et al., 1989) and in accordance with the generalizations of the model.

Storage of recalcitrant seeds over saturated solutions of various salts is sometimes used to achieve differential drying rates. Although the seeds will come to different equilibrium moisture contents, the initial rate of desiccation (possibly to below the lethal moisture content) may be quite similar (see, e.g. Figs 1 & 5, Probert and Longley [1989]). This would be equivalent to the situations illustrated in Figures 2b and c. (The slow drying treatment used by Probert and Longley [1989] involved immersion of the seeds in a solution of PEG 6000 of a water potential of ~10 MPa. It is not immediately apparent why those seeds took in excess of 90 days to approach equilibrium with this very concentrated solution).

Thus, in conducting experiments concerning the effects of differential drying rates on desiccation sensitivity, it is important that the drying rates and storage times used are commensurate with the rate of germination. An alternative approach is to test the desiccation sensitivity of the seeds (at the same rapid drying rate) after varying periods of storage under conditions that maintain their moisture content (Farrant et al., 1986). Maintenance of constant temperature during the drying treatments is also important, as is the stage of maturity at harvest or collection, if valid conclusions are to be drawn and comparisons made. Totality of germination alone following a particular treatment, reveals little about the vigour of the seeds. Use of a germination index, such as that of Czabator (1962) that considers rate of germination, is essential. For example, we have found that most seedlings produced by originally-debilitated seeds of Hevea brasiliensis, did not establish. Thus reporting on totality of germination assessed by root protrusion and growth, would have been entirely misleading. A final caveat: although the effects of microorganisms are recognised, these are seldom accorded more than passing comment. However, their incidence and the changing patterns of species composition with changing seed moisture status (Berjak, Farrant, Mycock and Pammeter, 1989a) merits far closer examination, as the microflora might constitute a significant variable in the behaviour of recalcitrant seeds under different conditions (Plate 8).

![A. marina: internal fungal proliferation during wet storage](image)
Although Tompsett (1982) reported that drying rate had no significant effect on lethal water content in Araucaria hunsteinii, viability being completely lost around 14% moisture content (wmb), when Pritchard and Prendergast (1986) rapidly dried down excised embryos of A. hunsteinii to around 13% moisture content in an air-stream, 84% of the root meristems survived in culture. Chin et al. (1981) reported loss of viability of intact Hevea brasiliensis seeds at a moisture content of 20% (wmb). From our studies on differential drying between embryonic axes and storage tissue, we estimate axis moisture content when viability is lost to be about 40% (wmb) for this species. However, Norman, Chin and Hor (1986) have reported that isolated embryos survive to a water content of 16% when dehydration occurs in three hours. Those results support the contention that, provided dehydration occurs before the onset of cell division and extensive vacuolation, the more rapidly a seed can be dried, the more desiccation tolerant it is. We have obtained similar results with another four recalcitrant seed species, Castanospermum australe, Scadoxus membranaceus, Landolphia kirkii and Camellia sinensis: extremely rapid drying (approximately one hour) of excised axes in an air-stream permitted viability retention to much lower moisture contents than did drying of whole seeds. We have termed this extremely rapid dehydration of excised axes, flash drying (Berjak, Farrant, Mycock and Pammenter, 1989b). However, it is impossible to dry large, intact seeds to similar moisture contents in a matter of a few hours.

An investigation has been carried out on the responses of excised axes of the recalcitrant seeds of Landolphia kirkii to flash drying (Berjak et al., 1989b). Intact seeds showed the germination in wet storage that is typical of moderately to highly recalcitrant material (Farrant et al., 1989). Relatively slow drying over silica gel reduced viability from 95% to 50% in 15 days, concomitant with a axis water content decline from 220% to 120% (dbm), and after 20 days, when water content had further dropped to 49%, only 7% of the seeds remained viable. In contrast, flash drying allowed the reduction of water content to 13% (dbm) within 60 minutes, with almost full retention of viability. Flash drying was shown to cause a marked compaction of the entire cell content, with no ultrastructurally-visible deterioration of membranes, at least to a water content of 13%. On the other hand, considerable subcellular damage occurred in axes of seeds dried over silica gel such that, even at a water content of 120%, many cells were extensively deteriorated (Berjak et al., 1989b).

Investigations on the recalcitrant behaviour of individual seed species are generally curtailed in any one season due to its brevity and, to state the obvious, the fact that the seeds cannot be stored in their original condition. We are extremely fortunate in having been able to obtain large consignments of hand-harvested fruits of Camellia sinensis (tea) at weekly intervals. This species has a fruiting season lasting some three months locally, enabling detailed studies on the behaviour and responses of the seeds to be carried out. While previous records had suggested that these seeds might be recalcitrant, this had not been unequivocally established (King and Roberts, 1980). Thus our first task was to establish whether or not the seeds of C. sinensis are recalcitrant. It has since been ascertained that these seeds do exhibit all the characteristics that have come to be associated with recalcitrance (Devey, 1989).
Moisture content of newly-harvested seeds changed during the season from 169% in the first month to 280% three months later (63 - 74%, wmb). Early-harvested seeds germinated slowly and could be wet-stored for a longer period, while those harvested from midway through the season germinated far more rapidly and had a proportionally shorter period for which they could be successfully stored. This was a major consideration in the assessment and comparison of the characteristics of the material investigated. Total viability loss occurred within eight days of storage under desiccating conditions for early-harvested material (Devey, Pammenter and Berjak, 1986). Wet storage maintained seed water content constant, during which the germination-associated progression of subcellular events took place. These processes occurred more slowly in wet stored seeds, but were similar in all respects to those occurring in newly-harvested seeds which were immediately planted out (Devey, Pammenter and Berjak, 1987; Devey, 1989). Viability ultimately declined in seeds after long-term wet storage (Devey, 1989), as has been found for all other recalcitrant species.

Mid-season seeds of *C. sinensis* were used for an in-depth comparison of responses elicited by flash drying of excised axes with those of intact seeds subjected to slower drying over five days. Viability was assessed by growth of isolated axes in tissue culture in both cases. Figure 3 shows that flash-dried isolated axes maintained viability to considerably lower water contents than those excised from intact seeds that had been dehydrated over silica gel, correlating with differential electrolyte leakage.

In common with the embryos from several other recalcitrant seeds we have examined, the axis is very small and shows no radicle development nor discrete root meristem. Instead, the distal portion of the axis is characterised by a narrow band of potentially meristematic cells which is continuous with the cambium of the hypocotyl as a whole. This band of cells was chosen for ultrastructural investigation.

![Figure 3](image-url)

**Figure 3.** The relationship of viability (left) and leakage (right) with water content, for flash-dried (—) axes of *C. sinensis* and those excised from slowly-dried, intact seeds (----).
Although these (and other) recalcitrant seeds are at their relatively most quiescent when newly-harvested, the subcellular organisation (Plates 9 & 10) indicated that they were metabolically active, which has been borne out by biochemical studies (Devey, 1989). The large nuclei, in which the nucleolus and patches of heterochromatin were well defined, dominated the cells, and fairly long rER profiles and polysomes were common. Golgi bodies occurred and were well-defined, although relatively undifferentiated plastids largely devoid of storage product, were common. Mitochondria were small and spherical, the relatively well-developed cristae and dense matrix suggesting their activity. Protein vacuoles with variably-depleted contents, were a feature of these cells.

The seeds dried over silica gel showed a precipitous decline in viability between the third and fourth day concomitant with a drop in water content below 170% (63% [wmb]) (Fig. 3). The major subcellular change occurring in the cells of still-viable axes sampled on day 3, was a marked dilution and irregularity of the vacuoles, accompanied by an apparent thinning of the bounding membrane (Plate 11). Organelle matrices had become dense, as had the chromatin. Similar features characterised those cells of non-viable (day 5) material that were still essentially intact. However, even in these cells areas of localised lysis were frequent (Plate 12) and there were many regions where total cell collapse and wall fragmentation had occurred.

Flash drying elicited a very different spectrum of subcellular events. After 10 minutes, when water content had declined to an average of 143% (58% [wmb]), a marked compaction of mitochondria and plastids had occurred and polysomes persisted in the generally denser cytomatrix. The most striking event was the close association between the rER and many of the vacuoles (Plate 13). Water content remained essentially constant (143 - 132%) despite a further 20 minutes of flash drying, during which similar rER associations developed with the other organelles as well. Most commonly, more than one plastid along with other subcellular constituents appeared to become closely enwrapped by the rER (Plate 14). There was a marked drop in water content to 76% (43% [wmb]) . Despite the fact that association was apparently lost between the plasmalemma and some of the frequent plasmodesmata, plasmalemma rupture was not observed ultrastructurally. Leakage from these axes was substantially lower than that from material slowly dried down to similar water contents and viability had not declined (Fig. 3). Plate 15 shows that the nucleus had become extremely compacted, as had the cytomatrix, and consequently the ER-enwrapped structures were contrasted by means of a negative staining effect. After 60 minutes of flash drying, such extreme compaction of the cells had occurred, that subcellular detail was almost obscured (see Plate 18). However, even in these cells it could be seen that vacuolar integrity had apparently been retained, and there were no visible manifestations of plasmalemma rupture. The low levels of electrolyte leakage are in accordance with this observation. Although survival had declined somewhat by 60 minutes of flash drying, significant loss of viability occurred only after this, when axis water content was reduced below 48% (32% [wmb]).

Thus for both L. kirkii and C. sinensis, flash drying of excised axes not only permitted retention of viability to lower water contents than did drying of intact seeds over silica gel, but also elicited different ultrastructural responses, particularly subcellular
C. sinensis: newly-harvested; axis mc ±280% (±74% wmb); p, Golgi body; v, vacuole

C. sinensis: newly-harvested; p, plastid; m, mitochondrion

C. sinensis: intact seed dried 3 d; axis mc 170 - 130% (63-56% wmb); v, vacuole

C. sinensis: intact seed dried 5 d; axis mc ±48% (±32% fmb); subcellular lysis

C. sinensis: 10 min, flash dried; mc ±143% (±59% fmb); note rER-vacuole association

C. sinensis: 20 min, flash dried; mc ±133% (±57% fmb); organelle enwrapping by ER

C. sinensis: 40 min, flash dried; mc ±76% (±43% wmb); plasmalemma contraction
compaction and maintenance of membrane integrity. It is not known whether these responses were the result of active processes or merely the passive consequence of rapid removal of water and the concentration of cellular contents.

The stabilisation of subcellular membranes in the dehydrated state is an important aspect of desiccation tolerance. In the hydrated state, water is considered to be firmly bound to the molecular components of the membrane surface. The involvement of polyols, and particularly oligosaccharides in membrane stabilisation upon dehydration (the 'water replacement hypothesis') is widely accepted (Crowe and Crowe, 1986a & b; Crowe, Crowe, Carpenter and Wistrom, 1987). While those authors describe trehalose as being primarily involved in animal cells, sucrose in conjunction with raffinose and stachyose is suggested to be the most likely combination of oligosaccharides acting to stabilise membranes in dry orthodox seeds (Leopold and Vertucci, 1986; Caffrey, Fonseca and Leopold, 1988; Koster and Leopold, 1988). It is generally agreed that on dehydration of desiccation tolerant tissues, sugars may bind to phospholipid of the cell membranes, so replacing water and maintaining headgroup spacing.

Eleven percent of the dry mass of the embryonic axes excised from newly-shed seeds of C. sinensis is composed of a combination of small sugars, of which sucrose constitutes about 45%. These values are of the same order as those of some orthodox seeds investigated by Koster and Leopold (1988). However, the ratio of raffinose plus stachyose plus cellobiose to sucrose in C. sinensis seeds (0.1:1) is lower than for the orthodox seeds. This ratio appeared to increase on flash drying and during the initial stages of slow drying of intact seeds, but it is not known whether this is significant.

The Lens culinaris agglutinin (Sigma) having an affinity for D-mannosyl and α-D-glucosyl residues was used for lectin gold labelling. In fresh material, the gold label was located predominantly within the vacuoles (Plate 16). Following 10 minutes flash drying frequency of the label in the vacuoles had increased and gold labelling also occurred in the extraplastomic space and plasmalemma invaginations. The label remained almost exclusively located within vacuoles, vesicles and extraplastomic space (eps) following longer periods of flash drying, seemingly never becoming closely associated with the subcellular membranes (Plates 17 & 18). In cells of axes excised from intact seeds after slower drying, the disposition of the label appeared somewhat different. Not only was there a noticeable intra-organellar location of the gold particles, but the label was considerably more aggregated (as chains or clumps) within the vacuoles (Plate 19). This situation, which had already occurred after one day during which axis moisture content had declined from 283 - 213% (74 - 68% wmb), persisted while viability was retained. Like the situation in flash-dried material, the label did not become membrane associated.

Although giving interesting indications, the data from this lectin gold labelling cannot be unequivocally interpreted, a similar approach with lectins having different, but related affinities being necessary. It cannot be assumed that sugars are definitely not bound to the membranes, as such interaction might well interfere with the complementarity required for recognition by the lectin used. However, the surfaces of membrane preparations from these axes lyophilized in the presence of sucrose/raffinose/stachyose mixtures in the mass ratios used by Caffrey et al. (1988), retained a considerable number of gold
particles. These preparations were processed for electron microscopy in exactly the same way as were the axes, thus there is nothing inherent in the techniques used that could have removed membrane-associated label.

It is possible that at a moisture content of 48 - 28% (32 - 22% wmb) there would be no tendency for water replacement by sugars. However, it has been suggested that sugars start to replace loosely-bound water on membranes in Phaseolus vulgaris seeds, at a water content of 33% (dmb) (Wolk, Dillon, Copeland and Dilley, 1989). In this regard, there are suggestions that an increase in the liquid-crystalline/gel phase transition temperature is initiated below a water content of 12 mol/mol phospholipid (Crowe and Crowe, 1986a, b). This represents a moisture content of 20 - 30% (dmb), assuming the cell water to be evenly distributed (Hoekstra and Van Rockel, 1988).

Wolk et al. (1989) make the point that, for the desiccation tolerant tissue with which they worked, the bound water fraction below 0.05g/g may not be replaceable, and Gaber, Chandrasekhar and Pattabiraman (1986) have indicated that some water remains bound to the phospholipid bilayer, no matter how severely desiccating the regime. Sorption isotherms for desiccation sensitive tissues have a hyperbolic form, as opposed to the reverse sigmoid form for desiccation tolerant tissues. This has been interpreted as indicating that strong water binding contributes only minimally, if at all, to the water sorption characteristics of these tissues (Vertucci and Leopold, 1987). However, the isotherms are such that water contents remain relatively high despite equilibration of the tissues at very low relative humidities (Leopold and Vertucci, 1986; Vertucci and Leopold, 1987). We have found that the sorption isotherm for embryonic axes of
Avicennia marina is also typically hyperbolic and it is apparently impossible to remove all the water, despite equilibration at very low relative humidities. Although considerably more water can be removed from axes of C. sinensis, the isotherm also has a hyperbolic form.

Perhaps one could re-interpret the significance of the hyperbolic isotherms of desiccation sensitive tissue in terms of a significant fraction of water that is, and remains, so strongly bound that it cannot be removed, no matter how low the relative humidity of the 'equilibration system. It might not be replaced by sugars, because of the very tenacity of its binding. Although this interpretation is conjectural, it highlights the possibility that under normal circumstances, membrane integrity in recalcitrant seeds (and germinating orthodox seeds) demands a degree of bound water, considerably in excess of the tenacious fraction, that becomes perturbed below a minimal moisture content. Additionally, as the events of germination proceed, the demand for structured water might increase with increasing subcellular organisation, so increasing the lethal water content (Farrant et al., 1989a).

There is another interpretation of the rôle of sucrose and the other oligosaccharides in desiccation-stressed material. Timasheff (1982) has suggested that a protein remains stabilised in a protein-water-cosolvent (including sucrose) system by exclusion of unfavourable interactions between its surface and the cosolvent molecules, so giving rise to higher concentrations of water at the protein surface. Williams and Leopold (1989) have presented evidence that both the lipids and the non-lipid components in corn embryos exist in a highly viscous glassy state even at ambient temperature, below water contents of 0.12g/g. Those authors draw attention to the probable rôle of sucrose and raffinoses, that together comprise 20% of the dry mass of the embryo, in vitrification of the non-lipid components. Glass formation is suggested to contribute to desiccation tolerance in terms of restricting molecular mobility and so imposing a stasis on biochemical activity (Williams and Leopold, 1989).

While the water content in the flash-dried axes of Camellia sinensis did not remotely approach 0.12g/g, there is no reason to assume that it was evenly distributed within individual cells, depending i.a., on the localisation of solutes. Viability was retained concomitantly with the apparent exclusion of much of the sugar from the compacted cytomatrix (Plates 17 & 18). It is possible (although difficult to ascertain) that while predominantly strongly-bound water persists in the cytomatrix and nucleoplasm, viscous sugar solutions occur within vacuoles, vesicles and the extraprotoplasmic space. While, in C. sinensis, these are unlikely to assume the glassy state, such a situation might minimise perturbation and biochemical reactivity during rapidly-imposed desiccation stress. In flash-dried axes of Landolphia kirkii, water content could be successfully reduced to 0.13g/g (Berjak et al., 1989b); here perhaps, the existence of glass, as suggested by Williams and Leopold (1989).

The ability of excised embryos to survive flash drying to remarkably low water contents poses the question as to whether this material is essentially orthodox in nature. Orthodoxy implies an inherent mechanism for desiccation tolerance, that might be expected to be expressed in conjunction with the appearance of novel proteins as a result of specific mRNA synthesis (Bewley, Kermode and Misra, 1989). Two-dimensional gel electrophoresis carried out on extracts of fully-viable fresh, flash-dried and slow-dried axes of Camellia sinensis have revealed that no new proteins appear as a result of
either drying regime. The controlled subcellular de-differentiation associated with maturation drying of orthodox seeds (Bain and Mercer, 1966; Klein and Pollock, 1968; Hallam, 1972) did not occur on flash-drying. Thus we consider as highly unlikely, the possibility of some inherent capacity that is not normally expressed. Rather, we are of the opinion that flash drying allows reduction of water content so rapidly that some vitrification might occur, permitting a measure of membrane stabilisation and imposing a stasis on metabolic activity. We have preliminary indications that flash-dried material may not be able to survive for any appreciable time at room temperature, and so may not be desiccation tolerant in the accepted sense. Presumably, if super-saturation or vitrification is a response to flash drying, crystallisation can subsequently occur. For desiccation tolerant organisms, the more rapid the dehydration, the more injurious its effects (Bewley and Krochko, 1982; Crowe and Crowe, 1986b). This is entirely contrary to the response of recalcitrant material to flash-drying. However, if flash-dried recalcitrant material is not truly desiccation tolerant, this would resolve the apparent conflict.

There are several facets of recalcitrant seed behaviour that have not been considered in this review, including: 1. Developmental control that ensures an orthodox seed entering the phase of maturation drying, and which is absent or non-operative in the recalcitrant situation; 2. The reaction of the cotyledons and/or endosperm to desiccation and the interaction between the storage tissues and embryonic axis under various conditions; 3. General biochemical characterisation of recalcitrance; 4. The nature of the lesion(s) resulting from desiccation; 5. The issue of chilling sensitivity; and 6. The possibility of cryostorage of flash-dried material. Although there is some information and speculation about certain of these aspects, much remains to be ascertained.

IN CONCLUSION – WHAT IS RECALCITRANCE?

A current working definition of recalcitrant seeds might be: seeds that are shed wet and cannot be dehydrated or stored. This working definition implies that there are two aspects to recalcitrance: intolerance to dehydration and inability to be stored. Both these aspects vary widely among recalcitrant seed species and there may be no numerical relationship between storage lifespan and desiccation sensitivity.

Lifespan in wet storage is related to the rate of axis development (if incomplete upon shedding) and germination (i.e. related to the time scale of the abscissa in Figure 2). Seeds that germinate rapidly when planted out will also undergo germination-associated events relatively rapidly in wet storage and will quickly reach the stage where additional water is required. Naturally slowly-germinating seeds will undergo these changes slowly in storage and so will have a longer lifespan.

Desiccation tolerance can be equated with the minimum water content to which tissue can be dried without viability loss. Sensitivity to dehydration of recalcitrant seeds increases with storage time and so inter-species comparisons should be made at the stage of shedding. It is our thesis that recalcitrant seeds cannot be dehydrated because they are well-differentiated at the subcellular level and either do not possess the genetic potential for de-differentiation (unlike orthodox seeds) or have it completely repressed. Flash-dried axes do show interspecific differences in the extent to which they can be dehydrated.
before marked viability loss (C. sinensis, 48%; L. kirkii, 13% [dmb]). If our hypothesis is correct, there should be a relationship between desiccation sensitivity (minimum lethal water content on almost instantaneous dehydration) and some measure of the degree of cellular differentiation, such as total membrane surface area.

As a result of all these factors it is extremely difficult to achieve a strict definition of recalcitrance, and perhaps it would be easier to reconsider the definition of orthodoxy. Orthodox seeds are those that are either shed in the dry state or can be dried and (this is the critical point) they can be successfully maintained in this state at moderate temperature for periods from months to years. A seed that fails to meet these criteria is not truly desiccation tolerant and is not orthodox.

In keeping with the terminology applied to plants in the vegetative phase, we propose the use of the term, poikilohydric, to describe seeds that can be maintained in equilibrium with ambient relative humidity for long periods (i.e. truly desiccation tolerant, or orthodox). Homoiohydric seeds, on the other hand, are those that cannot be so maintained (i.e. not truly desiccation tolerant, or recalcitrant).

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106


