CHAPTER 4

Orthodox and Recalcitrant Seeds

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The seeds of many species cannot be classified as orthodox, and this is particularly so in the case of tropical tree seeds. The views presented here favor a continuum of seed behavior that is based on a variety of characteristics. A suite of mechanisms or processes is discussed that embodies the properties currently thought to promote the acquisition of desiccation tolerance and to ensure survival of the desiccated condition in orthodox seeds. These include: cellular and intracellular physical characteristics; intracellular de-differentiation; the "switching off" of metabolism; the presence and efficient operation of antioxidant systems; the accumulation and roles of putatively protective molecules, including late embryogenic accumulating/abundant proteins (LEA's), sucrose, and certain oligosaccharides; deployment of amphipathic molecules; an effective peripheral oleosin layer around lipid bodies; the occurrence and operation of repair mechanisms during rehydration; and others yet to be identified. The presence of some of the mechanisms/processes, or their absence or partial expression, is considered in the context of the varied responses to dehydration shown by nonorthodox seeds. The factors that determine distinct variations in the behavior of recalcitrant seeds of individual species under the same conditions is given attention, with the effects of drying rate (i.e. the rate of water loss from tissues of desiccation-sensitive seeds) being stressed. Two different factors are distinguished in this regard: (1) damage that occurs at low water contents when nonfreezable water, which is held to stabilize intracellular structures and macromolecules, is removed, which is desiccation damage in the strict sense; and (2) damage that occurs during slow dehydration, when metabolic imbalances are proposed to cause the generation of damaging chemical species, e.g. free radicals, which is termed metabolic damage. Desiccation damage, in the strict sense, is attributed to the lack or inadequate operation of the processes/mechanisms held to protect desiccation-tolerant seeds in the dry state, while metabolic damage is considered in the context that nonorthodox seeds (especially those that are truly recalcitrant) do not possess the suite (or full suite) of mechanisms/processes that facilitate the acquisition and maintenance of desiccation-tolerance as exhibited by maturing and mature orthodox seed-types.

INTRODUCTION

Orthodox seeds (Roberts 1973) acquire desiccation tolerance during development and may be stored in the dry state for predictable periods under defined conditions. Unless debilitated by zero-tolerant storage fungi, orthodox seeds should maintain high vigor and viability at least from harvest until the next growing season (Berjak and others 1989) or for many decades at -18 °C (IBPGR 1976). Generally, such seeds undergo a period of drying during their maturation and are shed at low water content which is in equilibrium with the prevailing relative humidity (r.h.). The equilibrium water content at any particular r.h. is determined by seed composition, but all orthodox seeds can withstand dehydration to around 5 percent (0.053 g H_2O g^{-1} dry material [g g^{-1}]), even when maturation drying is not completed prior to shedding. *Any seed that does not behave this way is not orthodox*, and, in fact, the seeds of a great number of tropical species may accordingly be *nonorthodox*. Nonorthodox seeds have so far been described as either being *recalcitrant* (Roberts 1973) or *intermediate* (Ellis and others 1990a) according to their storage behavior.

Recalcitrant seeds are those that undergo little, or no, maturation drying and *remain desiccation sensitive* both during development and after they are shed. The situation is, however, far more complex than this because of the wide range of variability among recalcitrant seeds of different species and, indeed, of individual species under different conditions (Berjak and Pammenter 1997). Such seeds are shed hydrated, but the water content can generally be anywhere in the range from 0.43 to 4.0 g g^{-1}, which is 30 to 80 percent on a wet mass basis (wmb). Shedding water content is partly species characteristic, depending on the degree of dehydration that occurs late during seed development; this has been suggested to be correlated with the *degree of desiccation tolerance* developed by individual species (Finch-Savage 1996).

Recalcitrant seeds are not equally desiccation sensitive, in that variable degrees of dehydration are tolerated depending on the species. This implies that the processes or mechanisms (see below) that confer desiccation tolerance are variably developed or expressed in the nonorthodox condition. As diverse mechanisms have been suggested to be involved in the acquisition of desiccation tolerance and maintenance of the integrity of dehydrated orthodox seeds, it should be appreciated that any one of these may be absent, or present but ineffective, in recalcitrant seeds. Another important consideration is that desiccation tolerance is probably controlled by the *interplay* of mechanisms or processes, *and not by any one, acting in isolation*. Thus, the absence or incomplete expression of any factor proposed to confer dehydration tolerance could have profound consequences on the ability of a seed species to withstand a measure of dehydration below a particular level of hydration.

Differential desiccation sensitivity among recalcitrant seeds of various species is clearly shown by their different responses when subjected to the same drying regime–those of some species tolerating only a slight degree of dehydration, but others surviving to far lower water contents. There are also marked differences in the rates at which water will be lost from seeds of various species under the same dehydrating conditions (Farrant and others 1989). Other factors, too, influence the postharvest responses of recalcitrant seeds, e.g. developmental status (Berjak and Pammenter 1997, Berjak and others 1992, Berjak and others 1993, Finch-Savage 1996, Finch-Savage and Blake 1994) and chilling sensitivity (Berjak and Pammenter 1997).

In terms of desiccation sensitivity alone, therefore, it is not merely that a seed species *is* recalcitrant, but rather, *how recalcitrant* it is. This fact led to the proposal of a continuum of recalcitrant seed behavior, from species that are highly desiccation–and probably also chilling–sensitive, to those that will tolerate drying to the lowest water content still commensurate with recalcitrant seed behavior and will also tolerate relatively low temperatures (Farrant and others 1988).

The concept of a continuum of postharvest seed behavior (that is, dependent on preshedding developmental events) extends beyond the category of recalcitrant seeds. The continuum grades from extreme desiccation-sensitive types through the minimally recalcitrant types, to the intermediate seed species that do not react adversely to low temperatures, through those that are chilling sensitive when dehydrated (Hong and Ellis 1996), and finally to orthodox seeds that will tolerate less or more extreme dehydration (Vertucci and Roos 1990). It is also possible that there are seed species that behave in a manner that characterizes them as lying *between* the hitherto-defined categories: recalcitrant, intermediate, and orthodox. The idea of an extended continuum of seed behavior from the most desiccation tolerant of orthodox species, to the recalcitrant species that are most sensitive to even slight water loss, embodies many properties of seeds and their responses (Berjak and Pammenter 1994, 1997). It has its foundations in an appreciation of the physiological status of seeds at various water potentials (Vertucci 1993, Vertucci and Farrant 1995, Vertucci and Roos 1990) and the properties of water at the various hydration levels corresponding to specified water potential ranges (Vertucci 1993, Vertucci and Farrant 1995). It is more meaningful to consider seed responses to dehydration in terms of water potential rather than water content, but, since these two measures can be loosely correlated (Vertucci and Farrant 1995), the more familiar water content terminology is used here.

According to Vertucci and Farrant (1995): "Discrete changes in metabolic activity with moisture content are hypothesized to be associated with discrete changes in the physical properties of water… Thus upon the loss of water with certain properties, an essential function provided by [that] water is no longer possible. A tissue that is not damaged by the removal of a certain type of water has developed mechanisms to tolerate or avoid that particular stress." While the discussion that follows is not dependent on the reader's appreciation of the differences in the types of intracellular water, the basis of the arguments presented is that sequential removal of water with specific properties will have particular damaging effects on seed tissues that are not possessed of the appropriate mechanisms or processes to counteract that damage. We will, however, focus on the mechanisms or processes themselves.

MECHANISMS IMPLICATED IN DESICCATION TOLERANCE

It is most expedient to consider the processes or mechanisms listed below, which might confer protection against desiccation, and their deficiency or absence, which could contribute to the *relative degrees of desiccation sensitivity*.

- Intracellular physical characteristics such as
    - — reduction of the degree of vacuolation,
    - — amount and nature of insoluble reserves accumulated,
    - — integrity of the cytoskeleton,
    - — conformation of the DNA, chromatin, and nuclear architecture.
- Intracellular de-differentiation, which effectively results in the minimization of surface areas of membranes and probably also of the cytoskeleton.
- "'Switching off" of metabolism.
- Presence, and efficient operation of, antioxidant systems.
- Accumulation and roles of putatively protective molecules, including late embryogenic accumulating/abundant proteins (LEA's), sucrose and certain oligosaccharides, or galactosyl cyclitols.
- Deployment of certain amphipathic molecules.
- An effective peripheral oleosin layer around lipid bodies.
- The presence and operation of repair mechanisms during rehydration.

In the discussion that follows comparisons are made, as far as is possible, between desiccation-sensitive and orthodox seeds, and of the status of the processes or mechanisms that have been suggested to contribute to desiccation tolerance. Although the interrelationships among them are far from being resolved, these processes or mechanisms are those that have been implicated to date in the acquisition and maintenance of desiccation tolerance. However, it is important to realize that additional properties that contribute to the ability of seeds to withstand extreme dehydration are likely to be elucidated.

INTRACELLULAR PHYSICAL CHARACTERISTICS

Vacuolation and Reserve Deposition

In 1957, Iljin had already identified one of the major requirements of cells of desiccation-tolerant plant material: the ability to withstand mechanical stress. Vacuole volume reduction, whether by the shrinkage of the space occupied by these usually fluid-filled organelles or by their becoming filled with insoluble reserve material, is one of the mechanisms that would contribute to increased mechanical resilience of cells to dehydration. This aspect was examined by Farrant and others (1997) for (1) *Avicennia marina*, the highly recalcitrant seeds of which can withstand very little dehydration either before or after they are shed; (2) *Aesculus hippocastanum*, a temperate recalcitrant species, the seeds of which overwinter in the hydrated condition during which the necessary stratification occurs to facilitate germination the following spring; and (3) *Phaseolus vulgaris*, a typical orthodox seed that attains a low water content prior to shedding and is long-lived in this condition.

Avicennia marina seeds lose no water during development, and are as sensitive to dehydration before shedding as after abscission (Farrant and others 1992b). These seeds, at best, are unable to surive water contents lower than 0.5 g g^{-1} (33 percent wmb). The vacuoles ultimately occupy almost 60 percent on average of the volume across the cells of all axis tissues, and 90 percent of the cotyledonary cells when mature. At no stage do either the axial or cotyledonary vacuoles contain insoluble reserves, the little insoluble reserve material occurring as plastid starch. Seeds of *A. hippocastanum* naturally undergo a measure of dehydration during development, accompanied by an increase in relative desiccation tolerance (Tompsett and Pritchard 1993). The mature seeds are more desiccation tolerant than those of *A. marina*, being able to withstand dehydration to water contents in the range of 0.42 to 0.25 g g^{-1} (30 to 20 percent wmb). Vacuoles ultimately constitute only a small fraction of the intracellular volume, particularly in the axis cells at maturity. The cotyledonary cells contain many large, starch-filled plastids and protein bodies and are considerably less vacuolate than those of *A. marina*. In *P. vulgaris* seeds, which are orthodox and able to tolerate low water contents, vacuolar volume is reduced to an insignificant proportion in axis cells, and vacuoles in cotyledonary cells accumulate an amorphous, presumably insoluble, material. The differential degree of vacuolation and insoluble reserve deposition among the three species, in both developing and mature seeds, correlates with their degree of desiccation sensitivity. This is in accord with the concept that a high degree of vacuolation can lead to lethal mechanical damage upon dehydration (Farrant and others 1997).

Reaction of the Cytoskeleton

The cytoskeleton, the major components of which are microtubules and microfilaments, is not only an integrated intracellular support system, it also plays a major role in imposing organization on the cytoplasm and also the nucleus. Microtubules consist of polymerized a-tubulin, and microfilaments are composed of F-actin, which is a polymer of G-actin. We are presently investigating the status of the actin microfilaments in hydrated and variously dehydrated embryonic axes of seeds of *Quercus robur*, a temperate recalcitrant species. In the hydrated state, there is an extensive microfilamentous network in the cells of the root tip, which becomes dismantled as the seeds are increasingly dehydrated–a feature that is expected for orthodox seeds as well. In such desiccation-tolerant seeds, orderly reassembly of the elements of the cytoskeleton accompanies imbibition, but once the water content falls to damagingly low levels in *Q. robur*, the microfilaments are not reassembled when the seeds are subsequently rehydrated (Mycock and others 2000). The resultant lack of the intracellular support and structural organization afforded by the cytoskeleton would obviously be a major damaging factor upon rehydration of recalcitrant seeds. Additionally, certain cytomatrical (cytoplasmic) enzyme systems exist as multienzyme particles in plant cells (Hrazdina and Jensen 1992), the formation of which could occur because of the binding of key or anchor enzymes to the microfilaments of the cytoskeleton, as illustrated for glycolysis by Masters (1984). Thus, failure of the cytoskeleton to reassemble following deleteriously low levels of dehydration would have physiological as well as structural consequences in the cells of desiccation-sensitive seed tissues.

DNA, Chromatin, and Nuclear Architecture Conformation

Maintenance of the integrity of the genetic DNA material in the desiccated condition in orthodox seeds, and/or its rapid repair when seeds are rehydrated, is considered to be a fundamental requirement for desiccation tolerance. There is, however, little information on which to draw. DNA assumes different conformational states depending on water activity and, although this has not yet been demonstrated for seeds, it is considered that as water is lost (i.e. water activity is lowered) such conformational changes will occur (Osborne and Boubriak 1994). According to information reviewed by those authors, there is an increase in the number of base pairs per turn of the DNA helix as water is lost from the individually hydrated phosphate groups, and water bridges are formed instead as the conformation changes from the B to the Z form. Osborne and Boubriak (1994) have suggested that protein glycation (i.e. the nonenzymic addition of reducing sugars to [*i.a.*] histone proteins) is likely to occur, which could increase the incidence of DNA conformations appropriate to the dehydrated state. Those authors also discuss the possibility of nonenzymic methylation of cytosine occurring, which would favor the Z-form of the DNA.

However, besides the postulated necessity of conformational changes in the DNA occurring as desiccation-tolerant material is dehydrated, the structure of the chromatin itself must also be stabilized. The highly condensed state of the chromatin in dry, orthodox seeds (e.g. Crévecoeur and others 1976, Sargent and others 1981), which is reversed at the stage in germination when desiccation sensitivity ensues (Deltour 1985), is thought to be a visible manifestation of its stabilized condition. A major factor in chromatin stabilization in the dry state in orthodox seeds might be the change in the H1-histone:nucleosome ratio to 2:1 from the 1:1 ratio that typifies the hydrated condition (Ivanov and Zlatanova 1989).

Nuclear architecture is a further factor that is probably involved in chromatin stability. The structural framework of the nucleus has been convincingly demonstrated for plant cells and is based on intermediate-type filaments called lamins (Moreno Díaz de la Espina 1995). The nucleoskeleton, organized into the lamina (underlying and connected to the inner surface of the nuclear envelope) and matrix (ramifying throughout the nucleus) is suggested to support and localize the chromatin in discrete domains, imposing the topological organization and coordination of intranuclear processes (Moreno Díaz de la Espina 1995). It is implicit that during dehydration and in the desiccated state of orthodox seeds, orderly reorganization of the nucleoskeleton should occur with its restitution as a functional framework upon rehydration.

While little is known about the effects of dehydration on the DNA, chromatin, and nuclear architecture in desiccation-sensitive seeds, their stability in the dehydrated state clearly must be a prerequisite for desiccation tolerance. Maintenance of the integrity of the nucleus as a whole, and the genome in particular, may be imperfectly expressed, or the ability for this may even be totally lacking, in recalcitrant seeds. (For a fuller account of some of these aspects, see Leprince and others (1995) and Pammenter and Berjak (1999)). What is equally likely is that DNA repair mechanisms themselves are inadequate to restitute damage caused by dehydration of desiccation-sensitive seeds (see below).

INTRACELLULAR DE-DIFFERENTIATION

De-differentiation, a characteristic of maturing desiccation-tolerant seeds, is essentially a means by which intracellular structures are simplified and minimized (reviewed by Vertucci and Farrant 1995), which strongly suggests that membranes and cytoskeletal elements are vulnerable to dehydration. This phenomenon is reversed in orthodox seeds when water is taken up early during germination (Bewley 1979, Dasgupta and others 1982, Galau and others 1991, Klein and Pollock 1968, Long and others 1981).

An examination of the quantitative and qualitative status of mitochondria in seeds of *Avicennia marina*, *Aesculus hippocastanum*, and *Phaseolus vulgaris* showed that the proportion of cell volume occupied by these organelles was highest in *A. marina*, which is very desiccation sensitive, and substantially less for *A. hippocastanum*, which is in keeping with its less recalcitrant nature. In *P. vulgaris*, mitochondria occupied a significantly smaller proportion of the cell volume, even preceding the onset of maturation drying (Farrant and others 1997). Also, the mitochondria occupied a far greater proportion of the cell volume in the axis meristems of the two recalcitrant species than in the orthodox species, *P. vulgaris*. There were also marked differences in the structural complexity of the mitochondria among these three species: *A. marina* and *A. hippocastanum*, had well-developed cristae and a structure that was generally typical of an active, hydrated plant tissue; while in *P. vulgaris*, the mitochondria were almost completely de-differentiated even at tissue water contents comparable to those of the recalcitrant species at shedding (Farrant and others 1997). It thus seems that retention of organelles in the highly differentiated state is a major factor in the desiccation sensitivity of recalcitrant species, whereas the ability for ordered de-differentiation is, in fact, a prerequisite for seed survival in the dehydrated state.

There has long been uncertainty as to whether dehydration *causes* de-differentiation, or this intracellular minimization actually *precedes* the initiation of maturation drying (e.g. Vertucci and Farrant 1995). However, the observations on *P. vulgaris* reported by Farrant and others (1997), indicating that mitochondrial de-differentiation occurs, and that respiratory rate declines markedly (see also below) before maturation drying, support the idea that substantial qualitative and quantitative change actually occurs in advance of water loss.

"SWITCHING OFF" OF METABOLISM

Electron transport, albeit at a low level, has been recorded for dehydrated plant tissues, and respiration is measurable even at seed water contents as low as 0.25 g g^{-1} [20 percent, wmb] (Vertucci 1989, Vertucci and Farrant 1995). However, in the water content range 0.45 to 0.25 g g^{-1} (30 to 20 percent [wmb]), unbalanced metabolism may lead to the generation, and essentially uncontrolled activity, of free radicals (Finch-Savage and others 1994a, Hendry 1993, Hendry and others 1992, Leprince and others 1990b, Vertucci and Farrant 1995). It is therefore imperative that, during maturation drying, desiccation-tolerant seeds be able to pass through this water content range with the minimum of damage. The efficient operation of antioxidant systems (Leprince and others 1993, Puntarulo and others 1991), as well as the "switching off" of metabolism, would reduce such damage. Rogerson and Matthews (1977) recorded that a sharp decline in respiratory substrates precedes, and presumably causes, the fall in respiratory rate which, they suggested, is an essential event enabling an orthodox seed to withstand rapid loss of water. The observations of Farrant and others (1997), indicating that a decline in respiratory rate occurs while mitochondria become substantially de-differentiated *prior* to maturation drying in the orthodox seeds of *Phaseolus vulgaris*, support the data and suggestions of Rogerson and Matthews (1977).

In desiccation-sensitive seeds, lethal damage occurs in the water content range 0.45 to 0.25 g g^{1} (Vertucci and Farrant 1995) and, in some species, at considerably higher levels (Pammenter and others 1993). Death of relatively hydrated recalcitrant seeds (at *c.* 0.7 g g^{-1}, or higher [40 percent, wmb]) occurs when water is lost slowly. However, rapid dehydration rates allow survival to lower water contents (Farrant and others 1985). This observation led initially to the use of relatively rapid air-drying of excised embryonic axes to facilitate cryostorage (Normah and others 1986, Pritchard and Prendergast 1986) and later to the development of the flash-drying technique (Berjak and others 1990), by which the axes are dehydrated much more rapidly.

Flash-dried axes are *not* desiccation tolerant; on the contrary, they will not survive for longer than a day or two at best, under ambient conditions (Walters and others 2001) although they may be cryostored successfully (Wesley-Smith and others 1992). The desiccation sensitivity of recalcitrant material is the outcome of the fact that the axes (seeds) are actively metabolic, and the success of very rapid dehydration is that it minimizes the effects of this metabolism. This important point about drying rate is discussed in detail later.

Damage occurring in conjunction with unbalanced metabolism at these relatively high water contents should not be confused with desiccation damage in the strict sense. The latter describes the damage that occurs when water that is required to maintain the integrity of intracellular structures is removed (Walters and others 2001). Desiccation damage *sensu* *stricto* is the consequence of removing (any, or some, depending on the species) structure-bound, nonfreezable water (Pammenter and others 1991, Walters and others 2001). Lethal damage occurs upon loss of this water, even if flash-drying has successfully maintained axis viability to, or close to, this level of hydration (Pammenter and others 1991).

Another critical aspect of ongoing metabolism is cell cycling. The cell cycle describes the nuclear DNA content as 2C in cells that are not preparing for nuclear division, and as 4C in cells in which DNA replication has occurred, where the constant, C, denotes the DNA content of the haploid condition. During the cell cycle four distinct phases can be identified, *viz.* the G_1 phase (2C), which is followed by the S phase, during which DNA replication occurs; after this the cells enter the G_2 phase, during which the amount of DNA remains doubled (i.e. 4C) as a result of events in the S phase, and this is followed by the phase known as G_2M, when mitosis reduces the DNA content to the 2C level typical of somatic cells in the next G_1 phase. Brunori (1967) found that in orthodox *Vicia faba* seeds, most of the cells were arrested in G_1, and that DNA replication was one of the first events to be curtailed as the embryo cells lost water. S-phase replication is resumed only after several hours of imbibition, when water again becomes available to postharvest, orthodox seeds, as shown by Sen and Osborne (1974) for *Secale cereale* (rye): as soon as replication to 4C values occurs and the cells enter G_2M, desiccation tolerance is lost.

In the the highly recalcitrant seeds of *Avicennia marina*, there is only the most transient arrest of DNA replication in root primordia (meristems) of *Avicennia marina* lasting no more than 24 hours around shedding. This is the time when the seeds (although highly desiccation sensitive) are *relatively* most tolerant of water loss and least active. Ongoing cell cycling is associated with marked desiccation sensitivity of the DNA. When only 16 to 18 percent of the total water is lost from the *A. marina* material, there is a reduction of 70 to 80 percent in the nuclei that will incorporate thymidine, and after a 22-percent water loss, damage of the DNA cannot be repaired even when water is made freely available. Ongoing cell cycling, therefore, is another manifestation of the fact that metabolism is not “switched off,” at least in these highly recalcitrant seeds, which is considered to be a major factor accounting for their desiccation sensitivity. In related work on the temperate recalcitrant species *Acer pseudoplatanus*, however, cell cycling was found to be arrested, with over 60 percent of the cells in the 2C state (Finch-Savage and others 1998). However, seeds of *A. marina* are poised for immediate germination, while those of *A. pseudoplatanus* are dormant, requiring cold stratification before they will germinate. For seeds of *Azadirachta indica,* recorded as showing intermediate behavior, the 2C DNA level has been reported as occurring to the virtual exclusion of 4C (Sacandé and others 1997). These disparate results on the status of the cell cycle in three nonorthodox seed species serve to highlight the fact that different factors may contribute to the nature, and differing degrees, of desiccation sensitivity.

PRESENCE AND EFFICIENT OPERATION OF ANTIOXIDANT SYSTEMS

A range of antioxidant processes operate in orthodox seeds (e.g. Hendry 1993, Leprince and others 1993), and the role of such processes under conditions of water deficit and desiccation stress in plants has been reviewed by McKersie (1991) and Smirnoff (1993). As discussed above, it is particularly in the water content range from 0.45 to 0.25 g g^{-1} (30 to 20 percent, wmb), that unregulated metabolic events resulting in the first wave of free-radical generation are likely to occur (Vertucci and Farrant 1995). This implies that antioxidant systems (i.e. free-radical scavenging systems) should be maximally effective during maturation drying of orthodox seeds, and again when seeds take up water upon imbibition.

Reviews of metabolic damage associated with dehydration of recalcitrant seeds highlight the idea that free-radical generation may well be a major injurious factor (Berjak and Pammenter 1997; Côme and Corbineau 1996a, 1996b; Smith and Berjak 1995), particularly because protective mechanisms appear to become impaired under conditions of water stress (Senaratna and McKersie 1986, Smith and Berjak 1995). Rapid formation of free radicals and decreasing activity of antioxidant systems have been reported as occurring during dehydration of the seeds of the temperate recalcitrant species *Quercus robur* (Finch-Savage and others 1993). Lipid peroxidation, which is a major consequence of uncontrolled free-radical generation, with the ultimate accumulation of a stable free radical in the embryonic axes, has been shown to accompany dehydration of the seeds of three temperate, recalcitrant species—-*Q. robur*, *Castanea sativa,* and *Aesculus hippocastanum* (Finch-Savage and others 1994a)–and free radical formation has been reported to accompany viability loss in seeds of the highly recalcitrant, tropical species *Shorea robusta* (Chaitanya and Naithani 1994). While hydroperoxide formation has been shown to accompany dehydration at a range of temperatures of the recalcitrant seeds of *Zizania palustris*, significantly more was produced at 37 °C than at 25 °C, and tetrazolium tests revealed that viability was severely affected by water loss at the higher temperature (Ntuli and others 1997).

From the evidence reviewed above, there is no doubt that damage ascribable to uncontrolled free-radical generation occurs during dehydration in the recalcitrant seeds of a range of species that show differing degrees and manifestations of nonorthodox behavior. This implies not only that free radicals are produced as a consequence of water stress in these desiccation-sensitive seeds, but also that antioxidant systems are ineffective at curbing them. Together, then, these factors must be seriously considered as constituting one of the major causes of desiccation sensitivity.

ACCUMULATION AND ROLES OF PUTATIVELY PROTECTIVE MOLECULES

Late Embryogenic Accumulating/Abundant Proteins (LEA's)

LEA's (Galau and others 1986) comprise a set of hydrophilic, heat-resistant proteins associated with the acquisition of desiccation tolerance in developing orthodox seeds (Galau and others 1991 reviewed by Bewley and Oliver 1992, Kermode 1990, Ried and Walker-Simmons 1993). Their synthesis appears to be associated with the high ABA levels that peak during the later stages of seed development (Kermode 1990). The characteristics of LEA's and the conditions under which they appear have led to suggestions that they function as protectants, perhaps stabilizing subcellular structures in the desiccated condition (Close and others 1989, Dure 1993, Lane 1991).

The position of LEA's (or dehydrin-like proteins, as they may be termed) in nonorthodox seeds appears at first sight to be anomalous, as some species do not express these proteins while others express them to variable extents. Seeds of *Avicennia marina*, which are extremely desiccation sensitive, appear not to express LEA's at all (Farrant and others 1992a). In contrast, seeds of *Zizania palustris* (North American wild rice), which are recalcitrant (Vertucci and others 1994) but show differential responses to dehydration depending on temperature (Kovach and Bradford 1992a, Ntuli and others 1997), do express this type of protein (Bradford and Chandler 1992, Still and others 1994). Dehydrin-like proteins were shown to be expressed in a range of temperate, recalcitrant species (Finch-Savage and others 1994b, Gee and others 1994), but the absence of such proteins correlated with low ABA levels was found to characterize the mature, recalcitrant seeds of 10 tropical, wetland species (Farrant and others 1996). Those authors showed the presence of dehydrin-like proteins in other temperate and tropical recalcitrant (nonwetland) species, and suggested that their occurrence may be habitat-related, perhaps also providing protection against low-temperature stress. In a comparative study on mature seeds of two tropical tree species, neither of which occurs in wetlands, dehydrin-type proteins were absent in *Trichilia dregeana*, while accumulating in *Castanospermum australe* (Han and others 1997). The immature seeds and the seedlings of these two species were shown to differ in terms of production of such proteins in response to stresses imposed by dehydration, application of ABA, or exposure to cold, with *T. dregeana* not responding by the production of these putatively protective proteins (Han and others 1997).

Thus, it seems that the ability to express LEA's or dehydrin-type proteins cannot be taken as an indication that the seeds of a particular species will or will not withstand dehydration. This indicates clearly that desiccation tolerance must be the outcome of the interplay of more than one (and probably many) mechanisms or processes. Details of this, particularly pertaining to LEA's/dehydrins, sugars, and various stresses, have been reviewed by Kermode (1997). However, the variable expression of LEA's/dehydrins in recalcitrant seeds on a species basis may, in association with the presence or absence of other factors, account for the *degree* of nonorthodox behavior exhibited under a particular set of circumstances.

Sucrose, Oligosaccharides, or Galactosyl Cyclitols

The possible role(s) of nonreducing sugars in relation to desiccation tolerance in seeds has been extensively reviewed (e.g. by Berjak and Pammenter 1997, Horbowicz and Obendorf 1994, Obendorf 1997, Vertucci and Farrant 1995). Accumulation of nonreducing sugars, particularly of the raffinose series (Blackman and others 1992, Koster and Leopold 1988, Leprince and others 1990a) and/or galactosyl cyclitols (Horbowicz and Obendorf 1994, Obendorf 1997) has been implicated in the acquisition and maintenance of the desiccated state in orthodox seeds, generally in two major ways. These are in terms of the "Water Replacement Hypothesis" (Clegg 1986, Crowe and others 1992) and vitrification, otherwise referred to as glassy state formation (Koster and Leopold 1988, Leopold and others 1994, Williams and Leopold 1989).

Orthodox seed maturation invariably seems to be accompanied by the accumulation of nonreducing oligosaccharides which coincides with the reduction of monosaccharides, and maintenance of the desiccated state is associated with high levels of sucrose and other oligosaccharides. Evidence for the replacement of membrane-associated water (the Water Replacement Hypothesis, i.e. the replacement of water by sucrose to maintain lipid head-group spacing, thereby preventing gel-state transformation) is equivocal, and a recent critique questions its relevance in the desiccated state of orthodox seeds (Hoekstra and others 1997). However, the role of sucrose in the formation of intracellular glasses (i.e. vitrification) is more convincing. The metastable, glassy state occurs *at low water contents* in seeds, when sucrose and certain oligosaccharides or galactosyl cyclitols form high-viscosity, amorphous, super-saturated solutions (Obendorf 1997). The occurrence of glasses is held to impose a stasis on intracellular reactivity, protecting macromolecules against denaturation and possibly preventing or minimizing liquid crystalline gel phase transformations of the lipid bilayer of membranes (e.g. Leopold and others 1994).

Walters and others (1997) have suggested that a significant proportion of the sugars may be tightly associated with LEA's–these complexes acting to control and optimize the rate of water loss during dehydration of orthodox seeds. It should be noted, however, that this should not obviate the participation of either the LEA's or the sugars in the maintenance of orthodox seed viability in the desiccated state.

The formation of intracellular oligosaccharides occurs at the expense of monosaccharides, and confers the advantage that immediately available respiratory substrates are removed (Koster and Leopold 1988, Leprince and others 1992, Rogerson and Matthews 1977). This would serve to reduce the spectrum of damaging reactions that can occur as orthodox seeds pass through critical water content ranges favoring unbalanced metabolism, during maturation drying (see *"Switching off" of metabolism*, above).

Whatever the role(s) of sucrose and oligosaccharides or galactosyl cyclitols may be in orthodox seeds, *seeking parallels for desiccation-sensitive seeds is entirely inappropriate*. While sucrose and other oligosaccharides are produced in some of the few recalcitrant seed species that have been assayed (Farrant and others 1993, Finch-Savage and Blake 1994), glass formation will occur only at water contents *well below* the lethal limit. When recalcitrant seeds are dehydrated under ambient conditions (which is what would occur in the natural habitat), they lose viability at relatively high water contents–in the region of 0.7 g (or more) water per g dry mass [40 percent, wmb] (Pammenter and others 1991), which are *far* higher that those required for glass formation to occur (Bruni and Leopold 1992, Leopold and others 1994, Sun and others 1994, Williams and Leopold 1989). The same argument holds if water replacement by sugars is an operative phenomenon in orthodox seeds; this too would occur only at water contents of 0.3 g per g dry material (Hoekstra and Van Roekel 1988), which is well below the lethal limit for slowly drying recalcitrant seeds.

The one involvement of sugars in the variable desiccation sensitivity of recalcitrant seeds might be via the mechanism suggested by Walters and others (1997) for maturing orthodox seeds, *viz.* the modulating effect of sugar/LEA complexes on dehydration rate. Very marked variability occurs in the rate at which recalcitrant seeds of different species lose water under the same conditions (Berjak and Pammenter 1997, Farrant and others 1989) and it is possible that the significance of sugars and LEA's in embryos of recalcitrant seeds of some species lies in the modulation of the drying rate by complex formation. Walters and others (1997) have also suggested that LEA proteins in temperate recalcitrant seeds may play a role in their survival during overwintering.

DEPLOYMENT OF CERTAIN AMPHIPATHIC MOLECULES

It has been suggested that partitioning of endogenous amphipathic molecules (amphipaths) into membranes upon water loss may be a prerequisite for desiccation tolerance (Golovina and others 1998). Those authors have presented evidence of the movement during dehydration of both introduced, apolar spin probes and endogenous amphipaths into the bilayer of desiccation-tolerant pollen. This process, which was complete after dehydration to the relatively high water content of 0.6 g per g dry mass (37 percent, wmb), was reversed during rehydration, when the amphipaths repartitioned to the cytomatrix (aqueous cytoplasm). This reverse movement was suggested to account for the transient leakage that is invariably observed when desiccation-tolerant material (pollen and seeds) is imbibed from the dry state (Golovina and others 1998).

The partitioning of amphipathic molecules into the bilayer was suggested by those authors as serving to maintain the integrity of membranes in the dry state in desiccation-tolerant organisms, by substantially lowering the water content at which the phase change of membrane lipids occurs. Liquid crystalline to gel phase changes in membranes are well documented in response to dehydration, but the essential property for desiccation tolerance is that they must be reversible, reestablishing the membranes in a functional condition upon rehydration (Hoekstra and others 1992). This demands that integral membrane proteins retain their position in the desiccated state, a role that might also be ascribed to the amphipathic molecules.

If the partitioning of amphipaths into membranes is established as a universal phenomenon occurring during dehydration of orthodox seeds, it is possible that they are absent or, if present, incompletely functional or nonfunctional in desiccation-sensitive seeds. Dehydration of the embryos from recalcitrant *Camellia sinensis* seeds was found to induce a phase change in membrane lipids, which was reversible, but the proteins were irreversibly affected (Sowa and others 1991). It may be significant that at a water content of 0.6 g g^{-1}, when amphipath partitioning has been observed to be complete (Golovina and others 1998), slowly dried recalcitrant seeds, and even the flash-dried axes of certain species, will have lost viability (Pammenter and others 1991, 1993; also see below). In highly desiccation-sensitive recalcitrant seeds, it is possible that phase changes of the membrane bilayers might not be reversible, for example, if nonbilayer structures or hexagonal phases result (reviewed by Vertucci and Farrant 1995). Partitioning of endogenous amphipaths into the bilayer upon dehydration is unlikely to act in isolation; thus, even if such molecules are present in cells of recalcitrant seeds, they may well depend on another mechanism or process to achieve their reversible migration.

THE POSSIBLE ROLE OF OLEOSINS

The term *oleosin* refers to a unique protein type that surrounds the lipid (oil) droplets in plant cells (Huang 1992). Oleosins have a central, hydrophobic domain that interacts with the periphery of the lipid, and an amphipathic N-terminal domain that, with the C-terminal domain, facilitates interaction with the aqueous cytomatrix. The oleosin boundary of lipid bodies allows these hydrophobic masses to be accommodated as discrete entities in the aqueous cytomatrix under hydrated conditions, and it has been suggested that their role during dehydration prevents the bodies from coalescing in desiccation-tolerant seeds (Leprince and others 1997).

Leprince and others (1997) recorded a lack (or inadequate amount) of oleosins in desiccation-sensitive seeds of some species, and although little obvious change in the integrity of the bodies as a consequence of dehydration was observed, rehydration appeared to have deleterious effects on their stability. Coalescence of lipid bodies is a common abnormality accompanying deterioration, even in cells of stored, orthodox seeds (Smith and Berjak 1995). Although the effects of fungi associated with both stored orthodox and recalcitrant seeds in bringing about lipid body coalescence cannot be ruled out, the occurrence of this phenomenon could well be, at least partly, a consequence of some deficiency in desiccation-sensitive seeds. In view of the findings of Leprince and others (1997), the deficiency of an adequate oleosin sheath around the lipid bodies may underlie the inherent instability of these organelles during rehydration following damaging levels of desiccation of some recalcitrant seeds. However, it must be stressed that the presence of fully functional oleosins cannot, in itself, account for desiccation tolerance. Rather, it must be viewed as one of the mechanisms contributing to the spectrum of properties necessary if orthodox seeds are to survive extreme dehydration.

THE PRESENCE AND OPERATION OF REPAIR MECHANISMS DURING REHYDRATION

There is both indirect and direct evidence that repair mechanisms do come into play when dry orthodox seeds are rehydrated. For example, seeds that have been stored under adverse conditions, but are still 100-percent viable, typically show a lag before there are visible signs of germination, during which it is commonly accepted that repair processes are taking place. Ultrastructural studies on maize seeds have provided evidence supporting this contention, where mitochondrial repair was observed during the lag period (Berjak and Villiers 1972). Studies on rye seeds have shown that even in the dry state there is progressive deterioration of the DNA as a result of endo- and exonuclease activity during storage (Elder and others 1987), which cannot be repaired until the seeds are rehydrated (Boubriak and others 1997).

Much of the evidence for the operation of repair processes during rehydration comes from osmopriming experiments on low-vigor seeds. This process involves controlled rehydration to the end of phase II, which achieves a hydration level that facilitates repair but precludes germination proper (Bray 1995, Bray and others 1993). Those authors have shown that replacement of damaged rRNA occurs, and lesions in the DNA and protein-synthesizing systems are repaired, during priming.

It is generally agreed that free radical generation (see above) continues in air-dried orthodox seeds during storage (reviewed by Smith and Berjak 1995) and the ensuing damage obviously must be repaired on rehydration, arguing strongly for the presence and efficient operation of antioxidant systems at this stage. During dehydration of desiccation-sensitive seeds and seedlings, however, such systems have been shown to fail (Hendry and others 1992, Leprince and others 1992) and are assumed to remain ineffective when water is once again provided (Côme and Corbineau 1996a, 1996b).

When recalcitrant seeds or axes excised from such seeds are subjected to nonlethal dehydration, it is generally observed that there is an increase in the time taken for the onward growth of germination, which might be interpreted as facilitating repair. However, this is likely to be strictly limited; present studies have shown that after 22 percent of the water is lost from hypocotyl tips of *Avicennia marina*, dehydration-associated DNA damage can no longer be repaired when water is once again provided. DNA instability to dehydration is also shown by seedlings produced from orthodox seeds, once they have reached the stage when desiccation tolerance has been lost (Boubriak and others 1997).

Very little work that targets the aspect of possible repair of mature, dehydration-damaged recalcitrant seeds has yet been done. It is presently tacitly assumed that the necessary repair systems are present, but are themselves damaged by dehydration beyond certain limits–limits that might vary among seed species of markedly differing desiccation sensitivity. However, this aspect requires considerable investigation to obtain both qualitative and quantitative data to clarify the situation.

DRYING RATE - A VITAL FACTOR IN DETERMINING THE DEGREE OF DEHYDRATION THAT WILL BE TOLERATED

We now know that much confusion has occurred in comparative work on individual species of recalcitrant seeds because of conflicting data regarding "critical water contents," below which viability will be lost. This is because the dimensions of the *time* taken for water to be lost, or the temperature at which the drying experiments were carried out, have been ignored. While the effects of temperature will not presently be discussed, there are several publications focused on the seeds of *Zizania* spp. which show that this parameter can have very marked effects on the outcome of drying regimes and/or optimal storage water contents (Kovach and Bradford 1992b; Ntuli and others 1997; Vertucci and others 1994, 1995). The effect of the maturity status of the seeds–which is often extremely difficult to ascertain for recalcitrant types–also has significant effects on the degree of dehydration that will be tolerated (reviewed by Berjak and Pammenter 1997, Finch-Savage 1996) but also will not be taken further.

The aspect of the *time* taken for water to be lost is a variable that has been identified as having profound effects on the degree of dehydration that desiccation-sensitive seed material will tolerate. The more rapidly dehydration can be achieved, the lower is the water content to which the seeds or axes can be dried without damage accumulation that culminates in viability loss. This is particularly marked when excised axes are dried (Berjak and others 1993; Normah and others 1986; Pammenter and others 1991, 1993). Very rapid drying of excised recalcitrant axes (flash-drying) facilitates nonlethal dehydration to water contents in the region of 0.4 to 0.25 g g^{-1} dm, which is close to the hydration level where all the water is nonfreezable (generally structure-associated), although tolerance to such low water contents is not invariably the case (Pammenter and others 1993). It must be noted, however, that such rapid drying does *not* mean that the seed tissues are potentially desiccation tolerant; rather, the faster dehydration can be achieved, the less the time during which the axes are in the water content range that permits damaging, potentially lethal, aqueous-based reactions to occur. As discussed below, these are the processes that, given sufficient time, will cause viability loss at relatively high water contents when the tissues are dehydrated slowly (Berjak and others 1989, 1993; Pammenter and others 1998; Pritchard 1991). Far from actually being desiccation tolerant, axes from recalcitrant seeds will survive only for very short periods (hours to a day or two), at the lowest water contents attainable (Walters and others 2001).

Marked effects of drying rate on whole seeds are generally harder to attain, because seed size often prevents the achievement of suitably rapid dehydration. However, not all recalcitrant seeds are too large, or lose water too slowly, to facilitate the achievement of very different drying rates. The ability to achieve lower water contents while retaining viability has been recorded for whole seeds of *Avicennia marina* (Farrant and others 1985) and *Quercus rubra* (Pritchard 1991). We have recently carried out studies to ascertain the effects of drying rate on whole seeds of *Ekebergia capensis*, a tropical, meliaceous, recalcitrant species) for which markedly different drying rates can be achieved (Pammenter and others 1998). The results obtained illustrated the effects of drying rate dramatically: viability loss was already apparent in slowly dried seeds at high axis water contents [1.25 g water per g dry material (55 percent, wmb)] while those that were dehydrated rapidly showed unimpaired vigor and full germinability at an axis water content of 0.7 g g^{-1} (40 percent, wmb). Seeds dried at an intermediate rate retained viability to the intermediate axis water content level of *c.* 1.0 g g^{-1} (50 percent, wmb). Ultrastructural observations suggested that different damaging mechanisms bring about intracellular damage, depending on the drying rate. Advanced degradation of membranes, particularly of the plastids, and an abnormality of the lipid bodies occurred in axes from slowly dried seeds at water contents in the region of 1.1 g g^{-1} (52 percent, wmb) when viability had declined to 37 percent. The damage became steadily worse with slow drying to lower water contents, until, at 0.6 g g^{-1} (37 percent, wmb), only fragments of intracellular components remained. At a water content of 0.57 g g^{-1} (36 percent, wmb), axes from rapidly dried seeds (viability 80 percent) showed little signs of intracellular damage; it was only at considerably lower axis water contents that signs of deterioration were noted, which coincided with declining viability. At no stage did the extensive degradation that characterized axis cells from slowly dried seeds occur, supporting the proposal that if desiccation-sensitive material can pass quickly enough through water content ranges at which lethal reactions are prevalent, then it is possible to dry the material down to a far lower hydration level (see Vertucci and Farrant 1995 for discussion of the various hydration levels).

There will be a water content at which *rapidly dried* material that is desiccation sensitive will sustain injury, and, while the value varies from species to species, it is usually near the range where only structure-associated (nonfreezable) water remains (Pammenter and others 1991, 1993; Pritchard 1991). Damage occurring at such relatively low water contents is defined as desiccation damage in the strict sense (Pammenter and others 1998; Walters and others 2001) and is suggested to coincide with the perturbation of the nonfreezable water (Pammenter and others 1991). In contrast, desiccation-tolerant material can withstand the removal of a considerable proportion of this water (Pammenter and others 1991, Vertucci and Farrant 1995).

Slowly dried desiccation-sensitive material sustains damage at relatively high water contents, certainly those where solution (i.e. freezable) water prevails. This damage is suggested to result from aqueous-based, degradative reactions that are the result of unbalanced metabolism (Pammenter and others 2001; Walters and others 2001). Recalcitrant seeds (and, indeed, probably all nonorthodox types) are hydrated and metabolically active when shed (Berjak and others 1989, Berjak and Pammenter 1997). As water is slowly lost, metabolism will continue, but when the seeds are still at relatively high water contents, metabolism will become unbalanced or out-of-phase as a result of internal water stresses (Senaratna and McKersie 1986, Smith and Berjak 1995, Vertucci and Farrant 1995). A likely consequence of this unregulated metabolism will be the generation of free radicals and accompanying oxidative damage (Finch-Savage and others 1994a, Hendry 1993, Hendry and others 1992, Leprince and others 1990b). The severity of this type of damage, which is being termed metabolic damage (Walters and others 2001), is predicted to increase in inverse proportion to the drying rate, with viability loss occurring at increasingly high water contents.

CONCLUDING COMMENTS

It is proposed that nonorthodox seed behavior is a consequence of the lack of some, or perhaps all, of the suite of protective mechanisms or processes that together confer desiccation tolerance on orthodox seeds. There is likely to be a gradation in the presence and/or efficacy of the proposed processes/mechanisms among seeds of nonorthodox species, accounting for the variability of the responses to stresses, particularly that imposed by dehydration. The most desiccation-sensitive recalcitrant seeds are probably those that lack virtually all the protective and restitutional factors that facilitate the acquisition and maintenance of desiccation tolerance in orthodox seeds.

Two major factors are proposed to contribute to the loss of viability of recalcitrant seeds: (1) the consequences of unbalanced metabolism during dehydration [and possibly also when such seeds are stored in the hydrated condition (Smith and Berjak 1995)]; (2) desiccation damage in the strict sense, which occurs when water that is essential for the integrity of intracellular structures is removed; in recalcitrant seeds, this equates with nonfreezable water (Pammenter and others 1991).

We will probably be unable to account satisfactorily for nonorthodox seed behavior, particularly that of truly recalcitrant seeds, until complete understanding is gained of the apparently numerous interacting factors that enable desiccation-tolerance to be achieved.