Understanding poor storage potential of recalcitrant seeds

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Trees producing recalcitrant seeds are dominant among Asian (89%) and neo-tropical species (44%), but are rare among African tree species (9%). Among the families, Dipterocarpaceae is the most populous with 98% of recalcitrant species, followed by Meliaceae (17%), Moraceae (14%), Malvaceae (8%), Rubiaceae (7%) and Bignoniaceae (3%). Since desiccation-sensitive recalcitrant seeds are shed from the tree after maturation, with high moisture content, high metabolic activity and poor storage potential, it leads to significant challenge for *ex situ* conservation through seed-gene bank. A general understanding of the mechanisms underlying the poor viability potential of recalcitrant seeds is important to redefine our research programmes on these desiccation-intolerant seeds. There are three general categories of damage that may take place in recalcitrant seeds, eventually causing loss of viability: (i) mechanical damage, (ii) metabolism-induced damage, and (iii) macromolecular denaturation. This article discusses the occurrence of these three categories of damage, in the light of the various biochemical and structural changes reported in the different recalcitrant seeds, relative to (i) storage temperature, (ii) seed moisture content and (iii) rate of seed drying. Among these factors, maintenance of appropriate storage temperature is viewed as the most appropriate way to contain all the three types of damage. Storage of seeds in lowest suitable temperature is known to restrain the rate of moisture loss from seeds, thereby reducing mechanical damage and macromolecular denaturation; further, it can also minimize aberrant metabolism, eventually reducing metabolism-induced damage. Thus, the most practical way to extend the storage life of intact recalcitrant seeds is by storing them at lower temperatures, wherein the above damages can be reduced and at the same time chilling or freezing injury may not occur. Nevertheless, the 'lowest temperature' which is most suitable for seeds of a particular recalcitrant species should be standardized through dedicated research.

Keywords: Recalcitrant seed, seed damage, storage life, viability potential.

FORESTS act as a guardian for all the basic elements of this universe, viz. 'earth, water, air and fire', except the 'sky'. Forests purify the air, function as a reservoir of pure water and provide fuel wood besides nurturing the soil continuously. Trees have been central to the existence and civilization of human beings. Numerous descriptions of trees and their bond with the people of India are available in the Vedic literature. In Varahamihira's *Brihat Samhita* (c AD 700), detailed technical instructions have been provided on the kinds of trees which should be planted in a particular location[1]. According to Varahamihira the tank bunds should be shaded with mixed stands of *Terminalia arjuna* (Arjun; orthodox seed), *Ficus benghalensis* (banyan; orthodox seed), *Mangifera indica* (mango; recalcitrant seed), *Ficus religiosa* (Pipal; orthodox seed), *Syzygium cuminii* (Java plum; recalcitrant seed), *Mitragyna parviflora* (orthodox seed), *Borassus flabellifer* (Palmyra palm; recalcitrant seed), *Saraca asoka* (Asoka tree; orthodox seed), *Madhuca longifolia* (Mahua; recalcitrant seed), *Mimusops elengi* (Spanish cherry; orthodox seed), etc. Notwithstanding the cultural and historic emphasis on protecting forests and trees, deforestation of Indian forests was accelerated during European colonization due to intense colonial exploitation of timber resources. From 1850 to 1920, as much as 33 m ha of forests was cleared in India[2]. However, in the recent decades, India has been taking efforts to increase the current forest cover of 21.02% up to 33%, through the 'Green India Mission'. In order to achieve eco-restoration/afforestation in a period of 10 years, Rs 5.80 crores (2005–06) and Rs 4.64 crores (2006–07) were allotted to voluntary agencies, State Forest Departments and forest development agencies for raising quality seedlings in large-scale nurseries[3].

Success of a tree nursery critically depends on continuous supply of quality seeds. Many tree species produce seeds (or good seed crops) mostly in long intervals, ranging from a few to many years. Assured supply of seeds during the lean period can be achieved only from the seed stock held in storage[4]. Even if fruiting is regular and abundant every year, it may be more cost-efficient to collect surplus seed to cover the needs of few consecutive years[5]. Hence, efficient storage of seeds is indispensable to ensure continuous and cost-effective supply of tree seedlings, which is a prerequisite for the success of any massive afforestation programme. This apart, seed storage is also important for conserving the tree genetic resources which are ravaged by deforestation as well as by catastrophes such as forest fire, drought and floods.

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Storage potential of tree seeds is highly species-specific and large variation has been encountered across the tree species. Traditionally, based on their inherent storage potential, seeds have been grouped into two main categories, viz. recalcitrant and orthodox seeds[6].

Recalcitrant (desiccation-sensitive) seeds are metabolically active when shed from the mother plant and possess relatively high moisture content (0.4–4.0 g water/g). Even under ambient temperature and low relative humidity, their post-harvest life is very short, either days or months, depending on the species. Since they are sensitive to desiccation, seeds lose viability when their moisture content falls below 20–30%. Also, they cannot be stored in sub-zero temperatures due to ice formation[7,8].

Orthodox (desiccation-tolerant) seeds are not metabolically active when shed from the mother plant and possess relatively low moisture content (<15%). They can be dried to a very low moisture content and can retain viability for a long period of time under ambient temperatures and low relative humidity and at subzero temperatures (–18°C)[6]. Under ideal conditions, orthodox seeds can be stored for many years to decades or centuries, depending on the species[9].

Physiology of storage of orthodox seeds has been studied extensively. The loss of seed viability is generally attributed to free radical generation and lipid peroxidation, resulting in membrane damage as well as the generation of toxic by-products[10]. Oxidative damage to DNA and proteins is almost certainly involved in seed deterioration. Failure of repair mechanisms in cells, which comprise of a complex system of 'enzymatic and non-enzymatic' antioxidant defences to protect against the harmful consequences of activated oxygen species, is also likely to contribute to loss of viability[10]. Almost all the agricultural crops produce orthodox seeds. These seeds when sufficiently dried to safe moisture content have been successfully stored at least until next cropping season and at the most up to many years, depending on the storage conditions.

Another category of seeds which remain sensitive to dehydration both during development and when shed from the mother plant is termed as recalcitrant seeds. In horticultural crops, except vegetables, few species of fruits, most other seeds show recalcitrant behaviour, e.g. mango, citrus, nutmeg, avocado, jamun, litchi, curry leaf, etc.[11].

In the case of forest trees, orthodox seeds are more common than recalcitrant seeds. Of the approximately 8000 species for which data are available, more than 90% of seeds could tolerate drying to low water content[12]. Out of 225 species of a tropical seasonal forest in Panama, seeds of 32 species were desiccation-sensitive (recalcitrant) and 183 were desiccation-tolerant (orthodox)[13]. While summarizing the Kew database, it was found that, among the 345 species of tropical trees belonging to 15 families, more than 52% of the tree species (of 14 focal

families) were found to be recalcitrant[14,15]. Among the families, Dipterocarpaceae is one of the most populous with 98% of recalcitrant species, followed by Meliaceae (17%), Moraceae (14%), Malvaceae (8%), Rubiaceae (7%) and Bignoniaceae (3%). It was also reported that recalcitrant species are dominant among Asian trees (89%), followed by neo-tropical trees (44%), but rare among African tree species (9%)[14]. A detailed list of trees that produce recalcitrant seeds is provided in Table 1.

Recalcitrant seeds have a remarkably short life, particularly when stored in open air. Viability of certain species in families such as Dipterocarpaceae and Lauraceae lasts only for a few days or months, e.g. *Quercus*, *Populus*, etc. Seeds of *Shorea robusta*, which is common in both moist and dry forests, remain viable only for 7–10 days[16]. Most of the Malaysian dipterocarp species exhibit reduction in germination rate as soon as one week after dispersal[17]. The storage lifespan of recalcitrant seeds of tropical origin is quite short varying from two weeks to some months, while those of temperate origin like *Aesculus hippocastanum* or *Quercus robur* seem to have a longer storage lifespan of some months or 2–3 years respectively[18,19]. Since desiccation-sensitive seeds cannot

Table 1. List of popular horticultural and forest trees producing recalcitrant seeds

Common name	Botanical name	Family
Horticultural trees		
Citrus	*Citrus* spp.	Rutaceae
Nutmeg	*Myristrica fragrans*	Myristicaceae
Avocado	*Persea americana*	Lauraceae
Jack	*Artocarpus heterophyllus*	Moraceae
Jamun	*Syzygium cuminii*	Myrtaceae
Litchi	*Litchi chinensis*	Sapindaceae
Mangosteen	*Garcinia mangostana*	Clusiaceae
Durian	*Durio zibethinus*	Malvaceae
Rumbutan	*Nephelium lappaceum*	Sapincaceae
Curry leaf	*Murraya koenigii*	Rutaceae
Arecanut	*Areca catechu*	Arecaceae
Coconut	*Cocos nucifera*	Arecaceae
Cocoa	*Theobrama cacao*	Sterculiaceae
Coffee	*Coffea* spp.	Rubiaceae
Tea	*Camellia sinensis*	Theaceae
Clove	*Eugenia aromaticum*	Myrtaceae
Rubber	*Hevea brasiliensis*	Euphorbiaceae
Pepper	*Piper nigrum*	Piperaceae
Cinnamon	*Cinnamomum verum*	Lauraceae
Cassia	*Cinnamomum aromaticum*	Lauraceae
Forest trees		
Acer	*Acer* spp.	Aceraceae
Avicennia	*Avicennia* spp.	Avicenniaceae
Gurjan	*Dipterocarpus* spp.	Dipterocarpaceae
Giam	*Hopea* spp.	Dipterocarpaceae
Indian sal	*Shorea robusta*	Dipterocarpaceae
White oak	*Quercus alba*	Fagaceae
Shenbagum	*Michelia champaca*	Magnoliaceae
Arrayan	*Eugenia* spp.	Myristicaceae
Mangrove	*Rhizophora* spp.	Rhizoohoraceae

be dried safely, storage is only possible for short periods of time and therefore, poses a significant challenge for *ex situ* conservation through seed-gene bank.

Research on storage physiology of recalcitrant seeds has been carried out over many decades, although with little success in developing a viable storage method to extend their shelf life. Nevertheless, the spectrum of seed storage studies conducted has thrown ample light on two aspects: (i) the effect of storage atmosphere, viz. temperature and relative humidity, on seed viability percentage with the passage of time, and (ii) the underlying physiological causes for poor storage potential of desiccation-sensitive seeds. Excellent reviews by Berjak and Pammenter[20–23] are available on the physiological aspects of stored recalcitrant seeds; however, the main focal point of these reviews was to identify the crucial mechanisms or processes that may act as the critical cause for desiccation sensitivity. In this article, efforts have been made to specify yardsticks for fixing optimum storage atmosphere in terms of temperature and relative humidity, in order to extend shelf life of recalcitrant seeds on short-term basis.

Physiological basis for viability loss in recalcitrant seeds

The physiological basis of recalcitrant behaviour is not fully understood[24]. The loss of viability could be either due to the moisture content falling below a certain value or simply a general physiological deterioration with time[25]. Three possible categories of damage which may take place in recalcitrant seeds eventually leading to loss to viability have been suggested: (i) mechanical damage, (ii) metabolism-induced damage and (iii) macromolecular denaturation[18]. The type of damage reported in different recalcitrant seeds under specific conditions of storage is presented in Table 2. An overview in support of these biochemical and structural changes along with their implications on seed viability is given below.

Mechanical damage

Recalcitrant seeds are shed from the mother plant with high seed moisture content. Subsequently, with the passage of time, seed dehydration results in structural damage to the vacuoles, cytoskeleton and cellular membranes, culminating in loss of seed viability. Recalcitrant seeds possess prominent vacuoles both in the embryo and embryonic axis. In *Avicennia marina* seeds, the vacuoles ultimately occupy almost 60% of the average volume across the cells of the embryonic axis tissues and 90% of the cotyledonary cells when mature[26]. High degree of vacuolation can lead to lethal mechanical damage. Dehydration can lead to volume reduction and associated mechanical damage to the cells by way of collapse of the vacuoles, causing damage to both the embryos and

embryonic axes[27]. More the desiccation sensitivity, greater is the degree of vacuolation[28]. Mechanical damage of drying recalcitrant seeds is associated with volume reduction and collapse of vacuoles which act as an influential factor in causing damage to recalcitrant seeds[23].

Cytoskeleton which is constituted by microtubules and microfilaments plays a major role in imposing organization of the cytoplasm and nucleus[23]. The failure of the cytoskeleton of dehydrated recalcitrant seeds to reassemble following water imbibition is seen as a major damaging factor, because it leads to lack of intracellular support and structural organization. In the seeds of *Q. robur*, when the seed water content falls to very low levels, the microfilaments do not get reassembled when subsequently rehydrated[29].

Similarly, permanent damage to cell membrane systems of dehydrated recalcitrant seeds is seen as a major cause of seed viability loss. The increased demand for the bound water predisposes the seeds to desiccation sensitivity[7,30]. When the seeds of high moisture levels become dry, the membrane systems become irreversibly disrupted[18]. Therefore, unlike the orthodox seeds, upon water uptake, membranes of desiccation-sensitive seeds are not capable of reinstating the original structure of the cellular membranes to aid in complete reformation[31]. Consequently, when they are rehydrated during the imbibition stage of seed germination, solute leakage levels abruptly increase with simultaneous leakage of cellular contents ultimately resulting in cell death, which culminates in the loss of seed viability[32,33].

Taking into consideration all the above consequences of dehydration, it is proposed that the storage of recalcitrant seeds in conditions which favours 'very slow loss of seed moisture content', i.e. high relative humidity and low temperature, will help minimize mechanical damage to the seed vacuoles, cytoskeleton as well as seed membrane system, which will eventually result in extension of the seed viability period.

Metabolism-induced damage

In recalcitrant seeds, the embryos shed from the mother tree display high metabolic activity and high respiration rate. Even as the seed water is lost, aqueous-based metabolism continues but becomes unbalanced, leading to uncontrolled activity of free radicals as well as generation of reactive oxygen species (ROS) due to concurrent failure of the antioxidant system[34–36]. Free radical generation and associated generation of toxic by-products cause damage of cell membranes, proteins as well as DNA, resulting in the loss of seed viability[10]. This is termed as metabolism-linked damage[37]. The membrane-related physiological damages or an accumulation of by-products of biochemical enzymatic breakdown may be the basic cause for seed death[38]. In a study on physiological,

Table 2. Categories of seed damage occurring in different recalcitrant seeds at specific storage conditions

Recalcitrant species	Type of damage	Storage conditions	Reference
Mechanical damage			
Hopea hainanensis	Ultrastructure damage	15–20°C	49
Theobroma cacao	Desiccation damage to cell membranes	16°C; <80% RH	11
Antiaris toxicaria	Mechanical or physical damage	With silica gel at 15°C	96
Metabolic damage			
Camellia sinensis	High levels of ROS not effectively scavenged by increased levels of antioxidant enzymes	15°C; 15% RH	99
Carapa guianensis and *Carapa procera*	Lipid metabolism	27 ± 2°C; 40 ± 10% RH	39
Quercus alba	Damage to membrane lipids and protein secondary structure	Laboratory conditions	100
Ginkgo biloba	Unabated oxidative stress due to scarce regulation of antioxidants	25°C	101
Macromlecular denaturation			
Machilus thunbergii	Sub-cellular damages which include vacuolation and withdrawal of plasmalemma	25°C; 75% RH	50
C. guianensis and *C. procera*	Enlargement and accumulation of spherosomes	27 ± 2°C; 40 ± 10% RH	39

biochemical and ultra structural changes of *Carapa guianensis* Aubl and *Carapa procera* DC, for a period of 7–11 days, it was reported that seeds with intact moisture content revealed lipid composition metabolism leading to poor storage life[39].

In *Antiaris toxicaria* seeds, loss of desiccation tolerance was correlated with decline in the activity of antioxidases such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR), along with simultaneous increase in superoxide anion radical ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2) production, when subjected to slow drying[40]. Another report suggested that loss of metabolic balance may be the cause for loss of seed viability at intermediate levels of seed moisture content, due to production of ROS like superoxide and hydrogen peroxide culminating in lipid peroxidation[18]. Some examples of recalcitrant seeds which show free radical generation as the main cause of seed viability loss are *Q. robur, Castanea sativa, A. hippocastanum, S. robusta*, etc.[22,34,41].

Various reports have clearly established that free radical generation as well as ineffective antioxidant systems are instrumental in causing viability loss in recalcitrant seeds. Such aberrant metabolism is reported to take place under both high moisture content as well as in intermittent moisture levels reached during dehydration[42,43]. Therefore, it is obvious that curtailing aberrant seed metabolism of recalcitrant seeds is not possible by way of altering the seed moisture content. On the contrary, storage temperature is known to exert profound influence on the free radical formation in seeds. According to a study on the effect of temperature on seed storability of *Embelia ribes*, seeds stored at 0 ± 2°C, failed to germinate even after one week of storage, which might be due to freezing damage[44,45]. Among the remaining three storage temperatures, viz. 25 ± 2°C (ambient), 20°C and 10°C, seed germination was found to be better maintained at 10°C up to 4 weeks of storage (7%), while lower seed germination was recorded at 20°C (6%) and 25 ± 2°C (1%); the seed germination percentage recorded after the third week of storage was 12%, 10% and 3% respectively for the three temperatures. The results underscore that, lower temperatures – above freezing levels – are more effective in extending the seed viability period of recalcitrant seeds. Cold conservation has been reported to prevent oxidative damage which normally takes place during storage[46,47].

In the light of the findings discussed above, it can be concluded that metabolic damage inherent in the case of recalcitrant seeds cannot be controlled by altering the seed moisture content. However, storing the seeds at lower temperatures (above freezing temperatures) could be more effective in minimizing the seed metabolic activity and a possible method to minimize the aberrant seed metabolism and associated free radicle generation, leading to increased shelf life of seeds.

Macromolecular denaturation

Proper functioning of the cell organelles is a prerequisite for longer seed viability period. Among the cell organelles, mitochondrial integrity is an indication of cell respiratory potential, Golgi bodies are important for new membrane synthesis and polysome formation denotes the potential for *de novo* protein synthesis. Differentiation of mitochondria, membrane integrity and uninterrupted protein synthesis aided by the intact and efficient system of Golgi bodies and endoplasmic reticulum denote the able condition for cell metabolism or physiology[18]. In orthodox

seeds, which undergo maturation drying, all the cell organelles become de-differentiated and ultimately become simplified and minimized, thereby enabling seed survival in the dehydrated state. However, upon rehydration, the organelles regain the differentiation and effective functioning capacity. On the contrary, in recalcitrant seeds, which do not undergo maturation drying, organelles are retained in highly differentiated state, which upon dehydration become permanently damaged, unlike the orthodox seeds. Such damage to macromolecular structures is reflected in poor cell functioning or metabolism, which is a forerunner for poor viability potential. It was found that the embryonic axes of recalcitrant seeds undergo changes such as increase in cell size, extensive vacuolization, consumption of reserves and dysfunction of mitochondria, underscoring the damage caused to the cells by excessive drying in recalcitrant seeds[18].

Mechanical damage suffered by macromolecular structures due to dehydration is a physical damage (as discussed in the first section) and should not be confused with the macromolecular damage associated with the loss of cell metabolism (physiological damage), which occurs both during dehydration or during hydrated storage. For example, collapse of the vacuoles due to seed dehydration is termed as mechanical damage[27]. At the same time, formation of many vacuoles which become dilated and confluent after storage duration is termed as macromolecular denaturation[48].

A decrease in moisture content from 34.9% to 25.6% associated with various ultra structural changes, leading to decline in germination from 65% to 20% was observed in *Hopea hainanensis*[49]. Ultra structural studies in recalcitrant seeds of *Machilus thunbergii* by storing them for one or two weeks in sealed desiccators at 100% and 75% RH, and 25°C, revealed that in the metabolically active tissues of fresh mature embryo, the apical meristem of the plumule and radicle cells showed large nucleus with distinct nucleolus and only small valcuoles. The cytoplasm was typically compact with Golgi activity or polysome formation. Mitochondria showed well-defined cristae and relatively electron-transparent matrices. Plastids contained some starch grains and few oil droplets; however, in the cytoplasm no oil body was found. The germination percentage of such fresh seeds was about 94. When the seeds were stored in 75% RH for one and two weeks, the germination percentage decreased to 93 and 85 respectively. In such seeds, many subcellular damages were initiated, viz. increased vacuolation and withdrawal of plasma lemma. The damage was found to intensify as the storage days increased. After one week of storage, the mitochondria in the cells of plumule and radicle apical meristem were found to be less organized and there was a marked increase in vacuolation. After two weeks of storage, the cellular ultra structures were severely damaged. The results show that even at a relatively high relative humidity levels (75%) which enable slow dehydration of seeds, damage to macromolecules such as mitochondria, polysome, Golgi bodies, vacuoles, etc. could not be avoided, leading to acute damage to cell metabolism, culminating in the loss of seed viability[50].

The way in which hydrated storage affects the macromolecular structures and cell metabolic activity in recalcitrant seeds was studied in *A. marina*. Naked seeds of *A. marina* showed signs of incipient deterioration after 10 days of storage, which included 'unstacked' Golgi bodies, dilated endoplasmic reticulum (ER) cisternae and abnormal vacuoles. After 18 days the cells were found to be markedly deteriorated and by that time many vacuoles were formed and had become dilated and confluent[48].

Berjak and Pammenter[20] reviewed the reports published on ultra structure of recalcitrant seeds as affected by storage period, dehydration and hydrated storage. They envisaged that many of the ultra-structural developments that happen in recalcitrant seeds resembled the changes that occurred during early germination. They quoted the results of Berjak *et al.*[51], who reported certain curious changes in organelles, even after removal of 29% moisture from the seeds. The changes included the development of mitochondria and deposition of starch and dense material in the plastids, intracellular enhancement, mitochondrial development, appearance of Golgi bodies in root primordial and strong development of polysomes. All these changes suggested that in highly recalcitrant seeds of *A. marina*, immediately after being shed from the mother plant, germination metabolism was initiated. This reveals the lack of ability for 'metabolic switch-off' in recalcitrant seeds, which is however characteristic of orthodox seeds. Berjak and Pammenter[23] summarized that since such metabolism progresses without any obvious punctuation from seed development into germination, there is a strict reduction in the period for which the seeds can be stored. Therefore, even if recalcitrant seeds are stored in a hydrated state, at the water content at which they are shed, they will ultimately deteriorate due to initiation of germination metabolism, which cannot be 'switched-off'.

The above reports suggest that the seed ultra structure is prone to damage under both conditions of storage, i.e. normal and hydrated storage. Under normal conditions, wherein seeds undergo slow dehydration, macromolecular denaturation may occur by way of less organized mitochondria, increased vacuolation and withdrawal of plasma lemma, etc. as observed in *M. thunbergii*. In highly recalcitrant seeds of *A. marina* which are shed when seed moisture content is very high, the seeds may entrain germinative metabolism soon after shedding[52]. On the other hand, under hydrated condition and during very slow hydration, changes that happen in macromolecules resemble those that occur during early germination, which affect the lifespan of the stored recalcitrant seeds. These results underscore that irrespective of dehydration levels, macromolecular denaturation is bound to happen

in recalcitrant seeds, ultimately leading to loss in viability.

The studies on functioning of macromolecules in stored seeds of different recalcitrant species, have unequivocally established that ultra-structural damages cannot be curtailed either in hydrated or dehydrated conditions. However, Berjak and Pammenter[21] suggested that the metabolic progress which leads to denaturation of macromolecules can be limited by storing the seeds at low temperatures. In this context they proposed that, preliminary trials should be conducted to ascertain the lowest temperature which will limit the metabolic progress, so as to achieve extended period of seed viability.

Role of storage atmosphere in viability loss

The changes in seed physiology and shelf life of recalcitrant seeds under varied conditions of storage atmosphere need considerable attention because they form the basis for improving the shelf life of stored seeds. Seed storage in terms of temperature, moisture content and rate of drying has been researched at large and is reported to have serious implications on the maintenance of their shelf life. Roberts[7] reported that the viability of recalcitrant or desiccation-sensitive seeds is affected by moisture loss and/or cold temperature. According to Justice and Bass[53], the interrelations among temperature, moisture content and storage time impact the shelf life of seeds. However, the extent to which each of the factors acts as the basal or critical cause for seed deterioration is debatable.

Temperature

Seed storage temperature has been vastly researched to unearth its impact on viability potential of recalcitrant seeds. In many of the studies conducted, storability performance of seeds that were stored under ambient room temperature was compared with those stored at lower temperature. The effect of temperature and relative humidity on storage life of seeds *of Shorea javanica* K&V (Dipterocarpaceae) was studied by Umboh[54]. The seeds were stored at three temperatures: room temperature ($27 \pm 2°C$), air-conditioned room ($20 \pm 2°C$), and in a refrigerator ($<10°C$) by simulating three relative humidity conditions, viz. 20%, 66% and 86% respectively. At $27 \pm 2°C$ and 86% RH, the moisture content of the seeds was stable for even up to 30 days, but it showed a slow decrease under other RH conditions. At $20 \pm 2°C$, the moisture content of the seeds was stable up to 30 days under the high RH range 66–86%, while at 20% RH the moisture content drastically decreased even from day 7 onwards. Obviously, under $20 \pm 2°C$ (air-conditioned room), seed germination was maintained well above 80% irrespective the relative humidity, even after 30 days of storage. However, when the seeds were stored at a higher

temperature of $27 \pm 2°C$ (ambient), the seed germination percentage reduced to almost zero even within 14 and 30 days at relative humidity of 20 and 66 respectively. It is comprehensible that compared to ambient temperature ($27 \pm 2°C$), seeds stored in air-conditioned room ($20 \pm 2°C$) could better maintain the seed germination percentage, owing to lower levels of seed dehydration witnessed irrespective of the relative humidity.

Sivalingam et al.[44] conducted an experiment to compare the effect of ambient temperature ($25 \pm 2°C$) with that of lower temperatures such as 20°C, 10°C and $0 \pm 2°C$, evaluating the seed germination percentage of *E. ribes* at the end of 1, 2, 3 and 4 weeks of storage. Seeds stored at 10°C retained viability for 3 weeks with 12% germination. At this temperature alone the viability of seeds could be prolonged up to 4 weeks with 7% germination whereas with the increase in temperature there was decrease in seed germination percentage to the tune of 6 and 1 in 20°C and $25 \pm 2°C$ respectively. The results envisage that lower temperature is comparatively superior in maintaining the seed viability of recalcitrant seeds of *E. ribes*. On the contrary, seeds stored at $0 \pm 2°C$ failed to germinate even after 1 week of storage, which might be due to freezing damage[45].

Sukesh and Chandrasekar[55] compared the storage performance of *Vatica chinensis* by storing the seeds at $28 \pm 2°C$ (ambient), $20 \pm 2°C$, and $12 \pm 2°C$, after packing them in sealed polythene bags. The germination percentage was found to decrease to almost to 50 on the fifth day when stored at a higher temperature range of $28 \pm 2°C$ (ambient) and $20 \pm 2°C$; later on there was a drastic decrease leading to complete viability loss by the 19th and 21st days respectively. Among the two temperatures, ambient temperature ($28 \pm 2°C$) was found to be more detrimental to seed storage life. However, the results demonstrated that with further decrease in temperature to $12 \pm 2°C$, loss of seed viability was more rapid, leading to complete loss of viability even by the 15th day of storage.

The results obtained in all the above studies have shown that irrespective of the species, compared to ambient temperature ($25 \pm 2°C$), lower temperature helps improve seed viability potential in many species. But below a certain limit, low temperatures have been reported to cause detrimental effect on seed viability of many recalcitrant species. Chilling temperatures have been predominantly found to be detrimental to many recalcitrant seeds of dipterocarps such as *Drybalanops aromatic*[56] *Shorea curtesii*, *Shorea platycladus*[57], *Shorea ovalis*[58] and *Hopea odorata*[59]. However, temperatures well above 0°C have also been reported to induce total loss of viability in several species like mango[60] (5–10°C), mangosteen[61] (10°C) and rambutan[62] (6°C). Roberts[6], and Pritchard et al.[63] had also warned that low moisture content and sub-zero temperature storage will not reduce, but in most cases accelerate loss of viability of recalcitrant

Table 3. Species-specific optimum seed storage temperature for extending the storage

Species	Optimum temperature (°C)	Viability period*	Reference
Shorea javanica	20 ± 2	30 days (54.2%)	54
Vatica astrotricha	25	3 months (15.5%)	102
Hopea hainanensis	15	6 months (70.5%)	102
Acmena acuminatissma	15	3 months (50.5%)	102
H. hainanensis	15	14 months (~90%)	79
Carica papaya	28	3 months (32%)	103
Embelia ribes	10	4 weeks (7%)	44
Vatica chinensis	20 ± 2	15 days (29.5%)	55
Ginkgo biloba	4	12 months (7%)	101
Livistona chinensis	15	6 months (45%)	104

*Figures in parentheses indicate seed germination percentage.

seeds. Low temperatures may become detrimental to seed viability in two ways, i.e. freezing injury and chilling injury. With slow freezing, cells may damage the seeds due to desiccation[64], while rapid freezing may be lethal to the seeds due to formation of ice crystals[45,65]. Besides, the sub ambient temperature may also cause chilling injury to the seeds since damages such as protein denaturation[66] or changes in membrane thickness and permeability[67] may occur resulting in loss of seed viability.

Optimum temperature for extending the seed viability period was found to largely vary with the species. Seeds of *Hopea* species could tolerate 4°C (ref. 68); *Trichilia emeria* seeds were damaged at 6°C (ref. 69) and *Theobrama cacao* succumbed to temperatures below 10°C (ref. 19). The way a particular recalcitrant species responds to storage temperature has been attributed to aspects such as ecology as well as seed maturity levels. In *H. odorata* Roxb, seed viability decreased during storage at 10°C (ref. 59) while *H. quisumbingian*, *H. beccariana*, *H. montana*, *H. dyeri*, *H. altocollina* and *Hopea pterygota* could tolerate storage temperatures lower than 10°C by prolonged longevity[70]. It was inferred that species which were adapted to high altitudes (1000–1600 m amsl) tolerated storage temperatures lower than 15°C and 20°C. Chin and Roberts[19], and Yap[17] found that sensitivity to lower temperatures was higher in tropical recalcitrant seeds with high moisture content. Goveia *et al.*[71] found that seed maturity status strongly influences the optimum storage temperature. According to them, *Trichilia dregeana* seeds which are shed from the trees before complete development stored well for several months at 16°C.

Thus both higher ambient temperature (25 ± 2°C) as well as very low temperatures (0–10°C) were detrimental to storage life of recalcitrant seeds. However, the exact range of temperature at which the seed viability is affected is highly species-specific. It is thus recommended that further efforts should be devoted to standardize the lowest compatible storage temperature, specific to a particular species so as to achieve maximum storage life of recalcitrant seeds. The criteria for defining the optimum

temperature should focus on (i) attaining lowest desiccation rate to minimize mechanical damage to the seed vacuoles, cytoskeleton as well as seed membrane system; (ii) reducing the extent of free radical generation by minimizing the metabolism-induced damage, and (iii) avoiding freezing damage by formation of ice crystals.

Moisture content

High seed moisture content is characteristic of any recalcitrant seed, since seeds do not undergo maturation drying before they are shed from the mother plant. It has been reported that germination of desiccation-sensitive seeds declines rapidly as seed moisture content decrease[72–75]. Seeds of *Hopea parviflora* Beddome and *Hopea pomga* (Dennst.) Mabb., lost their germination potential when dried to 26% and 28% moisture content (wet mass basis) respectively[76]. For storage of *H. hainanensis* seeds, relatively high moisture content (33–38%) and temperature (15–20°C) were recommended because when stored at 18°C and 80% RH, seeds with 36–38% moisture content were found to be viable even after one year[77].

Asomaning *et al.*[78] studied the relationship between seed moisture content and germination capacity of recalcitrant seeds of *Garcinia kola*. They observed that with 58% moisture content, the seeds recorded 80% germination, but as the moisture content was reduced to 25–27%, the seed germinability almost reduced to 10% or less due to gradual loss of viability. Lan *et al.*[79] demonstrated that fresh seeds harvested at maturity and not subjected to a drying treatment have much higher germination and production of normal seedlings than seeds that have been dried for 1–2 days and stored at 4°–20°C.

When the moisture content of *H. hainanensis* seeds was reduced from 34.9% to 25.6%, the germination percentage declined from 65 to 20, accompanied by various types of ultrastructural damage[49]. Farrant *et al.*[28] concluded that high degree of vacuolation is the main reason for the occurrence of lethal mechanical damage in recalcitrant seeds upon dehydration. Asomaning *et al.*[78] found

that in *G. kola*, as seeds were dehydrated from 50% moisture content to lower moisture levels, they recorded higher electrical conductivity revealing significant damage to the cellular membrane upon dehydration.

Obviously, reduction in seed moisture content is closely linked to seed decay and death of recalcitrant seeds. However, it has been proposed that seeds can be dried to a certain level without much damage to their physiological potential. The safe limit up to which seeds can be dried without apparent damage is termed as 'critical water content'[80]. Tompsett[81] found that the lowering of seed moisture up to the lowest safe moisture content (LSMC) does have much effect; however, below this level the seed viability decreases drastically. Many reports suggest that generally critical moisture content is slightly higher than the level of water which is non-freezable (≤ 0.35 g g^{-1}; refs 82–86).

Rate of drying: The proclamation that 'drying' of seeds is deleterious to viability of recalcitrant seeds is unanimous; however, the opinion on the effect of 'rate of drying' on viability potential is highly variable. The effect of drying rate on desiccation sensitivity of recalcitrant *A. marina* seeds was first studied by Farrant *et al.*[30]. Since then many studies have been made on the effect of drying methods on desiccation tolerance and cryopreservation of recalcitrant seeds or excised axis[1,82,83,87–90]. Rapid dehydrations has been reported to facilitate seed survival at lower water content compared to slow drying[30,51,83]. Pammenter *et al.*[89] suggested that if dehydration is slow, the seeds will have to spend extended time at 'intermediate' water content, wherein aqueous-based metabolism-linked damage accumulates overwhelming results in loss of seed viability even at relatively high water content, e.g. around 1.0 g g^{-1} for *Ekebergia capensis* axes.

According to Liang and Sun[91], when recalcitrant seeds are subjected to very slow drying conditions, seed axes may be damaged by various deleterious processes ranging from the disruption of metabolic regulation to the failure of antioxidant systems. Further, it was also hypothesized that uneven distribution of water in the seed tissues could improve the desiccation tolerance in the fast-dried seed axes[75,89]. Tang *et al.*[92] found that loss of viability of *Mangifera persiciformis* seeds was faster during slow drying than during rapid drying. In the rapid drying treatments, seed moisture was reduced to 24.7% by 24 h and 94% of seeds germinated. However, seed viability decreased gradually to 53.2% when dried down to 18.2% moisture content and then sharply decreased with further desiccation. The moisture content of non-viable seeds dried for 96 h was 13.7%. In comparison, during slow drying, loss of viability was faster. Moreover, the moisture content resulting in 50% viability loss was higher in slow than fast-dried seeds (about 24% versus 20%).

On the contrary, Liang and Sun[91] reported that rapid drying at low RH and slow drying at high RH were both

harmful to *T. cacao* seed axes. They observed that electrolyte leakage began to increase and axes viability began to decrease at high water content. They suggested that under low RH the water potential of seed axes will change rapidly. As a result, the uneven and rapid volumetric change would inevitably induce great damage within the well-organized seed tissues (and also cells), unless the seed tissues are able to withstand such enormous mechanical stress. Thus, the effect of drying rate on desiccation tolerance is associated not only with the regulation of metabolism (the physico-chemical aspects), but also with the physical process of dehydration itself (the mechanical aspects)[91]. Based on the studies on effects of dehydration rate on *T. cacao* and *G. biloba* axes, Liang and Sun[93] also concluded that desiccation damage under rapid drying appeared to be mainly resulting from 'mechanical or physical stress', while damage under slow drying is likely to be a consequence of physio-chemical damage induced by 'metabolic alteration and damages'.

Berjak and Pammenter[23] confessed that it seemed paradoxical to discuss the effects of drying rate when addressing the implications of the lack of desiccation tolerance of recalcitrant seeds. They also concluded that, neither the embryo nor embryonic axis of recalcitrant seeds is desiccation-tolerant nor does rapid dehydration infuse such tolerance. Even when dried rapidly, recalcitrant seeds will not survive for longer than a day or two under ambient or above zero-refrigerated conditions[37]. However, such dehydrated tissues may be amenable for cryopreservation.

Relative humidity: Higher the relative humidity, higher will be the moisture content of the seeds[53]. It is also known that as temperature is increased by exposing the seeds to a constant relative humidity, the seed moisture content decreases. Kadam *et al.*[94] monitored the viability and germination of *Citrus limonia* seeds for six weeks in open and refrigerated conditions (10°C; 45% RH). Both parameters showed decline during storage that was least when the seeds were refrigerated at 10°C with 45% RH.

Umboh[54] reported the effect of temperature, relative humidity and moisture content on *Shorea javanica*. According to him, at 20°C, irrespective of the RH, viz. 60%, 66% and 86%, seeds recorded very good germination capability (above 80%) even after 30 days of storage. When the relative humidity decreased drastically to 20%, the moisture content decreased and it negatively influenced the germination capacity at both 27°C and 20°C. The results showed that rapid drying is deleterious to the shelf life of *S. robusta*.

Silica gel: Asomaning *et al.*[78] compared the viability loss of *G. kola* when subjected to drying over silica gel (relatively fast drying) and in shade (slower drying). They observed that the rate of drying did not alter the critical moisture content. In all cases, whether seed or

seed parts were dried in silica gel or under shade, viability was drastically reduced below this critical moisture content (30–32%). Bonner[95] found that at 27°C, rate of desiccation made no difference in the lethal moisture level for intact seeds of *Quercus nigra*. Pammenter *et al.*[52] reported that rapid drying favoured viability retention in excised embryos of *A. marina* Forsk. However, excised embryos do not always react in the same manner as intact seeds during desiccation[95].

Xin *et al.*[96] studied the relation between oxidative damage and viability loss of excised embryonic axes of *Antiaris toxicaria* when subjected to rapid drying with silica gel at 15°C. They reported that viability loss of axes of *A. toxicaria* under rapid drying with silica gel appeared to be associated with mechanical or physical damage, rather than metabolic damage.

Irrespective of the species, researchers have claimed that recalcitrant seeds which are shed with high moisture content undergo two types of damage: (i) mechanical or physical stress, and (ii) physiological damage due to metabolic activity. Storage studies upheld that slow drying at ambient temperature may help delay the time taken to cross the critical seed moisture content, thereby reducing the mechanical or physical damage to the seeds. However, at such seeds with moisture content are predisposed to physiological damage due to metabolic activity, which is equally detrimental to seed viability. Thus, it is proposed that slow drying at lower temperature may be adopted to reduce both mechanical damage as well as metabolic damage. Rapid drying at ambient temperatures, however, may be experimented to achieve cryopreservation of embryonic axes of recalcitrant seeds.

Concluding remarks

The three major factors which influence the damages caused to stored recalcitrant seeds are (i) seed moisture content, (ii) rate of drying, and (iii) storage temperature. Since the recalcitrant seeds have high seed moisture content, maintenance of seed volume by retaining high seed moisture content is quintessential to avoid mechanical damage to the cell membrane as well as macromolecules. However, at high level of moisture content, seeds are exposed to yet another type of damage, i.e. metabolism-linked damage caused by ROS-mediated lipid peroxidation, protein and DNA denaturation. Obviously, this conflicting role of seed moisture content may be the reason for divergent reports on critical seed moisture content for various recalcitrant seeds. Roberts[6], and Pammenter *et al.*[82] reported that critical moisture content normally varies between 21% and 31%. However, several species require higher levels of moisture content, e.g. *Symphonia globulifera* (67%), *H. ponga* (54%), *Simarouba amara* (39%), *H. odorata* (33%)[97]; *V. chinensis* (62%)[55]; *H. hainanensis* (35–38%)[49,77]; *H. helferi* (47%)[98] and *H. ver-*

vosa (43–50%)[58]. Since reduction in moisture content *per se* is deleterious to seed shelf life, 'rate of drying' cannot augment extension of seed storage life. The third factor that influences recalcitrant seed viability period is storage temperature. Maintenance of appropriate storage temperature is viewed as the only way to contain all the three types of damage caused to recalcitrant, seeds, viz. (i) mechanical damage, (ii) metabolism-induced damage, and (iii) macromoecular denaturation. Storage of seeds at the lowest temperature will help minimize moisture loss[21], thereby preventing mechanical damage and macromoecular denaturation; further it can also minimize aberrant metabolism, eventually reducing metabolism-induced damage. Cold conservation has been reported to prevent the oxidative damage which normally takes place during storage[46,47]. Thus, the most practical way to store intact recalcitrant seeds is by keeping them at lower temperatures, wherein the above-mentioned damages can be reduced, seed associated mycoflora can be minimized and freezing injury may also be avoided. However, the lowest temperature which can be tolerated by the seeds may vary with the tree species, which obviously necessitates dedicated research. Further, there is a need to sub-classify seed recalcitrance into high, moderate and low levels of desiccation tolerance. Subsequent studies on recalcitrant seeds should focus on deciphering the specific effects of storage temperature and relative humidity vis-á-vis the level of desiccation tolerance with respect to all the three categories of damage discussed here. This will help develop more precise storage technologies, specific to each species by containing the type of damage to which a particular species is more susceptible. Thereby the post-harvest life of such recalcitrant seeds can be effectively extended.

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