

Storage of recalcitrant seeds: a case study of the Chinese fan palm, *Livistona chinensis*

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Summary

Various seed storage techniques were applied to the recalcitrant seeds of the Chinese fan palm (*Livistona chinensis* [Jacq.] R. Br.). Fully hydrated seeds were stored in perlite at 5 moisture levels at 15°C, and partially dried seeds were stored at 6 moisture levels at 4 temperatures. Cryopreservation was attempted with both intact seeds and isolated embryos. It was found that *L. chinensis* seeds can not survive sub-zero temperature storage for even one week, but short-term storage at above-zero temperature is feasible. Moist storage of fully hydrated seeds did not impair seed viability, but led inevitably to germination and fungal growth after one month. Partially dried seeds began to lose viability after 3 months storage. Intact seeds can not survive cryo-exposure at any moisture content. By contrast, embryos were successfully stored at liquid nitrogen temperature after desiccation to below 20% moisture content, and no significant viability loss was found after 2-year cryostorage. The advantages and disadvantages of these storage methods are evaluated.

Introduction

Storage of seeds is widely practiced in horticulture and germplasm conservation, and arguably the most effective and efficient method for the *ex situ* preservation of plant genetic resources (Pritchard, 1995). However, seed storage behaviour varies from one species to another. 'Orthodox' seeds can be routinely stored for long term in seedbanks with practical and economic benefit; whilst 'recalcitrant' seeds cannot because they are always sensitive to dehydration and freezing which are the conditions for storage of orthodox seeds. Low moisture content and sub-zero temperatures storage will not reduce, but in most cases accelerate, loss of viability of recalcitrant seeds (Roberts, 1973; Pritchard, 2004). The long-term preservation of such seeds is still an unsolved problem for most species.

In the past three decades, seed scientists have developed several methods to store recalcitrant seeds, such as 'moist' storage, partially dry storage and cryostorage, but all these methods have their own limitations (Chin, 1989). The efficacy of recalcitrant seed storage depends on both the method used and the attributes of the species. However, for the vast majority of recalcitrant seeds a general method for long-term storage has not yet been developed. So it is necessary to evaluate the feasibility of a storage method for a recalcitrant seed species on a species-specific basis.

The Chinese fan palm, *Livistona chinensis*, native to China, is a monocotyledonous angiosperm tree species. It is useful as it produces good fibre which can be made into ropes, while its leaves have been made into traditional Chinese fans since ancient times. Because of its elegance, it is probably the most popular of all fan palms as a tub plant for indoor use, and is also widely grown as an outdoor plant in tropical and subtropical areas. However, its seeds are short-lived, which makes its germplasm conservation a problem. This study characterizes the effect of temperature and moisture content on seed survival.

Materials and methods

Plant materials

Fruits of Chinese fan palm, *L. chinensis*, were collected from trees growing in Menglun, Mengla, Xishuangbanna, China, where Xishuangbanna Tropical Botanical Garden of the Chinese Academy of Science is located. Because of the influence of the southwest monsoon, the collecting location (21°41' N, 101°25' E; altitude 580 m) has distinct dry and rainy seasons with an annual mean temperature of 21.8°C and rainfall of 1493 mm per year, 84% of which falls during the rainy season, May–October (Wen and Song, 2007).

L. chinensis produces new inflorescences from November to April and mature fruits are available from the following October until May. Flowers in the same inflorescence all open at about the same time. Fruits from whole inflorescences were collected; seeds were removed from the fruits manually and stored in polyethylene bags at 15°C until use up to one week after collecting.

Moisture content determinations

Moisture content of plant material was determined gravimetrically after oven drying at $103 \pm 2^\circ\text{C}$ for 17 h. Eight single fruits or seeds or eight sets of 5 embryos were used for each of these determinations. Moisture content was expressed on a percentage fresh weight basis.

Seed viability evaluation

Germination tests using six replicate samples, each of 10 or 15 seeds, were carried out in 12 cm Petri dishes on 1% distilled water agar. They were incubated in a temperature-controlled incubator at $30 \pm 1^\circ\text{C}$, 14 h photoperiods with light provided by white fluorescent tubes light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$). Scoring was made weekly for six months and those with at least 5 mm radicle extending from the testa were scored as germinated. Ungerminated seeds were carefully cut open to check if the embryos were healthy before finishing the germination trial.

Seed desiccation

Large desiccators containing plenty of activated silica gel were used to dehydrate seeds at 25–28°C. The seeds were buried in silica gel with alternating layers of seed and gel, for a few hours to a few days. After desiccation, moisture content was determined immediately and the dehydrated seeds were transferred to subsequent experiments.

Storage of fully hydrated seeds

Perlite was oven-dried at $103 \pm 2^\circ\text{C}$ for 17 h and cooled down before use. Fifty g dry perlite was dispensed into plastic jars and mixed with 60, 75, 90, 105 or 120 g distilled water. Then 98 seeds were put into each jar, shaken up, and stored at 15°C with the top covered loosely. One jar at each moisture content was sampled for viability evaluation after storage for 1 week or 1, 3, 6, 9 or 12 months. In order to reduce fungal growth, the seeds were cleaned and surface air-dried before storage.

Storage of partially dried seeds

Trials to investigate the effects of three factors, seed moisture content, temperature and duration of storage, on viability of partially dried seeds was designed following that of Pritchard *et al.* (1995). Seeds were dehydrated to six moisture levels between 33% and 18% using silica gel as described above.

From each moisture level, a sample of seeds was taken to determine the viability, and the rest divided into 20 groups of 60 seeds each in aluminum foil packets. Five packets were then stored at 15°C , five packets at 4°C , five at -20°C and five at -70°C . After storage for one week or 1, 3, 6 or 12 months, one package was taken from storage at each temperature, warmed to ambient temperature and seed viability evaluated. The same was done at all six moisture levels.

Cryopreservation of intact seeds

This experiment was conducted twice using two seed lots at different maturity. A previous study (Wen and Song, 2007) showed that embryos at the mid stage of seed development (between 27-36 weeks after flowering (WAF)) had relatively higher tolerance to desiccation and freezing, so seed cryopreservation was tried separately on two seed lots. Seed lot 1 was from mature but unripe, dark green, fruits at about 33WAF; seed lot 2 was from blue, ripe fruits at about 42WAF.

Fresh seeds were dehydrated in activated silica gel to eight moisture levels with the lowest being 13% as described above. Sixty seeds at each moisture level were sown for viability evaluation and another 60 seeds were sealed in an aluminum foil package and immersed in liquid nitrogen. They were removed from liquid nitrogen after 3 days storage and re-warmed under ambient condition before viability evaluation.

*Desiccation and cryopreservation of isolated embryos**Preparation of isolated embryos*

Newly harvested seeds were cleaned, surface-sterilized with 75% ethanol for 90 seconds, 0.1% HgCl_2 for 20 minutes and then rinsed 5 times with sterilized de-ionized water prior to removing embryos under aseptic conditions. One by one, the isolated embryos were placed in small containers (2 cm in diameter) made of aluminum foil in sterile Petri dishes.

Dehydration and cryopreservation of isolated embryos

In a first experiment to examine the interaction of moisture content and freezing on viability, isolated embryos in small aluminum foil containers were dehydrated over silica

gel for 0-12 h within a closed desiccator at 25-28°C to eight different moisture contents between 68% and 10%. Half at each moisture content were preserved in liquid nitrogen, and the other half were used as control. For cryopreservation, embryos were put into 2 ml cryovials and then plunged into liquid nitrogen. After one week storage at -196°C, the cryovials were taken out of liquid nitrogen and immersed in 40°C sterilized water for 1 min to rapidly thaw the embryos, which were then cultured on sterile medium. Control embryos were directly cultured on medium to assess viability after desiccation alone.

In a second experiment to monitor the effect of long-term storage on embryo viability, embryo samples at three moisture levels were prepared in the same way as described above. They were placed in liquid nitrogen and removed for viability evaluation after cryostorage for 1, 3, 6, 12 or 24 months.

Culture of isolated embryos

Embryos were cultured on the modified Murashige and Skoog (MS) medium described by Chin *et al.* (1988). This comprised basal MS medium modified by the addition of 0.17 g l⁻¹ NaH₂PO₄, plus 2 g l⁻¹ activated charcoal, 30 g l⁻¹ sucrose, 0.2 mg l⁻¹ 2-naphthyl acetic acid (NAA) and 0.1 mg l⁻¹ 6-benzylaminopurine (BAP), and solidified with 7 g l⁻¹ agar. Medium was dispensed into 55 mm Petri dishes after autoclaving at 121°C for 15 min. All cultures were maintained at 24 ± 2°C, with 40-50% relative humidity and a photoperiod of 14 h light (66 µmol m⁻² s⁻¹) and 10h dark for 6 months. They were examined regularly. 'Emergence' was scored as the percentage of embryos showing root and/or shoot formation during this period; 'survival' was scored as the percentage of embryos showing evident elongation. 'Survival' therefore includes 'emergence'.

Statistical analysis

Seed germination percent were expressed as means ± SD of 6 replicates of 10 or 15 seeds, while survival and emergence of embryos were expressed as means ± SD of 4 replicates of 10 embryos. Analysis of variance was carried out and Duncan's test was applied at significance level P = 0.05 after arcsine transformation.

Results

Chinese fan palm fruits usually contain a single seed composed of a large endosperm and a small embryo. Mean dry weight per seed and per embryo at maturity are about 840 mg and 3.5 mg, respectively. The seeds when shed have high moisture contents varying between seed lots and ranging from 32% to 39% as can be seen in the various tables of results below. Some seeds are viviparous at their last developmental stage (figure 1a).

Storage of fully hydrated ['moist'] seeds

The effect of moisture level of the storage medium on the survival of fully hydrated seeds under storage at 15°C for up to 12 months was investigated. Seeds placed in storage had an initial moisture content of 36±3% and initial viability of 76±7%. An analysis of variance shows that there is no significant effect on viability of moisture level of the medium or of storage duration. ($F_{\text{moisture content}} = 0.15$; $F_{\text{duration}} = 0.34$) (table 1).

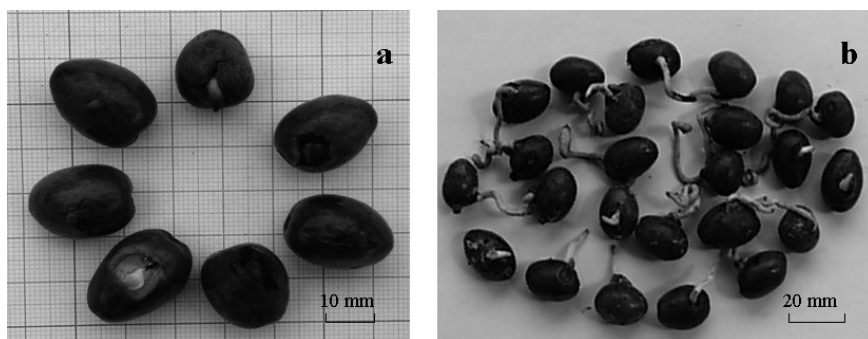


Figure 1. Germinating *L. chinensis* seeds (a) from viviparous fruits on trees and (b) under storage in polyethylene bags.

Up to one month storage under any moisture conditions investigated did not reduce seed viability essentially, but severe fungal growth was recorded after one month. Seeds began to germinate under these storage conditions after two months, and no further seeds germinated after three months storage. After three months some seeds had just initiated germination and some had 5cm long radicles penetrating the testa. After six months, seedlings with longer roots and shoots formed. After one year, seedlings were found to be yellow and withering. In this study, fungal growth was severe but it seems not to be lethal because it did not impair seed germination nor cause seedling death. Possibly this should be ascribed to rapid seed germination and active seedling growth, because fungal growth was no more severe after seed germination began.

Chinese fan palm seeds were shed with moisture content high enough to support their own germination without an extra water supply. Even when stored in a moist medium, seed moisture content remained between 35% and 39% and, as shown below, there was no measurable imbibition of water. Hence, it was the high initial moisture content of seeds, not imbibed water, which allowed germination during storage. This conclusion was confirmed further by the following observations: (1) the seeds in the initial stage of germination had a moisture content of $38 \pm 2\%$, close to the initial moisture content of the seed lot, $36 \pm 3\%$, showing that no water had been imbibed; (2) since 36 WAF on, seed germination inside fruits on trees was observed, and more seeds germinated with seed maturity increased (figure 1a); (3) both seeds inside fruits and seeds removed from fruits germinated after storage in polyethylene bags, within which there was of course no extra water supply (figure 1b).

Hence storage of fully hydrated 'moist' seeds of *L. chinensis* is possible for three months but not longer. This length of time is not affected by the moisture level of the storage medium.

Storage of partially dried seeds

Seeds used in this study had an initial moisture content of $32 \pm 2\%$ and initial viability of $78 \pm 3\%$. Seed moisture content, storage temperature and storage duration, all had a significant influence on seed viability (table 2).

Table 1. Seed viability under moist storage conditions at 15°C.

Medium moisture level (%, dw)	Seed viability (%)					
	1 week storage	1 month storage	3 month storage	6 month storage	9 month storage	12 month storage
120	74 ± 4	82 ± 4	82	80	76	81
150	78 ± 4	79 ± 2	79	78	71	84
180	80 ± 3	86 ± 4	80	81	83	81
210	79 ± 5	73 ± 5	79	73	78	84
240	77 ± 3	87 ± 2	80	74	78	76

Note: viability values are expressed as means ± SD of germination percentage of 6 replicates of 15 seeds, or as the percentage germination of 98 seeds after three months and longer

Sub-zero temperatures are lethal to *L. chinensis* seeds. Seeds survived neither -20°C nor -70°C even for the shortest duration investigated. At above-zero temperatures no seeds survived 12 months storage (data now shown). Table 2 shows that seeds survive longest at and above 30% moisture content, and in this state it makes no difference whether they are stored at 15°C or 4°C. As with fully hydrated seeds, viability is unchanged up to three months, but, unlike the fully hydrated seeds, at 15°C and 4°C storage is possible for up to six months though with reduced viability. Below 30% moisture content, survival drops off dramatically, both in the short and the long term, and there is no significant interaction between moisture and temperature.

Hence storage of *L. chinensis* seeds can be extended to six months by above-zero low temperatures, for example, at 4-15°C, but partial drying, even the few percent drying, did not increase the length of survival in storage.

Desiccation tolerance and cryopreservation of intact seeds

Two seed lots at eight moisture levels were used to investigate the possibility to cryopreserve intact seeds. As expected of a recalcitrant seed, no seeds survived freezing, whatever their hydration state. Seed cryopreservation is a relative simple procedure, but success is difficult to obtain. So only the data from dehydration alone are presented (table 3). It can be noticed that these two seed lots differed in desiccation tolerance depending on their age although they both are sensitive to desiccation and freezing. Seed lot 1 had higher initial moisture content and higher viability, exhibited higher desiccation tolerance than Seed lot 2. In addition, the embryo had much higher moisture content than the coving seed structures at all stages of desiccation in both seed lots.

Cryopreservation of isolated embryos

This study used embryos from fruits at about 30 WAF, which are dark green, mature but unripe, the seeds had an initial moisture content of $39 \pm 2\%$.

The first experiment to examine the interaction of moisture content and freezing on viability shows that desiccation alone did not reduce embryo viability, except that at a moisture content of 11% there was a small but significant fall in emergence (figure 2). Hence, isolated embryos had much higher desiccation tolerance than intact seeds.

Table 2. Viability of seeds stored in the partially dried state.

Seed MC (%, fw)	Storage temperature	Viability (%)				
		Before storage	One week storage	One month storage	Three months storage	Six months storage
32 ± 0.2	15°C		65 ± 3 (ab)	72 ± 9 (a)	57 ± 6 (bc)	15 ± 4 (f)
	4°C	78 ± 3 (a)	70 ± 4 (a)	73 ± 2 (a)	72 ± 7 (a)	38 ± 4 (cd)
30 ± 0.5	15°C		60 ± 5 (ab)	72 ± 8 (a)	67 ± 3 (a)	45 ± 3 (bcd)
	4°C	70 ± 3 (a)	68 ± 9 (a)	80 ± 5 (a)	62 ± 8 (ab)	20 ± 4 (ef)
28 ± 0.3	15°C		45 ± 3 (bcd)	50 ± 1 (bcd)	32 ± 2 (def)	10 ± 3 (f)
	4°C	65 ± 8 (ab)	57 ± 4 (bc)	70 ± 4 (a)	45 ± 12 (cd)	1.7 ± 1.7 (g)
24 ± 0.5	15°C		37 ± 4 (cd)	32 ± 5 (de)	12 ± 6 (f)	0 (g)
	4°C	52 ± 6 (bc)	25 ± 6 (e)	28 ± 6 (de)	28 ± 7 (e)	13 ± 4 (f)
21 ± 0.4	15°C		20 ± 9 (ef)	13 ± 5 (f)	8 ± 3 (fg)	0 (g)
	4°C	28 ± 5 (de)	20 ± 6 (ef)	13 ± 3 (f)	18 ± 5 (ef)	0 (g)
19 ± 0.2	15°C		12 ± 5 (f)	7 ± 3 (fg)	3 ± 3 (g)	1.7 ± 1.7 (g)
	4°C	17 ± 3 (ef)	13 ± 2 (f)	13 ± 3 (f)	7 ± 3 (fg)	0 (g)

Note: 1) Seeds stored at -20°C and -70°C all died. MC (MC=Moisture content, the same for the following tables) values are expressed as mean ± SD of 8 replicates of single seed. Viability values are means ± SD of the germination percentage of 6 replicates of 10 seeds.

2) Analysis of variance was used to assess the relative effects and interactions between moisture content (m), storage temperature (t) and storage duration (d). ** indicates significant differences at P = 0.01.

For moisture content, $F_m^{**} = 157$; for storage temperature, $F_t^{**} = 7.53$; for storage duration, $F_d^{**} = 251$; and $F_{m \times t} = 0.92$, $F_{m \times d}^{**} = 8.62$, $F_{t \times d} = 1.61$, $F_{m \times t \times d}^{**} = 2.02$. Those data marked with same letter are not significantly different at P = 0.05.

Table 3. Seed viability after dehydration.

Lot 1	Seed MC (%)	34 ± 5	30 ± 4	27 ± 8	24 ± 5	20 ± 3	17 ± 9	14 ± 2	13 ± 2
	Embryo MC (%)	64 ± 9	52 ± 3	47 ± 2	45 ± 3	28 ± 2	25 ± 1	23 ± 2	22 ± 3
	Viability of seeds	92 ± 5	97 ± 3	93 ± 3	73 ± 6	35 ± 4	20 ± 4	10 ± 4	0
Lot 2	Seed MC (%)	32 ± 2	31 ± 7	29 ± 4	27 ± 5	25 ± 9	22 ± 4	19 ± 4	15 ± 3
	Embryo MC (%)	61 ± 3	46 ± 8	42 ± 4	37 ± 9	34 ± 8	28 ± 2	24 ± 4	25 ± 1
	Viability of seeds	87 ± 6	77 ± 6	75 ± 6	77 ± 3	70 ± 7	23 ± 3	12 ± 2	5 ± 4

Moisture content values are expressed as mean ± SD of 8 replicates of a single seed or of 5 embryos. Viability values are expressed as means ± SD of germination percentage of 6 replicates of 10 seeds. Note: 1) Seed lot 1 – from dark green, mature but unripe fruits. Seed lot 2 – from blue, ripe fruits. 2) For seed lot 1, $F^{**} = 56$; for seed lot 2, $F^{**} = 33$.

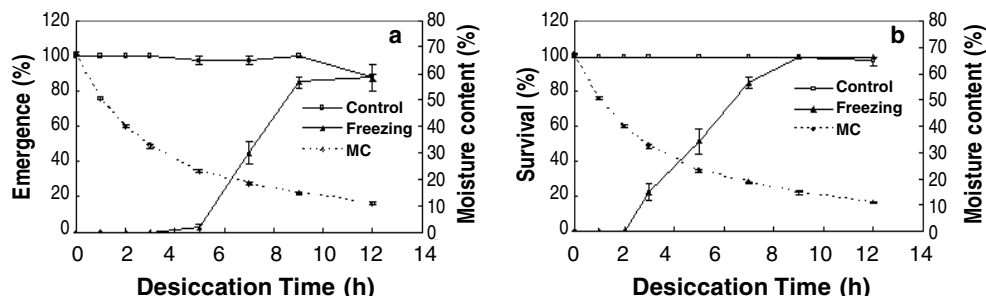


Figure 2. Emergence (a) and survival (b) of frozen and control embryos and their moisture contents after desiccation treatment. Embryos isolated from fresh seeds were immediately dehydrated over activated silica gel to the indicated moisture contents. At each moisture level, half the isolated embryos were then cultured to assess viability. The other half was stored at liquid nitrogen temperature for one week, then thawed and cultured to assess viability. Embryos showing root and shoot formation after culture were scored as 'emergent', and those showing only elongation or root and shoot formation after culture were scored as 'survived'. All values are means $\pm$ SD of four replicates of 10 embryos each.

Embryos at their initial moisture content, 68%, failed to survive freezing. But dehydration of excised embryos prior to freezing solved this problem. Dehydration of 3 hours saved a quarter of the samples though none of them emerged. From 32% down to 14.5%, every fall in moisture content increased post-thaw viability until dehydration itself began to harm viability when drying was extended to 12 hours. The key to successful cryopreservation was rapid drying of the isolated embryos. Maximum preservation at liquid nitrogen temperature was obtained with embryos dehydrated for 9 and 12 hours, with a moisture content of less than 20%. Under these conditions all embryos survived and emergence was over 80%.

Thus, although intact seeds were sensitive to desiccation and freezing, isolated embryos can be successfully preserved in the frozen state when given desiccation treatment before storage.

The second experiment using embryo samples at 3 moisture levels between 23% and 14% to monitor the effect of long-term storage on embryo viability shows that long-term cryostorage of excised embryos of *L. chinensis* is feasible, with high post-thaw viability and long-lasting stability. There is no loss of viability of embryos at liquid nitrogen temperatures for up to 2 years. The dehydration of excised embryos to moisture contents between 10% and 15% confers resistance to freezing (table 4).

Discussion

Although it has long been known that the Chinese fan palm produces short-lived seeds there are different opinions on its seed storage behaviour category (Chin *et al.*, 1989; Tweddle *et al.*, 2005). Recently, Wen and Song (2007) reported that its excised embryos had the same developmental pattern of desiccation tolerance and cryotolerance as typical recalcitrant seeds, and vivipary was observed in some seeds on trees. In the present study, seeds of the Chinese fan palm exhibited high sensitivity to desiccation and freezing and

Table 4. Viability of embryos after long-term cryogenic storage at different moisture contents.

Embryo MC (% fw)		1 week	1 month	3 months	6 months	1 year	2 years
Emergence	23 ± 0.6	2 ± 2	5 ± 2	8 ± 4	2.5 ± 2.0	8 ± 6	13 ± 6
	18 ± 0.4	45 ± 5	43 ± 6	30 ± 6	36 ± 13	28 ± 2	41 ± 16
	15 ± 0.6	85 ± 3	92 ± 4	70 ± 9	80 ± 9	90 ± 6	83 ± 4
Survival	23 ± 0.6	51 ± 6	38 ± 6	50 ± 8	60 ± 6	48 ± 4	43 ± 6
	18 ± 0.4	85 ± 2	68 ± 7	82 ± 7	80 ± 3	85 ± 4	80 ± 7
	15 ± 0.6	100	100	98 ± 2	98 ± 2	93 ± 4	88 ± 5

Note: 1) Moisture content values are expressed as means ± SD of 8 replicates of 5 embryos, and emergence and survival values as means ± SD of 4 replicates of 10 embryos. 2) Analysis of variance (values not shown) shows that there is no significant effect of length of storage on viability of stored embryos

failed to survive conventional seedbank storage. High moisture at shedding, low tolerance to desiccation and freezing, vivipary, and inter- and intra-seasonal variation in seed quality between individuals and seed lots observed in this research, are all typical of recalcitrant seeds. These results indicate that Chinese fan palm seeds are recalcitrant.

Recalcitrant seeds cannot be stored routinely in seedbanks. Differing from orthodox seeds, which dry and become quiescent at maturity, recalcitrant seeds maintain high moisture and active metabolism after shedding and continue progress towards germination, and this makes seed storage difficult. Intensive efforts have been made in the last three decades to find methods for storage of recalcitrant seeds. Among them, ‘moist’ storage, ‘partially dry’ storage and cryostorage have each achieved limited success.

‘Moist storage’ involves storage of seeds in media with some moisture-retention capacity to prevent dehydration, such as perlite, vermiculite, sawdust, coconut dust, damp charcoal and moist sand. This minimizes seed deterioration resulted from dehydration stress, and provides sub-optimal conditions for seed germination. Details of this method include: mixing seeds and the media appropriately; adjusting the medium moisture level so that the stored seeds will neither dehydrate too quickly and die, nor imbibe so much water as to germinate quickly; allowing necessary ventilation; and possibly maintaining a low temperature but without causing chilling injury. The suitable low temperature for moist storage of recalcitrant seeds can be determined through calculation of the base temperature for germination rate as Pritchard *et al.* (1995) did using *Araucaria hunsteinii*. Seeds of *Hopea hainanensis* (Song *et al.*, 1984), *Podocarpus milanjanus* and *Prunus africana* (Schaefer, 1991) have been reported to live longer under these conditions. The two general problems of moist storage are fungal growth and early germination of seeds. Anti-fungal sprays and other antimicrobial treatments can reduce microbial contamination (Finch-Savage *et al.*, 2003), but application of natural germination inhibitors like abscisic acid (ABA) has largely failed to prevent germination of recalcitrant seeds in storage, because recalcitrant seeds usually are not sensitive to ABA. Thus ‘moist storage’ of recalcitrant seeds is only useful in the short term.

If recalcitrant seeds germinate during moisture storage, as *L. chinensis* seeds did after 2-3 months (table 1), then seed storage becomes seedling storage, which is considered as another storage method, practiced and recommended by some authors (Corbineau and Côme, 1986, Krishnapilly, 2000). The main disadvantage of handling seedlings rather than seeds is that seedlings are more fragile and easily damaged during handling and planting. However, storage of seedlings is still very short-term and makes no contribution to germplasm survival.

Storage of partially dried seeds is a second method for recalcitrant seeds. Seeds of *Hevea* survived for up to 13 months with approximately 20% germination using this method (Noor and Chin, 1989). The key to this method is the determination of the Lowest Safe Moisture Content (LSMC) (Tompsett, 1984), which approximates to the Critical Moisture Content of King and Roberts (1979). For *L. chinensis* this value is about 27% (tables 2 and 3). The viability of recalcitrant seeds responds non-linearly to water loss. Above LSMC, reducing the moisture content of recalcitrant seeds has little effect, but below this level there is a drastic loss of seed viability. The successful partially dried storage of recalcitrant seeds involves air-drying for a few days or drying above silica gel for a few hours, to decrease seed moisture to levels just above the LSMC, followed by sealed or ventilated storage at as low a temperature as possible (provided chilling does not damage the seeds). Under partial drying storage, fungal growth and early germination of seeds would happen also if the recalcitrant seeds were stored at too high moisture content. Additionally, a severer problem with this storage method is rapid loss of seed viability. The principle of this method is that partial drying can reduce seed metabolism and fungal growth on seeds, and deterioration and damage to seeds are reduced in consequence. The efficacy of partial drying to prevent germination in storage and to prolong the duration of storage has also been shown by others (Fu *et al.*, 1990, Tompsett and Pritchard, 1998). Pritchard *et al.* (1995) reported that pre-emergence still occurred to *Araucaria hunsteinii* seeds stored near their base temperature for germination rate when not partially dried. The length of time that recalcitrant seeds can last in partially dry storage is still short, varying from months to at most several years.

Both the moist and the partially dried storage methods store actively metabolising seeds, they prolong lifespan of recalcitrant seeds through limited reduction of moisture content or storage temperature or both. Seeds under these conditions are still metabolically active and metabolism-related deterioration will unavoidably accumulate and impair seed viability, so neither of them can preserve recalcitrant seeds for long term. Although some limited extension to the period of storage can be expected through careful selection of conditions in the future, they cannot meet the purpose of long-term conservation of genetic resources.

Cryopreservation is the most promising method for long-term storage of recalcitrant seeds and there is no alternative to cryopreservation for this purpose (King and Roberts, 1979, Robert *et al.*, 1984, Chin, 1989; Englmann, 2000, 2004, Berjak and Pammenter, 2001, 2004). The basic concept of cryopreservation is that, at liquid nitrogen temperature, all metabolic processes are suspended. Consequently, all sources of deterioration that are metabolically related are greatly reduced, and it is often taken for granted that samples under cryogenic conditions can survive indefinitely. However, molecules remain sufficiently

mobile at low temperature to allow aging reactions to proceed slowly, but nevertheless seeds appear likely to survive for some thousands of years (Walters *et al.*, 2004). It is reasonable to believe in the potential of cryopreservation to preserve even recalcitrant seeds for the long term. This is the most attractive attribute of cryopreservation. With regard to non-orthodox seeds, several temperate recalcitrant or sub-orthodox species have been recovered with 80-100% survival from embryo axes cryostored for nearly 10 years (Pence, 2004). In the present study of *L. chinensis*, the most significant finding is that there is no significant loss in the viability of embryos even after 2 years cryostorage.

However, cryostorage of recalcitrant seeds is not straightforward. It has been tried on recalcitrant seeds since this category of seeds was defined more than 30 years ago, but only limited success has been achieved on a few species (Englmann, 2000, 2004). This technique still remains unfeasible for most recalcitrant seeds up to date.

Cryopreservation of recalcitrant seeds is still experimental (Berjak *et al.*, 1996; Berjak and Pammenter, 2004), with selection of plant materials and sophistication of the operators' manipulation sometimes making the difference between success and failure. In this study, the replacement of intact seeds by isolated embryos as the cryopreservation target turned the failure into success in *L. chinensis* cryopreservation, as what happened to cryopreservation of oil palm (Grout *et al.*, 1983). This resulted mainly from rapid dry and *in vitro* recovery method employed in cryopreservation of isolated embryos. As showed by Pritchard (1991) using *Quercus rubra*, embryos of *L. chinensis* possessed significantly higher moisture content than the other seed tissue at equilibrium and during dehydration (table 3). We think that the higher moisture content of the embryos is the main reason why both seed lots did not survive cryo-exposure in this study— even when the seeds appear highly dehydrated, the embryos remain hydrated, and therefore when exposed to liquid nitrogen lethal intracellular crystals would inevitably form and the embryos would be damaged. Intact seeds can not, but isolated embryos can be dehydrated safely to low moisture content and facilitate cryopreservation because rapid dry achieved after removal of the covering seed structures. So cryopreservation of isolated embryos can have different results from that of intact seeds.

The validation of these storage methods largely depends on the species-specific traits of recalcitrant seeds. Thus it is necessary to conduct a range of seed storage experiments on each species using different storage methods to find the method best suited to a given purpose. Both 'moist' storage and partially dried storage are simple, easy and cheap, but of only short duration. Moist storage is recommended for seeds which will be stored for planting, especially when germinated seeds are acceptable, because the method gives highest viability. Partially dried storage is better when seeds need to be kept ungerminated for relatively short periods. Both are widely practiced in forestry and horticulture, used for temporary seed storage during transport from the seed source to a prepared planting site, and for keeping seeds until the next planting season. Cryostorage is the only method that can ensure long duration of preservation, but is rather complex, laborious and relative expensive, and currently feasible only for a few species. For this reason, it is as important to improve techniques to prolong longevity of recalcitrant seeds under conventional storage conditions as it is to improve cryopreservation procedures to suit further recalcitrant seed species.

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