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ACQUISITION AND LOSS OF CRYOTOLERANCE IN *Livistona chinensis* EMBRYOS DURING SEED DEVELOPMENT

Bin Wen[1,2]* and Songquan Song[1]
[1]Xishuangbanna Tropical Botanical Garden, the Chinese Academy of Sciences, Mengla, Yunnan, 666303, China.
[2]Graduate School of the Chinese Academy of Sciences, Beijing, 100039, China
*Author for correspondence (E-mail address: wenb@xtbg.org.cn)

Abstract

Changes in desiccation tolerance and cryotolerance of Chinese fan palm (*Livistona chinensis* [Jacq.] R. Br.) embryos were studied during seed development from 15 to 45 weeks after flowering (WAF). Acquisition and then progressive loss in both desiccation tolerance and cryotolerance was observed within this period. Survival (apparent elongation of embryos) and emergence (formation of root and/or shoot) of embryos following dehydration increased progressively with development of seeds until 33 WAF, and then decreased up to 45 WAF. Similar changes occurred in the minimum moisture content at which 90% of embryos survived or emerged. Cryotolerance of embryos was nil at the early stages of seed development, until 21 WAF. Embryos acquired slight cryotolerance at 23 WAF and cryotolerance increased gradually from 27 to 36 WAF, then decreased by 45 WAF. Survival and emergence of post-thaw embryos were closely related to their moisture contents prior to freezing. However, this correlation between cryopreservation and moisture content was notably influenced by the embryos' developmental stage. Embryos at stages with greater cryotolerance gave higher post-thaw survival and emergence at a given moisture content, and the moisture content range allowing embryos to avoid cryo-damage was widened at both the lower and upper limits. Greater than 50% post-thaw emergence was observed only in embryos with moisture contents below 20% (fresh weight) at developmental stages between 27 and 36 WAF, although more than 90% of embryos could be dehydrated to < 20% moisture contents without loss in survival and emergence as early as 21WAF. Nearly 80% embryos could be dehydrated safely to 20% moisture content as late as 45 WAF.

Keywords: cryopreservation, cryotolerance, desiccation tolerance, recalcitrant seed, seed development, palm.

INTRODUCTION

It has been predicted that cryopreservation might be the most promising method for long-term storage of recalcitrant seeds (6,16,20), and to date there is no alternative to cryopreservation for this purpose (4,5,10). However, after nearly 30 years of effort, relatively

few successes have been achieved in the cryopreservation of recalcitrant (desiccation sensitive) seeds and their embryos or embryonic axes (10,11). Even in successful cases, some limitations remain, including: survival after freezing is extremely variable; regeneration is frequently restricted to callusing or incomplete development of plantlets; the number of accessions tested per species is generally very low; non-reproducible results with different seed lots and contrasting results from different laboratories are often obtained from work on the same species (10,11).

Seed maturity may contribute to these variable results. Embryonic axes from immature jackfruit seeds could be cryopreserved successfully using slow cooling after partial dehydration and treatment with cryoprotectant, while those from mature seeds could not, although they could be cryopreserved using a vitrification protocol (17, 23). Similarly, embryos from immature coconut seeds could be cryopreserved, but those from mature seeds failed to survive (1, 2). For these two species, embryos or embryonic axes before full maturity were the best choice for cryopreservation. In preliminary experiments on *Livistona chinensis* embryos we observed that those harvested at the last seed development stage, i.e. the most mature, were the worst for cryopreservation. Overall, these results suggest that embryos or embryonic axes from immature recalcitrant seeds have a higher cryotolerance than those from mature recalcitrant seeds and that cryotolerance may be reduced just before seed shedding. The objectives of this study are to explore how cryotolerance develops in recalcitrant seeds and what the relationship is between cryotolerance and desiccation tolerance of recalcitrant seeds. We investigated the changes in desiccation tolerance and cryotolerance in Chinese fan palm (*Livistona chinensis* [Jacq.] R. Br.) embryos at different developmental stages.

MATERIALS AND METHODS

Plant materials

Fruits of Chinese fan palm (*Livistona chinensis* [Jacq.] R. Br.) were manually collected between 4 and 45 weeks after flowering (WAF) from trees growing in Menglun, Mengla, Xishuangbanna, China in 2004. The collecting location in Menglun (21°41'N, 101°25'E; altitude 580 m) has distinct dry and rainy seasons because of the influence of the southwest monsoon. According to the records between 1958 and 1998 at the weather station of Xishuangbanna Tropical Botanical Garden, the annual mean temperature is 21.8°C; mean winter minima and mean summer maxima are 12.5°C and 32.5°C respectively. Rainfall is 1492.9 mm per year, only 15.9% of which occurs during the dry season (November-April), and 84.1% during the rainy season (May-October).

After manual removal from fruits, seeds were stored in polyethylene bags at 15ºC until use within one week after collecting. The embryos were excised from the seeds and treated immediately as described below.

Desiccation treatments

Embryo dehydration was achieved by placing embryos in small containers (2 cm in diameter) made of aluminum foil. The containers were then put over activated silica gel for different times (between 0.25 and 12 h, depending on embryo maturity stage) within a closed desiccator at 25-28°C. Each desiccator contained 20 embryos and 90 g activated silica gel to three quarters of its volume.

Moisture content determinations

The moisture content of plant materials was determined gravimetrically after oven drying (103 ± 2°C for 17 h). Eight samples of a single fruit, single seed or 5 embryos were used for each of these determinations. Moisture content was expressed on a fresh weight basis.

Embryo cryopreservation

The dehydration protocol of Engelmann (10) was employed in this study, modified as follows. Prior to excision of embryos under aseptic conditions, the seeds were washed with tap water, surface-sterilized in 75% ethanol for 90 s and in 0.1% $HgCl_2$ for 20 min, then rinsed five times with sterile de-ionized water.

After dehydration to different moisture contents the excised embryos were divided into two sub-samples. Half of the embryos were used for cryopreservation and the other half were used as controls. For cryopreservation, embryos were put into 2 ml cryovials and then plunged quickly in liquid nitrogen (LN_2). After one week storage at -196°C , the cryovials were taken out, immersed in 40ºC 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 recovery after desiccation.

Embryo culture and viability evaluation

The modified Murashige and Skoog (MS) medium described by Chin *et al.* (8) was used. This comprised basal MS modified slightly by the addition of 0.17 g l^{-1} NaH_2PO_4, supplemented with 2 g l^{-1} activated charcoal, 30 g l^{-1} sucrose, 0.2 mg l^{-1} NAA and 0.1 mg l^{-1} BAP and solidified with 7 g l^{-1}agar. After autoclaving at 121ºC (1 kgf cm^{-2}) for 15 min the medium was dispensed into 55 mm-diameter Petri dishes. All cultures were maintained at 24 ± 2ºC, with 40%-50% relative humidity and an alternating photoperiod of 14 h light (66.25 µmol m^{-2} s^{-1}) and 10 h dark for 6 months. Embryos were observed regularly and 'survival' scored as the percentage of embryos elongating; 'emergence' was scored as the percentage of embryos showing root and/or shoot formation during this period. In this assessment, emergence was included in the total survival estimate. Both survival and emergence were expressed as means ± SD of 4 replicates of 10 embryos

RESULTS

Changes in seed development

The Chinese fan palm is a monocotyledonous angiosperm. The seeds have a thin testa, large endosperm and small embryo (Fig. 1a). Dry weight per seed and per embryo was about 840 and 3.5 mg, respectively, at 45 WAF; the proportion of embryo to whole seed was about 0.004. The moisture content of fruit, seeds and embryos was about 73%, 68% and 85% respectively at 15 WAF. From 15 to 45 WAF, the moisture content of fruit, seeds and embryos gradually decreased by 17, 46 and 18%, respectively (Fig. 2a). At the latter developmental stages the moisture contents of embryos and fruits was much higher than that of the seeds.

Fruit and seed dry weight increased slowly in the early stages of development, until 13 WAF, and then increased more rapidly, from 15 to 27 WAF (Fig. 2b). The embryos were very small, and dry weight per embryo increased from less than 1 mg at 15 WAF to 3.5 mg at 27 WAF. The dry weight of fruits, seeds and embryos was essentially constant from 27 to 45 WAF (Fig. 2b).

Pericarp color and seed texture changed during development. The epicarp colour gradually changed from light green to dark green and finally to blue, the mesocarp colour from light green to orange, and the endocarp changed from white to blackish. In addition, the

endocarp became markedly thicker and formed an osseous layer covering the seeds after 21 WAF. At the last developmental stage the radicle was observed to protrude through the testa and epicarp in some seeds / fruits (Fig. 1b).

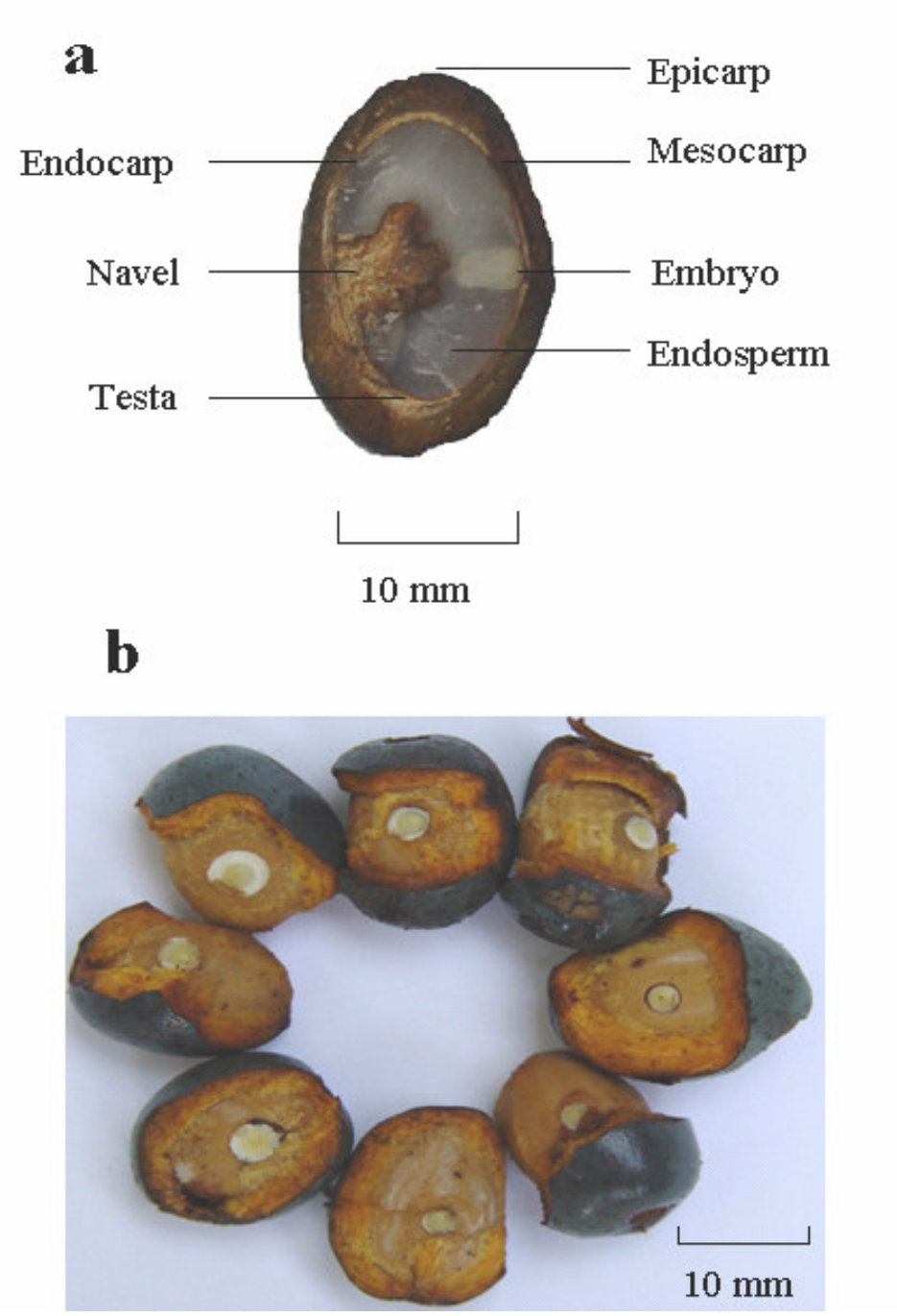

Figure 1. Fruit and seed structure (a) and seed vivipary of *Livistona chinensis* (b). Note the white dots on the seeds in (b) are embryos protruding through the testa whilst inside the fruit.

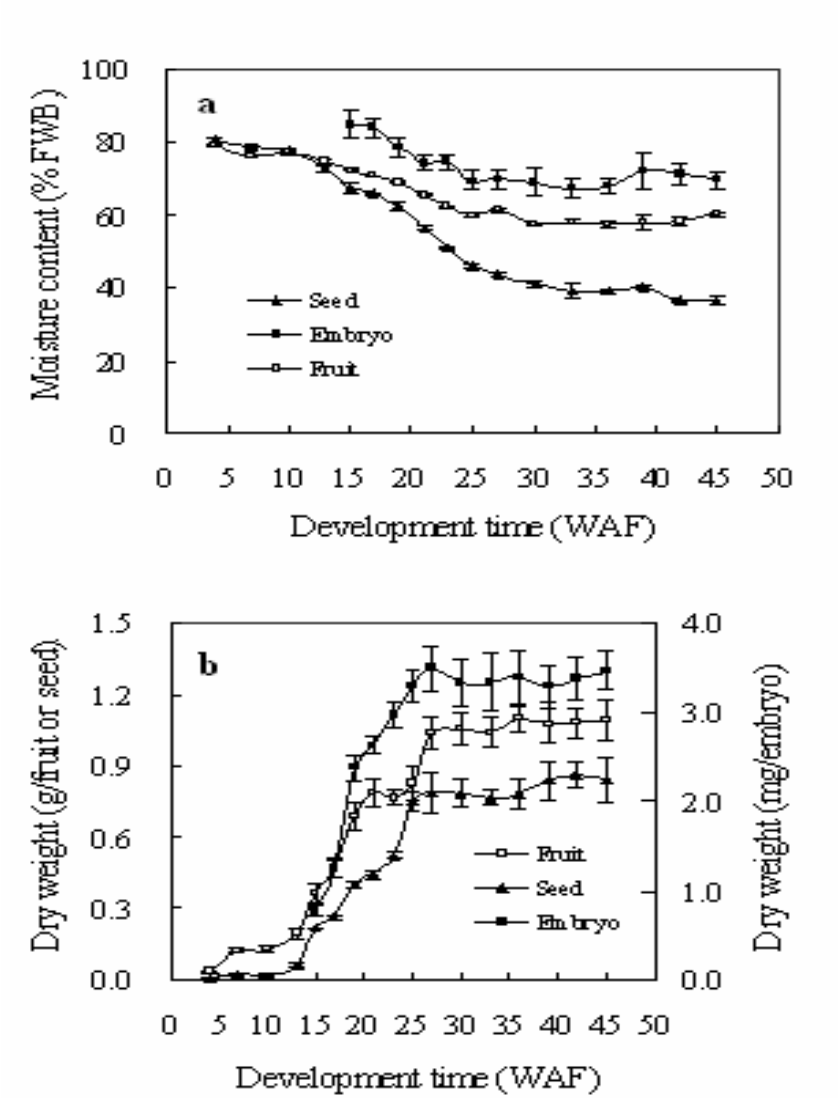

Figure 2. Changes in moisture content (FWB)(a) and dry weight (b) of fruits, seeds and embryos of *Livistona chinensis* during fruit development. All moisture content values are means ± SD of eight replicates of a single fruit, single seed, or 5 embryos each, and dry weight values are means ± SD of eight replicates of 50 fruits, 50 seeds or 10 embryos each.

Changes in desiccation tolerance of embryos during seed development

To assess and quantify the changes in desiccation tolerance during seed development, embryos excised at various developmental stages were dehydrated to different moisture contents over activated silica gel and then cultured on nutrient medium. Desiccation tolerance of embryos, as measured by emergence (Fig. 3a and b) and survival (Fig. 3c and d) both at a given moisture content and as a whole, was slight at the early developmental stages. Emergence and survival progressively increased with seed development; for example, after dehydration to about 16% MC their values were 0% and 3% at 15 WAF, 23% and 38% at 17 WAF, 85% and 90% at 19 WAF, respectively (Fig. 3a and c). Desiccation tolerance peaked between 25 and 33 WAF, and was gradually lost afterwards. Survival and emergence of embryos notably declined by the late developmental stages. For embryos at 30 WAF, dehydration to 12 % MC had little effect on emergence and survival. However, dehydration to the same MC depressed survival to 10% and emergence to nil at 45 WAF (Fig. 3b and d).

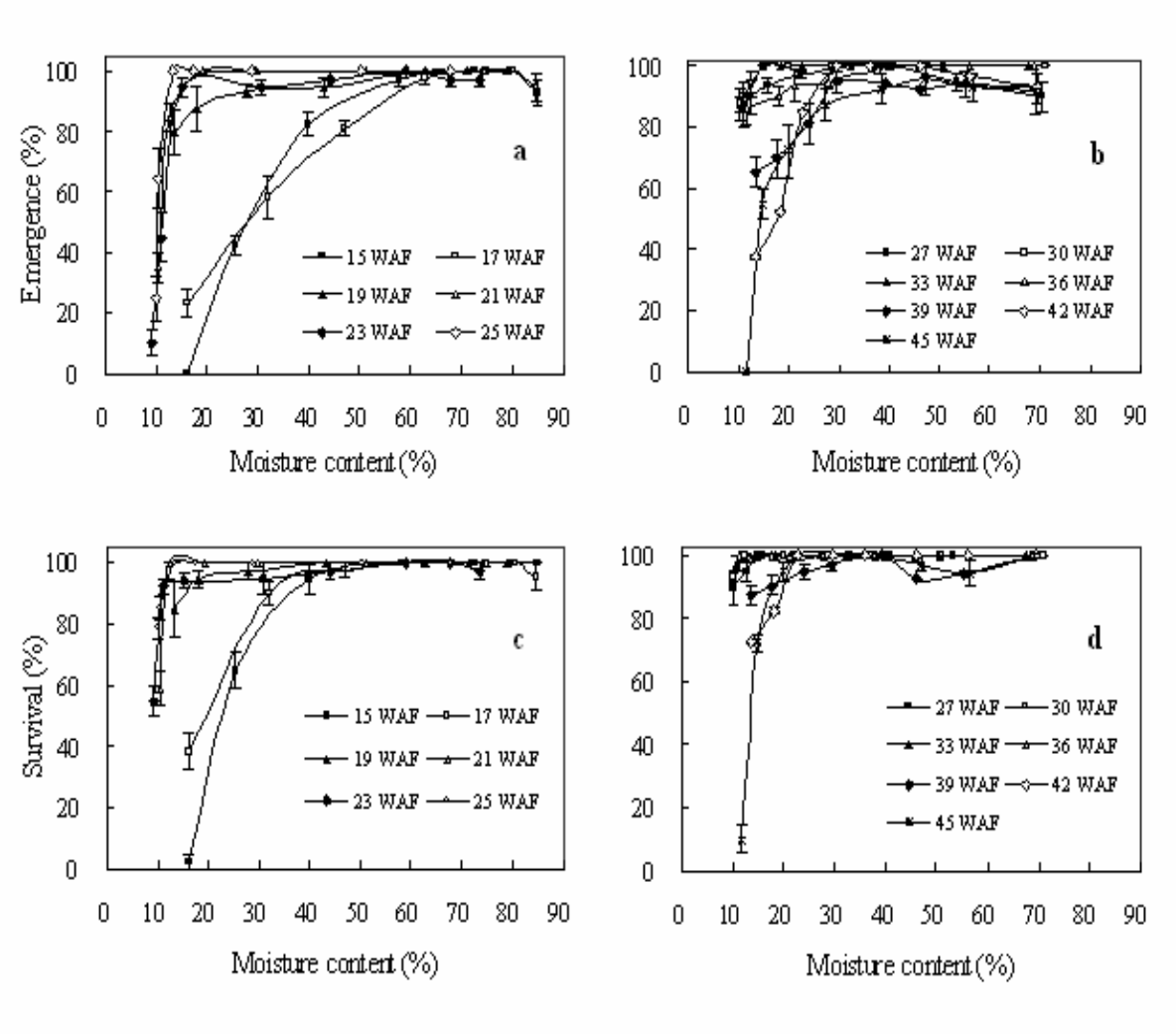

Figure 3. Changes in emergence (a, b) and survival (c, d) of *Livistona chinensis* embryos at different developmental stages following dehydration to different moisture contents. Embryos excised from fresh seeds were immediately dehydrated over activated silica gel at 25-28°C to the indicated moisture content, and then were cultured for 6 months under an alternating photoperiod of 14 h light (66.25 μmol m^{-2} s^{-1}) and 10 h. Embryos showing root and shoot formation after culture were scored as 'emergence' , and those showing an apparent elongation after culture were scored as 'survival'. All values are means ± SD of four replicates of 10 embryos each.

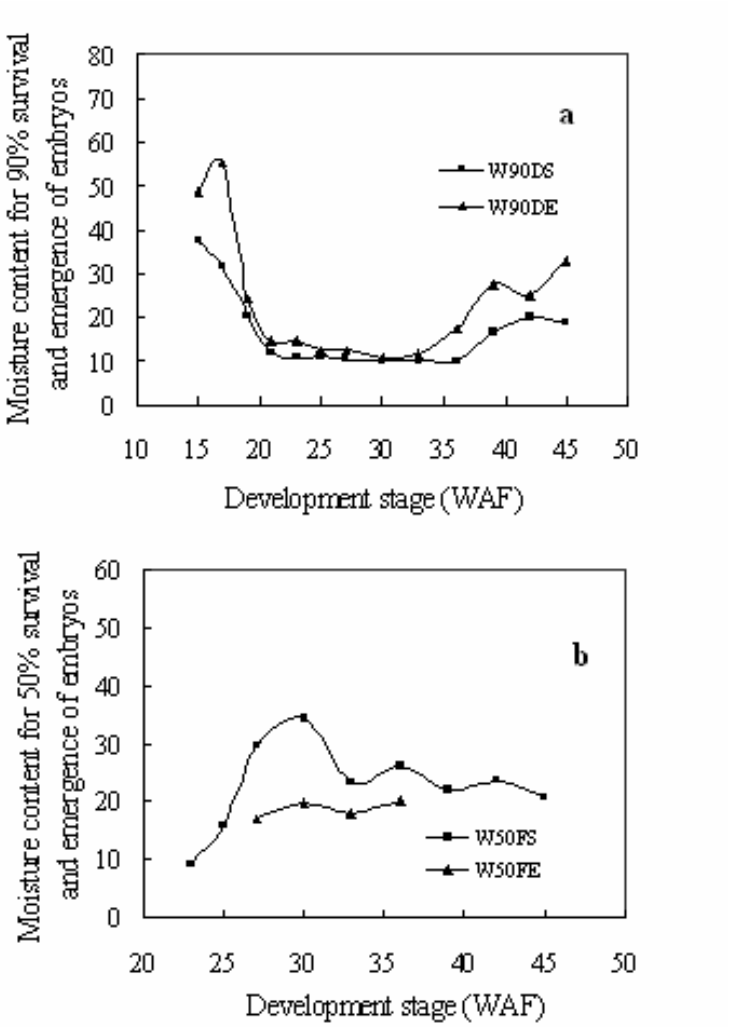

Figure 4. Changes in W_{90DS} and W_{90DE}, moisture contents allowing 90% *Livistona chinensis* embryos to survive and to emerge at different developmental stages following dehydration to different moisture contents (a), and changes in W_{50FS} and W_{50FE}, the maximum moisture contents allowing 50% post-thaw embryos to survive and to emerge at different developmental stages following dehydration to different moisture contents prior to freezing (b).

The term W_{90DE} and W_{90DS}, calculated by interpolation using emergence curve in Fig. 3a and 3b, survival curves in Fig. 3c and 3d, were used to describe the moisture contents at which 90% of embryos can still emerge and survive after dehydration, respectively. Both W_{90DE} and W_{90DS}, being higher at the early development stages, decreased gradually with seed development (Fig. 4). W_{90DE} reached its minimum between 25 and 33 WAF, and W_{90DS} had a minimum between 21 and 36 WAF; thereafter both measures increased during the later developmental stages. Furthermore, W_{90DE} was higher than or close to W_{90DS} (Fig. 4).

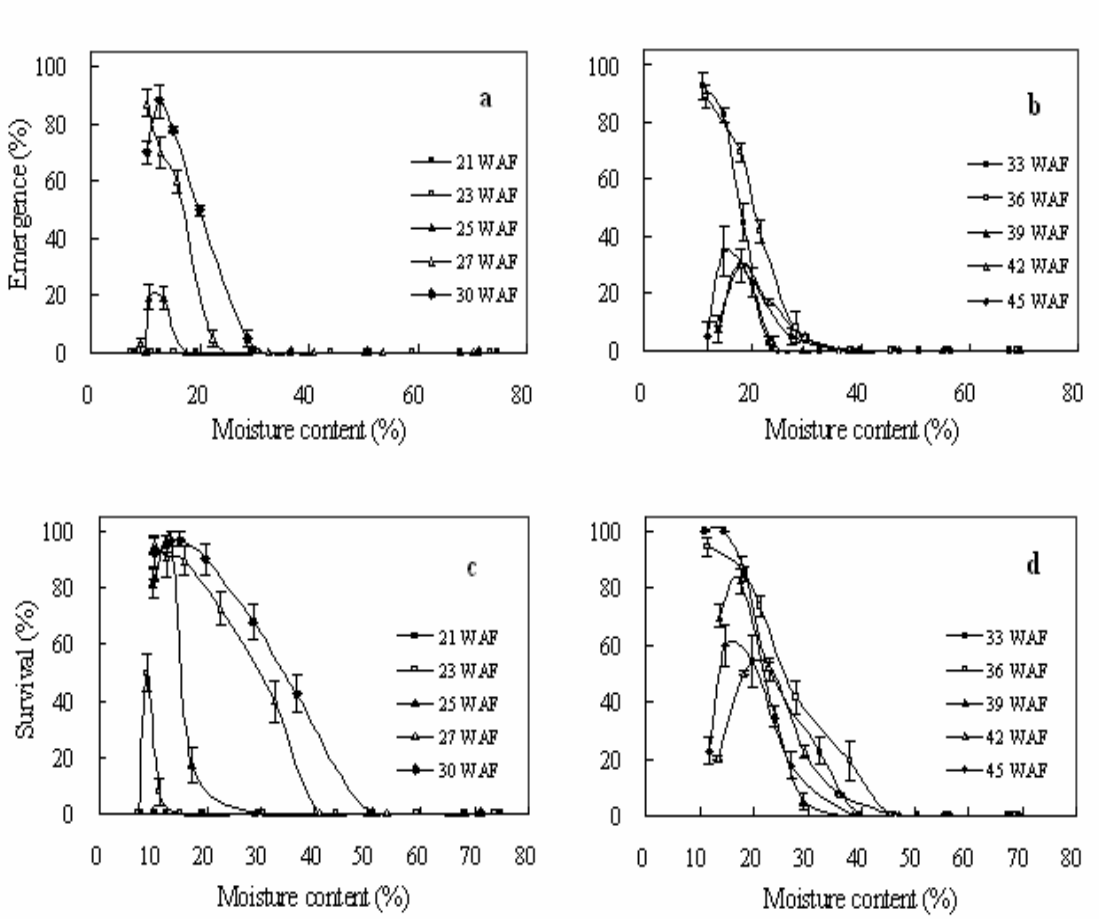

Figure 5. Changes in emergence (a, b) and survival (c, d) of *Livistona chinensis* embryos at different developmental stages after dehydration and cryopreservation. Embryos excised from fresh seeds were dehydrated to the indicated moisture content, stored in liquid nitrogen for one week, and then cultured for 6 months under an alternating photoperiod of 14 h light (66.25 μmol m^{-2} s^{-1}) and 10 h dark. Embryos showing root and shoot formation after culture were scored as 'emergence', and those showing an apparent elongation were scored as 'survival'. All values are means ± SD of four replicates of 10 embryos each.

Changes in embryo cryotolerance during seed development

After dehydration to different moisture contents, embryos at different developmental stages were plunged into liquid nitrogen, stored for one week, then rewarmed quickly and cultured. Cryotolerance, expressed as survival and emergence of post-thaw embryos, was nil at early stages of seed development up to 21 WAF (data not shown before 21WAF). Embryos acquired some cryotolerance at 23 WAF, reached higher levels by 27-36 WAF, and then decreased by 45 WAF (Fig. 5). When cryotolerance increased, survival and emergence of post-thaw embryos both at a given moisture content and as a whole increased. Alternatively, moisture contents allowing a given percentage of survival and emergence increased also. The reverse happened when cryotolerance fell during the last stage of development (Fig. 5). It was noted that a high level of survival and emergence of post-thaw embryos was obtained only when dehydration resulted in moisture contents prior to freezing below 20% for emergence and 35% for survival. At the same developmental stage, the moisture contents required for

embryo emergence were lower than those for embryo survival; while for the same developmental stage and moisture content, survival of embryos was higher than emergence after cryopreservation (Fig. 5).

Embryos acquired desiccation tolerance earlier than cryotolerance during seed development, and both the developmental stage and moisture content of embryos before freezing notably influenced the cryopreservation results (Fig. 5). The MC windows allowing 50% past-thaw embryos to emerge and to survive were calculated by interpolation from the emergence curves in Fig.5a and 5b and the survival curves in Fig. 5c and 5d. The upper limit of these windows were expressed as W_{50FE} and W_{50FS}, which indicated the highest moisture contents allowing 50% past-thaw embryos to emerge and to survive. Both W_{50FE} and W_{50FS} were low at the early developmental stages. Moisture contents allowing 50% embryo survival after cryopreservation increased with development, reached a peak at about 27-30 WAF, and then decreased (Fig. 4b). Post-thaw emergence ≥50% was observed only for embryos with moisture contents below 20% at developmental stages between 27 and 36 WAF (Fig. 4b). However, > 90% of embryos could be dehydrated to moisture contents below 20% without loss in either survival or emergence as early as 21 WAF, and nearly 80% embryos could be dehydrated safely to 20% as late as 45 WAF (Fig. 5).

Figure 6. Seedlings produced from desiccated (a) and cryopreserved embryos (b). Embryos excised from fresh seeds at 27 WAF were dehydrated over activated silica gel at 25-28℃ to a moisture content of 10% (fresh weight basis) and directly cultured for assessment of desiccation tolerance or transferred to liquid nitrogen for one week before culturing for assessment of cryotolerance. All cultures were maintained for 6 months at 24±2ºC with 40%-50% RH and an alternating photoperiod of 14 h light (66.25 μmol m^{-2} s^{-1}) and 10 h dark.

DISCUSSION

The storage behaviour of seeds of Chinese fan palm is largely uncertain. It has been known for some time that this species produces short-lived seeds, and its seeds were thought to be recalcitrant (desiccation sensitive) because of their large size and 1000-seed weight (7). However, more recently it was suggested that this species might have orthodox or intermediate rather than recalcitrant seed (25). In our experiments, this species produced short-lived and desiccation-sensitive seeds. When intact seeds at 27 WAF were dehydrated rapidly by activated silica gel in a desiccater, the moisture content at which 50% of seeds were killed was about 22% (fresh weight basis), and seed germination fell to 8% as seed moisture content decreased to 17% (data not shown). In addition, the radicle was found to protrude through the testa and epicarp in some seeds at the last developmental stage (Fig. 1b), i.e., they exhibited vivipary, which is also a feature of some recalcitrant seeds. Based on these characters and according to opinions from Chin *et al*. (7), Farnsworth (12) and Tweddle *et a*l. (24), Chinese fan palm seeds in our experiments can be considered as recalcitrant.

Like the oil palm seeds investigated by Grout *et al* (14), excised embryos of Chinese fan palm exhibited much greater tolerance to dehydration than intact seeds, and could be cryopreserved successfully (Fig. 6). Embryos displayed only slight desiccation tolerance at 15 WAF; their survival and emergence following dehydration progressively increased with development until 25 WAF. Desiccation tolerance remained at its highest between 25 and 33 WAF, and then declined at latter developmental stages. These results are comparable to those for *Clausena lansium* seeds (13), which displayed a similar pattern of acquisition and loss in desiccation tolerance during seed development. When expressed as W_{90DS} and W_{90DE}, desiccation tolerance of Chinese fan palm embryos reflected the same behaviour; it decreased with development, until 21 WAF for survival and 25 WAF for emergence, and then increased after 33WAF. The progressive improvement in desiccation tolerance of Chinese fan palm embryos during the early development stages is probably a consequence of physiological and morphological changes that take place gradually as development proceeds, perhaps including the synthesis of specific protective substances. The decline in desiccation tolerance of embryos during the last developmental stages might be the result of the initiation of germination-associated events, as seeds become more sensitive to dehydration once germination is initiated (3).

For cryopreservation and subsequent seedling regeneration from recalcitrant embryos or embryonic axes, desiccation- and cryo-tolerance are closely related throughout seed development. It has been suggested that selecting embryos at the right developmental stage is of critical importance for the success of cryopreservation (10,11), but only a few recalcitrant seed species have been tested at two or three different maturity stages, including jackfruit (17,23) and cocoa (1,2). In this study, the cryotolerance of Chinese fan palm embryos was nil before 21 WAF, but gradually acquired from 23 WAF onwards, reached its highest level at 27-36 WAF and then decreased through to 45 WAF. This developmental pattern of acquisition and loss of cryotolerance was concomitant with that of desiccation tolerance during seed development. To some extent, changes in cryotolerance of Chinese fan palm embryos can be attributed to those noted in desiccation tolerance during seed development.

However, freezing and dehydration are fundamentally different stress vectors (9). Not all changes in cryotolerance can be attributed to those observed in desiccation tolerance during seed development. In this study, embryos acquired desiccation tolerance much earlier during seed development than cryotolerance. Thus, embryos at 21 WAF had almost reached the highest desiccation tolerance, whilst those before 23 WAF could not survive cryoexposure and the highest cryotolerance was not acquired until 27 WAF.

For embryos at the same developmental stage, the moisture content before cryopreservation markedly influenced survival and emergence after cryopreservation. Embryos suffered cryo-damage if they were plunged in liquid nitrogen at their initial moisture content, which was always greater than or close to 70%, but they could survive and emerge well when dried to moisture contents below 20% prior to freezing. Mazur (18) considered that lethal intracellular ice crystal formation might be the major factor causing cryopreservation failure. Therefore, all cryopreservation protocols are designed to either remove freezable, intracellular water or to avoid ice formation through vitrification. Because of the vital role of moisture content in cryopreservation, the upper limit of moisture content range for avoiding ice crystal injury was defined as the TFMC (Threshold Freezable Moisture Content) by Hor *et al.* (15). In plant germplasm cryopreservation it is a common procedure to lower the moisture content of samples to be preserved below TFMC prior to freezing.

However, embryo developmental stage notably influenced the correlation between moisture content and cryopreservation success, since embryos with higher moisture contents retained high survival or emergence when they were at developmental stages displaying high cryotolerance. The moisture content limit for ≥50% embryo survival after cryopreservation increased with seed development, reached a peak at about 27-30 WAF, and then decreased. However, the moisture content limit for ≥50% emergence in surviving embryos was only observed in 27-36 WAF embryos with moisture content below 20%. Nonetheless, > 90% of the embryos could be dehydrated to moisture contents below 20% without loss in either survival or emergence as early as 21 WAF, and nearly 80% embryos could be dehydrated safely to 20% as late as 45 WAF (Fig. 5).

A number of processes or mechanisms have been suggested to confer, or contribute to, desiccation tolerance. These processes may protect against the consequences of water loss at different hydration levels, and the absence or ineffective expression of one or more of them could determine the relative degree of desiccation sensitivity of seeds of individual species (19). The main mechanisms involved in desiccation tolerance include cellular de-differentiation, metabolic 'switch off', vitrification, protective molecule synthesis, and the presence and efficient operation of antioxidant system (19, 21, 22). The mechanisms involved in cryotolerance are so far unknown, and deserve further research. It is particularly intriguing to study how Chinese fan palm embryos with higher moisture contents at intermediate developmental stages are protected from freezing damage, and what the protective mechanism is. This species produces many fruits per plant and it is easy to collect them at the same developmental stage. These characteristics make it an excellent species for further research on the mechanisms of desiccation- and cryo-tolerance and for studying how these parameter change during seed development.

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