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Effects of Dehydration on Freezing Characteristics and Survival In Liquid Nitrogen of Three Recalcitrant Seeds

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ABSTRAK

Biji benih 'rekalsitran' seperti rambutan (Nephelium lappaceum), *durian* (Durio zibethinus) *dan cempedak* (Artocarpus integer) *mempunyai kelembapan kritikal yang tinggi iaitu 27.0%, 26.0% dan 37.9% masing-masing. Kelembapan kritikal embrio pula lebih tinggi iaitu 39.0% untuk rambutan, 53.9% untuk durian dan 43.2% untuk cempedak. Analisis 'terma bezaan' (DTA) embrio mengesahkan bahawa ambang kelembapan (threshhold moisture) lebih rendah daripada kelembapan kritikal. Ambang kelembapan untuk rambutan, durian dan cempedak masing-masing adalah lebih kurang 30%, 32% dan 33%. Dicadangkan bahawa kegagalan percubaan menyimpan embrio rekalsitran dalam cecair nitrojen pada masa dahulu disebabkan oleh ketiadaan julat yang sesuai antara kelembapan kritikal dengan ambang kelembapan. Ini menyebabkan kerosakan beku ditahap kelembapan tinggi dan kerosakan kering ditahap kelembapan rendah. Teknik-teknik yang potensi untuk mengatasi masalah ini dan membaiki kaedah penyimpanan embrio spesies 'rekalsitran' secara kriogenik dibicangkan.*

ABSTRACT

The recalcitrant seeds rambutan (Nephelium lappaceum), *durian* (Durio zibethinus) *and cempedak* (Artocarpus integer) *have a high critical moisture content (below which rapid loss of viability occurs) of 27.0%, 26.0% and 37.9%, respectively. The critical moisture contents for embryos were higher at 39.0% for rambutan, 53.9% for durian and 43.2% for cempedak. Differential thermal analysis of the embroyos confirmed that their threshhold moistures (below which there is no freezable water) were lower than their critical moistures. The threshhold moistures for rambutan, durian and cempedak embryos were approximately 30%, 32% and 33% respectively. It is suggested that unsuccessful attempts at cryopreservation of embroyos of recalcitrant seeds in the past may be due to the absence of a safe window between the high critical moisture content and the threshhold moisture. This results in freezing injury at the higher moistures and dehydration injury at the lower moistures. Potential techniques to overcome this and improve cryopreservation of recalcitrant seed embryos are discussed.*

INTRODUCTION

Cryopreservation is acknowledged as an important tool in present day genetic conservation. Many seeds have been successfully frozen in liquid nitrogen and stored for many years (Stanwood, 1984). At the low temperature of -196° C, it is claimed that deterioration is essentially absent and seeds can theoretically be stored for long periods.

Nearly all seeds which have so far been successfully cryopreserved were orthodox seeds. Only few reports are available on successful cryopreservation of recalcitrant seeds (Bajaj, 1985; Normah et al. 1986). This is because

many of these have high moisture content when ripe and do not survive even moderate dehydration to relatively high moisture of 28% (Hor, et al. 1984; Roberts, 1973). At these high moistures, cryopreservation is lethal and ice formation can occur when the tissues are frozen in liquid nitrogen.

Successful protocols for cryopreservation of recalcitrant seeds generally utilise the embryonic axes, and commonly involve some degree of dehydration to reduce freezing injury (Normah et al, 1986; Bajaj, 1985). However, the critical moisture content (below which rapid loss of viability occurs) of many recalcitrant seeds and their embryos have not been clearly elucidated; and the moisture content at which freezable water is totally removed from these embryos (threshhold moisture content) has not been measured. Bewar *et al.* (1983) investigated some tropical seeds, but none of these were truely recalcitrant. In this study, the critical moisture content of the seed and the attached embryo of three recalcitrant species were determined. Additionally, the ability of the dehydrated embryos to survive cryopreservation in liquid nitrogen was elucidated. Lastly, differential thermal analysis (DTA) of the excised embryos was conducted to determine their threshhold moisture content, and to help explain whether unsuccessful cryopreservation of these embryos was due to dehydration or freezing injury.

MATERIALS AND METHODS

Seeds of three recalcitrant tropical fruit species, namely, rambutan (*Nephelium lappaceum L*), durian (*Durio zibethinus Murr.*) and cempedak (*Artocarpus integer* (Thunb.) Merr.) were extracted from the ripe fruits and the surrounding fleshy tissues or pulp removed. The clean seeds were dusted with 0.02% w/w of an equal benomyl-thiram mixture and dried in an air-conditioned room (22°C,55% relative humidity). After various periods of dehydration, batches of seeds were removed to measure seed moisture, embryo moisture and germination. The viability of the attached embryos with and without exposure to liquid nitrogen was also determined. All treatments were replicated three times.

Moisture and germination tests were conducted according to the International Rules for Seed Testing set by the International Seed Testing Association (1985); ten and 50 seeds were used per replicate respectively. For moisture, the 103 C oven method was used while for germination the in-sand method was employed.

The ability of embryos from dehydrated seeds to survive liquid nitrogen exposure was evaluated using three replicates of 20 seeds. Seeds after various periods of dehydration were sterilised in 1% w/w chlorine for 10 min before the embryos were aseptically excised and put into sterilised polypropylene vials. The vials were then tightly wrapped in alluminium foil, plunged into liquid nitrogen, and left immersed overnight for at least 16 hr. After retrieval from liquid nitrogen and thawing in air for 30 min, survival of the cryopreserved axes was assessed by culture on agar medium (Murashige and Skoog 1962) supplemented with $1 mgl^{-1}$ each of kinetin, indoleacetic acid and gibberellic acid. The ability of the embryos to expand and develop a root and shoot system was used as a measure of viability. For the control, 20 axes were similarly excised from the seeds after each period of dehydration and cultured without immersion in liquid nitrogen.

The presence or absence of freezable water in dehydrated axes was monitored separately using differential thermal analysis (DTA). For cempedak and rambutan, the whole embryonic axis was used but for durian, only a longitudinal quadrant was utilised as the axis was too large. The method was as described by Hor and Standwood (1987).

RESULTS AND DISCUSSION

Freshly harvested seeds of the three species have high moisture contents ranging from 32% to 49% (*Fig. 1*). Fresh embryonic axes have even higher moisture contents of 44% to 65%. Such high moisture is characteristic of many tropical recalcitrant seeds and points to the greater damage that can result during dehydration since more moisture has to be removed causing greater instability within the seed tissues. Among the three seed types, rambutan showed the fastest rate of drying

followed by durian. Cempedak was relatively slow and there was little loss of moisture from the seeds and embryonic axes for the initial four days. This could possibly be due to the testa being more impermeable to moisture especially when it is still moist. Desiccation of the attached embryo generally followed the same pattern as moisture loss from the seed in all three species.

With dehydration, seed germination and embryo viability were rapidly lost (*Fig. 2*). Germination was reduced to 75% in rambutan, durian and cempedak when seed moisture was decreased to 27.0%, 26.0% and 37.9% respectively (Table 1). Germination was totally lost when seed moisture decreased further to 12.7% for rambutan, 20.1% for durian and 30.2% for cempedak. The effect of dehydration on the attached embryo was similar to that of the seed and although moisture was relatively high, their critical moisture (when viability was reduced to 75%) was also higher than the seed. Thus in rambutan, durian and cempedak significant reduction in embryo viability was evident at moisture contents of 39.0%, 53.9% and 43.2%, respectively. The relatively high critical moisture of these seeds confirms the difficulties in storing them using conventional methods. At most, the seeds can only be stored for a few months before rapid decrease in germination occurs (Hor, 1988). For embryos, storing them by the slow growth method did not extend their storage life significantly and was reported to be impractical (Normah, 1987).

TABLE 1

Percentage moisture of seed and embryo at various levels of survival based on the polynomial plots of Figure 2.

	Seed moisture			Embryo moisture		
Seed	75% germ	50% germ	0% germ	75% viab.	50% viab.	0% viab.
Rambutan	27.0	22.6	12.7	39.0	32.0	14.4
Durian	26.0	23.5	20.1	53.9	48.1	40.1
Cempedak	37.9	34.8	30.2	43.2	36.0	25.7

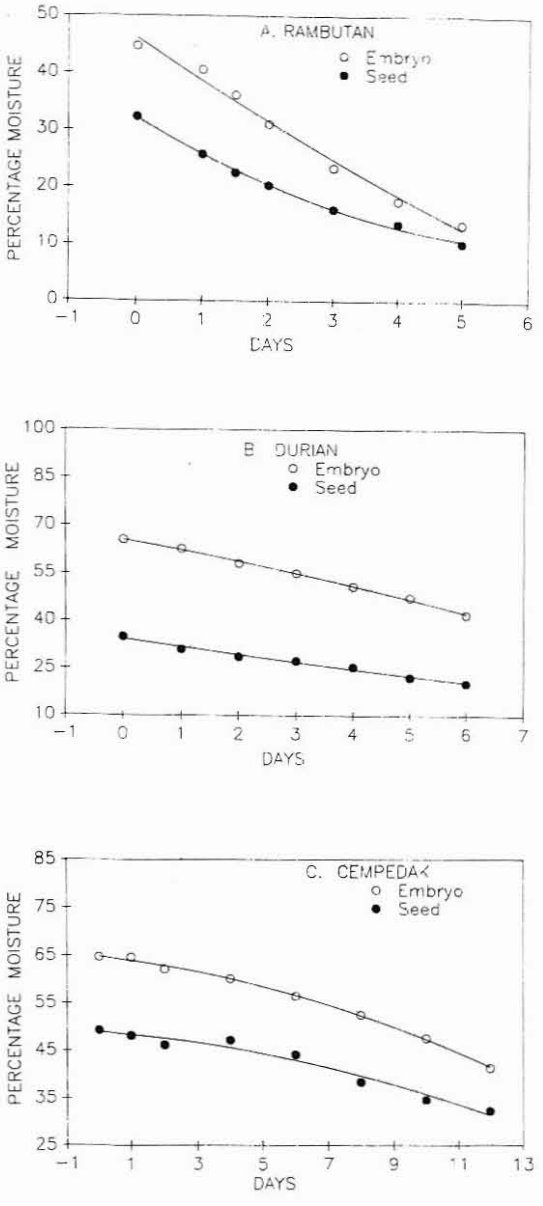

Fig. 1: Moisture changes in the embryo and seed of (a) Rambutan (b) Durian and (c) Cempedak with days of seed dehydration

The appropriate regression equations are :

a. Rambutan :

$Y = 46.13 - 7.73\ X + 0.21\ X^2$ $r^2 = 0.99$ *(embryo)*

$Y = 32.16 - 6.84\ X + 0.51\ X^2$ $r^2 = 0.99$ *(seed)*

b. Durian :

$Y = 65.59 - 3.14\ X - 0.12\ X^2$ $r^2 = 0.99$ *(embryo)*

$Y = 34.38 - 2.16\ X + 0.05\ X^2$ $r^2 = 0.98$ *(seed)*

c. Cempedak :

$Y = 64.83 - 0.78\ X - 0.1\ X^2$ $r^2 = 0.99$ *(embryo)*

$Y = 48.95 - 0.53\ X - 0.08\ X^2$ $r^2 = 0.97$ *(seed)*

Cryopreservation of fresh and dehydrated embryos of rambutan, durian and cempedak by the direct plunge method was unsuccessful. Excised embryos from fresh or dehydrated rambutan and cempedak seeds did not expand but gradully turned whitish and then brown. The durian embryos turned brown directly after thawing and often released a mucous exudate.

Death of the embryos after liquid nitrogen exposure may be partially explained by differential thermal analysis of the embryonic axes of the three fruit species. Typical exothermal peaks of rambutan, durian and cempedak axes at various moisture contents are shown in *Fig. 3*. The exotherms were produced when moisture within the embryo froze and their presence indicate the existence of freezable water within the tissues. The exotherms exhibited by embryos of the three fruit species were narrowly spiked and slightly skewed towards the lower temperature. It suggests that in all cases, the supercooled freezable moisture was very rapidly frozen at the temperature when freezing was initiated. At any particular moisture, the exothermal peaks exibited by durian embryos were relatively large while those of rambutan were relatively small. Although this suggests the presence of more freezable water in durian compared with rambutan at a specific moisture, it is more a reflection of the size of the tissues used in the differential thermal analysis. In durian, the longitudinal embryonic quadrant was approximately 4-6 times larger than the excised rambutan axis so that the total amount of water at any particular moisture content was higher compared to rambutan embryos.

Within each species, the size of the exotherms was progressively reduced as dehydration progressed indicating the reducation of freezable water. At the same time, the freezing temperature was generally lowered. Further dehydration resulted in the removal of all freezable water and the absence of an exotherm (Fig. 4). At this stage, no lethal ice formation occurred and the tissues could theoretically be frozen without injury. For rambutan, this threshhold moisture was reached when the embryo was dehydrated to approximately 30%. For durian and cempedak, the corresponding moistures were 32% and

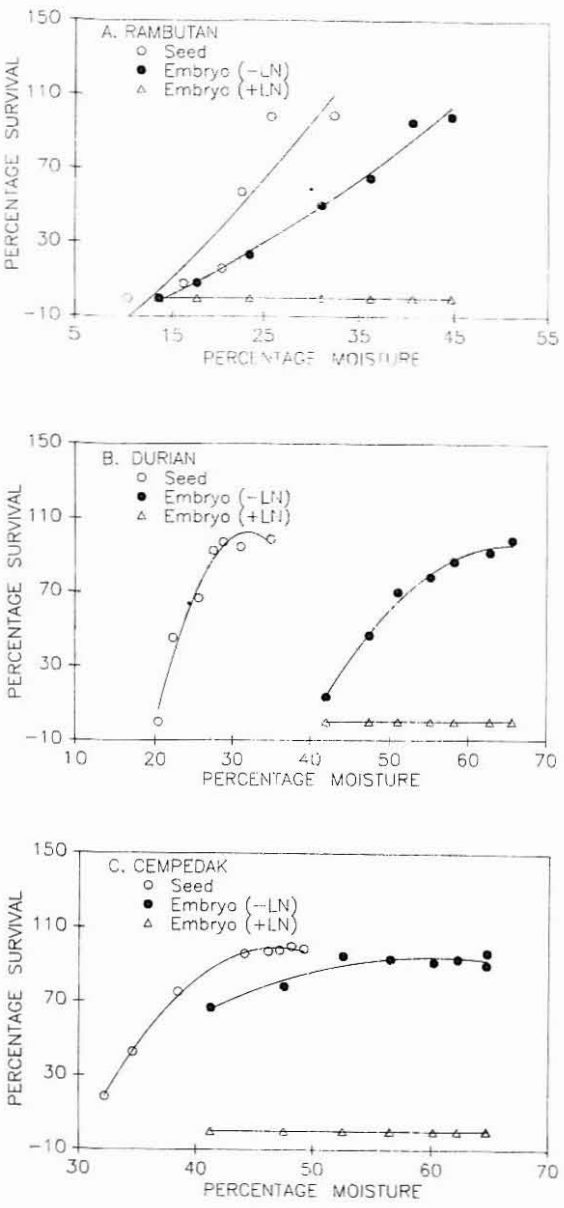

Fig. 2: he relationship between seed/embryos survival and their respective moisture content for (a) Rambutan (b) Durian and (c) Cempedak

The appropriate regression equations are :

a. Rambutan :

$Y = -27.05 + 1.45\ X + 0.03\ X^2\ r^2 = 0.99$ *(embryo)*

$Y = -49.63 + 3.27\ x + 0.05\ X^{2\ 2} = 0.92$ *(seed)*

b. Durian :

$Y = -534.05 + 19.55X - 0.15\ X^2\ r^2 = 0.99$ *(embryo)*

$Y = -629.66 + 45.82\ X - .72\ X^2\ r^2 = 0.91$ *(seed)*

c. Cempedak :

$Y = 198.36 + 9.77\ X - 0.08\ X^2\ r^2 = 0.91$ *(embryo)*

$Y = -720.56 + 35.02\ X - 0.37\ X^2\ r^2 = 0.99$ *(seed)*

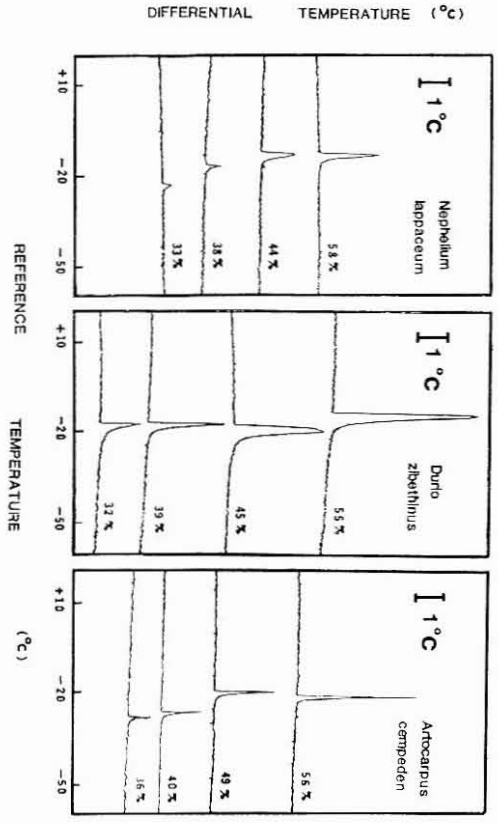

Fig. 3: DTA cooling profiles of rambutan (Nephelium lappaceum), durian (Durio zibethinus) and cempedak (Artocarpus cempeden) embryos dehydrated to various moisture content. The exotherm temperature (arrowed) is the temperature at which freezing of the moisture in the axes commenced.

33%, respectively. However, in all three species there was no survival when these embryos were frozen in liquid nitrogen even though their freezable water has been removed (*Fig. 2*).

Comparison of the threshhold and critical moisture of these recalcitrant seeds explains why this is so. For rambutan, embryos above the threshhold moisture of 30% were killed as a result of lethal ice formation since freezable water was present. At moisture contents below the threshhold level, no freezable water was present but the embryos were already killed by dehydration since the critical moisture at 39.0% was higher than the threshhold moisture. For durian and cempedak, the embryos were similarly killed as their critical moisture content of 53.9% and 43.2% respectively were higher than their threshhold moisture of 32% and 33%.

The results suggest that when undetached embryos of the three recalcitrant seeds were subjected to dehydration, there was no safe window between the threshhold and critical moisture at which they can survive cryopreservation. This could be a common phenomenon in many recalcitrant seeds where the critical moisture content of embryos dried undetached is relatively high. This may be one of the main reasons why many of them have not been successfully cryopreserved. However, it should be noted that in this study, the embryos were dehydrated while they were still attached to the seeds. They were then excised and directly plunged into liquid nitrogen without exposure to cryoprotectants. Perhaps, the use of more controlled dehydration coupled with

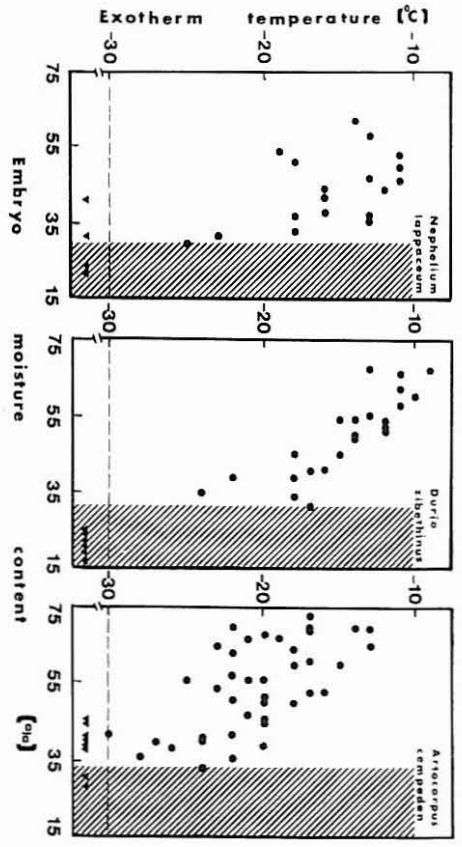

Fig. 4: Exothermal temperature of rambutan (Nephelium lappacium), durian (Durio zibethinus) and cempedak (Artocarpus cempeden) embryos as a function of embryo moisture content. The shaded portions denote embryo moistures where no exotherm was detected).

the use of cryoprotectants and a slower rate of freezing may create a narrow window where embryo survival after cryopreservation is possible. This could come about either through increased tolerance of the axes to desiccation thereby lowering their critical moisture content, or by prevention of ice formation in moist tissues thereby effectively raising their threshhold moisture content. The potential of such techniques was demonstrated by Chin *et al.* (1988) when the critical moisture content of embryos of the recalcitrant seeds of rambutan, jackfruit (*Artocarpus heterophyllus*) and coconut were reduced to below 14%-15% when they were dehydrated as excised rather than attached embryos. Although these embryos did not survive direct exposure to liquid nitrogen, some survival (10-40%) was achieved when they were imbibed in cryoprotectants and partial dehydration were also demonstrated by Bajaj (1985) using coconut embryos. Although these embryos were recalcitrant, 17% to 25% of the partially dried embryos survived freezing when they were cryoprotected in a mixture of 7% dimethylsulphoxide (DMSO) and 4% sucrose and proliferated after a lag period of up to four months. Similar results were obtained by Normah *et al* (1986) using the recalcitrant seed, *Hevea brasilliensis*. Cryoprotected embryos partially dehydrated to 13%-19% moisture showed a 20%-69% survival rate after cryopreservation, and in many cases survival was further increased through slower freezing using a stepwise freezing method (Normah, 1987). Collectively, these studies indicate there is optimism for the successful cryopreservation of excised embryos of many recalcitrant seeds. However, the effects of various factors on survival need to be systematically elucidated for each seed species before a practical method of cryopreservation with high survival and minimal genetic changes could emerge.

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