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Desiccation and Cryopreservation of Embryonic Axes of *Hevea brasiliensis* Muell. — Arg.

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ABSTRAK

Paksi embrio Hevea *telah dikeringkan selama 1 – 5 jam dan kandungan kelembapan ditentukan selepas setiap jam pengeringan. Sekumpulan paksi embrio yang lain pula dikeringkan dengan cara aseptik untuk masa yang sama sebelum dikrioawetkan untuk 16 jam di dalam nitrogen cecair (– 196°C). Pada kandungan kelembapan antara 14 – 20% (pengeringan selama 2 – 5 jam), 20 – 69% paksi embrio hidup selepas krioawetan dan membentuk akar dan pucuk yang normal. Oleh kerana ketidaknormalan dapat dicerap pada beberapa anak benih, teknik ini perlu diperbaiki.*

ABSTRACT

Hevea *embryonic axes were desiccated for a period of 1 – 5 hours and the moisture content was determined at the end of each hour of desiccation. Another set of embryonic axes were aseptically desiccated for the same period before they were cryopreserved for 16 hours by direct immersion in liquid nitrogen (– 196°C). At a moisture content between 14 – 20% (desiccation for 2 – 5 hours), 20 – 69% of the embryonic axes survived cryopreservation and formed seedlings with normal roots and shoots when cultured* in vitro. *Abnormalities were deteched in some seedlings however, hence, refinement of the technique is needed.*

INTRODUCTION

Rubber or *Hevea* seeds are classified as recalcitrant seeds (Roberts, 1973). Recalcitrant seeds are killed if their moisture content is reduced below some relatively high critical value (12 – 31%) (Roberts, 1973). Moreover when such seeds are maintained in a moist condition, their longevity is relatively short, varying from a few weeks to a few months (King and Roberts, 1980). *Hevea* seeds are also killed by freezing temperatures (Chin *et al.*, 1981). At present there are no methods available for the storage of *Hevea* seeds for longer than a year. Although conventional seed storage methods will satisfy the requirement for many species, an alternative method is required for vegetatively propagated plants and species with recalcitrant that in (Withers, 1980). Bajaj (1985) suggested that in such cases where the seeds are short-lived, germplasm could possibly be conserved through the cryopreservation of excised embryos or their segments. It has also been reported that materials dehydrated by drying in an oven (Sun, 1958) or under vacuum (Withers, 1979) exhibit remarkable resistance to cryogenic damage. Grout *et al.* (1983) have shown that embryos of oil palm *(Elaesis guineensis)* seeds which were formerly classified as recalcitrant, can be successfully cryopreserved after desiccation. In the present study, attempts were made to desiccate embryonic axes of highly recalcitrant *Hevea* seeds by a slow drying method in order to deter-

mine a suitable moisture content for successful cryopreservation.

MATERIALS AND METHODS

Hevea seeds (clone PB 260) were obtained from Prang Besar Research Station in Prang Besar, Malaysia. For moisture determination, fresh seeds were cracked open and embryonic axes were excised. The axes were then dried in a laminar flow hood at 26 – 28°C for 0, 1, 2, 3, 4 and 5 hours after which the moisture content was determined by drying the axes at 105°C for 16 hours. Moisture content was expressed as a percentage of fresh weight. Four replicates of 10 embryonic axes were used for moisture content determination.

Seeds were sterilized in 20 – 30% Clorox solution containing a few drops of Teepol for 30 minutes, and rinsed with sterilized water. The seeds were cracked open and the excision of the embryonic axes was done by removing the endosperm and cotyledons completely. The excision and desiccation of embryonic axes was carried out under aseptic condition. After desiccating the embryonic axes in a laminar flow hood for 0, 1, 2, 3, 4 and 5 hours, three replicates of 10 embryonic axes of each treatment were enclosed in tightly folded, sterilized aluminium foil envelopes, immersed directly into liquid nitrogen and stored for 16 hours. Thawing was carried out by immersing the aluminium foil envelopes in a water bath at 37 ± 2°C for 5 minutes. Each embryonic axis was then cultured on a modified Murashige and Skoog (1962) medium supplemented with 0.6 – 0.7 μM kinetin, 1.0 μM naphthaleneacetic acid (NAA), 1.4 μM gibberellic acid (GA), and 4 g l^{-1} activated charcoal. Cultures were incubated in the light (3000 lux) under a 12 hour photoperiod. Observations were made on the growth of the embronic axes. The axes were recorded as viable when they expanded and turned green, and as having survived cryopreservation when a fully developed seedling (with normal shoot and root) was obtained.

A similar number of embryonic axes cultured immediately following each desiccation hour (no cryopreservation), were used as the control.

RESULTS

Freshly excised embryonic axes had a moisture content of approximately 55% (Table 1). The moisture content of axes decreased rapidly within the first two hours of desiccation under the condition employed but the decrease was more gradual thereafter. Embryonic axes with no desiccation showed 100% viability and survival when cultured *in vitro*. However, with increasing duration of desiccation (1 – 5 hours),

TABLE 1
Percentage viability and survival of embryonic axes of *Hevea* following cryopreservation in liquid nitrogen for 16 hours at various initial moisture contents

Duration of desiccation (hour)	Moisture content (%)	Viability (%)	Survival (%)
0	54.51 ^{a}	0 ^{c}	0 ^{d}
1	35.21 ^{b}	0 ^{c}	0 ^{d}
2	19.17 ^{c}	20 ^{b}	20 ^{cd}
3	16.23 ^{cd}	87 ^{a}	69 ^{a}
4	15.28 ^{cd}	80 ^{a}	25 ^{bc}
5	13.69 ^{d}	80 ^{a}	33 ^{b}

Values in the same column having the same superscript are not significantly different at p = 0.05 based on Duncan Multiple Range Test.

there was a decrease in viability down to 50% at 5 hours.

Following cryopreservation, none of the axes with moisture content 55% and 35% (0 and 1 hour desiccation) were viable. More than 50% viability was obtained when the cryopreserved axes contained less than 20% moisture. Satisfactory survival *in vitro* was only obtained when the moisture content of the axes was 16% or below. The best percentage viability (87%) and survival (69%) was obtained when embryonic axes were cryopreserved at a moisture content of approximately 16%. Moisture contents below this significantly decreased the percentage survival of seedlings. Various abnormalities in seedling growth were observed in some of the treatments, particularly in those which developed from axes cryopreserved at the two lowest moisture contents used.

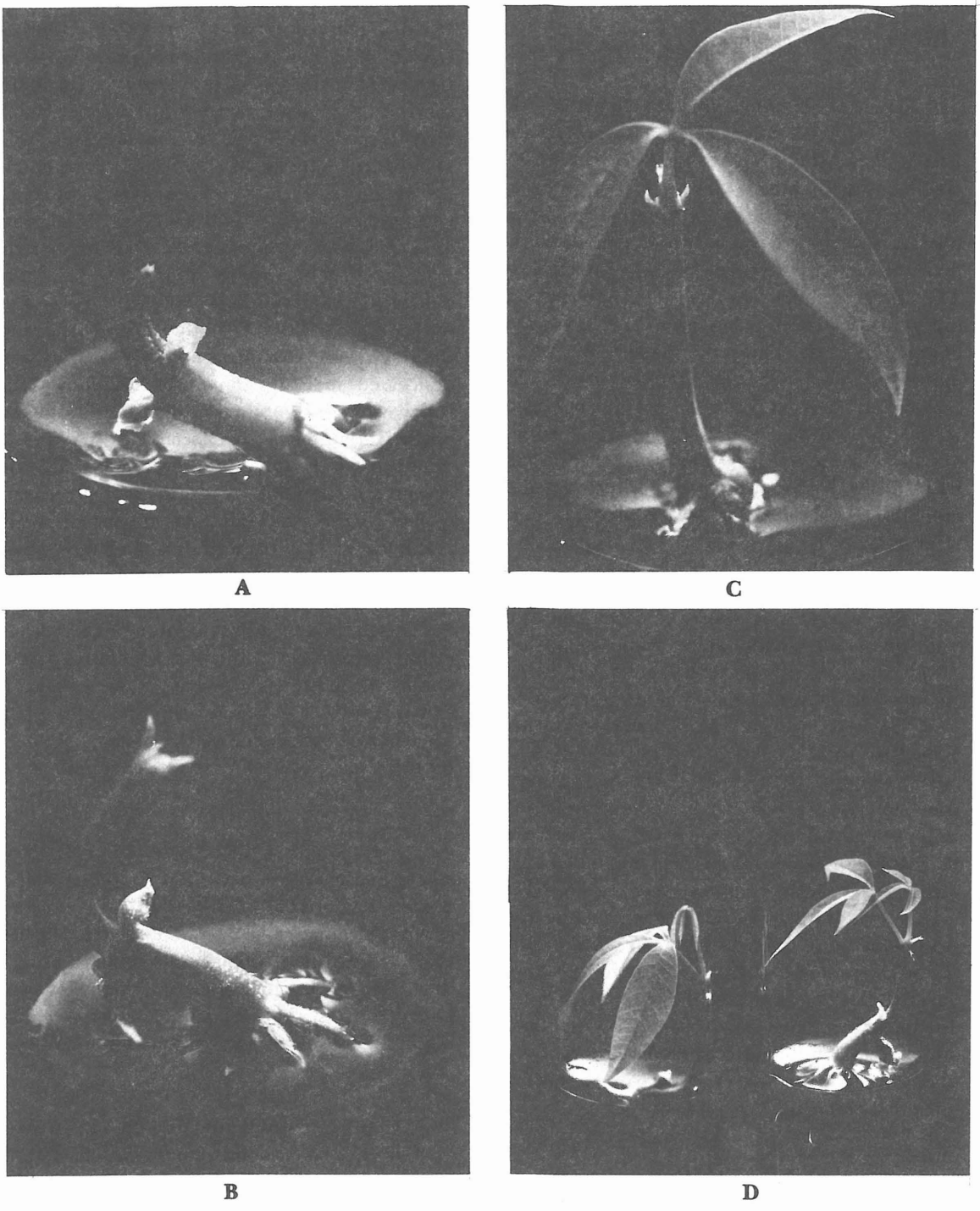
Plates 1 A, B, C show the development of the embryonic axes after cryopreservation. Viable axes swelled and turned green within 7 – 10 days of culture. Shoot and root emergence was observed between 2 and 3 weeks in culture. The first trifoliate leaves formed within 3 – 4 weeks. *Plate 1D* shows a seedling from a control axis (no cryopreservation) and a seedling from a cryopreserved axis. The hypocotyl of the control seedling elongated and swelled more than the hypocotyl from the cryopreserved axis. Compared to the control, the development of the root and shoot of the cryopreserved axis was slower by 2 weeks. However, after the first trifoliate leaves formed, both types of seedlings were very similar in appearance. Thus, the cryopreserved embryonic axes took a longer time to develop root and shoot, but once these had developed, normal growth continued.

DISCUSSION

Recalcitrant seeds are believed to be sensitive to desiccation. The critical moisture content of rubber seeds is reported to be between 15 – 20%; below this level the seeds are killed (Chin *et al.*, 1981). However, it has been reported that zygotic embryos of citrus (Mumford and Grout, 1978) and oil palm (Grout *et al.*, 1983) withstood freezing after being subjected to partial desiccation. Withers (1979) also reported the successful survival of somatic embryo-derived carrot plantlets subjected to desiccation. The present study is believed to be the first to demonstrate the potential of desiccation and cryopreservation of *Hevea* embryonic axes. The study suggests the existence of a critical optimum moisture content of the axes in order to survive cryopreservation. The dehydration optimum may vary with species and tissues (Sun, 1958 and Withers, 1979). Though the *Hevea* embryonic axes used in this study were desiccated, moisture content of the axes was still relatively high compared to that used in the cryopreservation orthodox seeds. Bajaj (1985) stated that although desiccation may enable the cryopreservation of embryos of recalcitrant species, such procedure should be carefully manipulated, as subcellular damage would occur below the critical moisture level for each species. Thus, the high moisture in the axes in the present investigation might have caused subcellular damage resulting in the formation of abnormal seedlings. Lowering the moisture content further however did not reduce the occurrence of abnormalities, suggesting the need for further improvements in the technique.

Attention should be therefore given to all stages of the cryopreservation procedure, since apparently minor modification can improve survival dramatically (Withers, 1982). Withers (1979) also suggested that slow cooling, using a dry freezing method and slow thawing and recovery on a charcoal-supplemented medium might be suitable for large organs and plantlets.

At the temperature of liquid nitrogen (– 196°C) all metabolism would be considerably reduced and essentially "indefinite" preservation could be achieved (Stanwood, 1985). Although improvement in the technique is still necessary, cryopreservation of embryos should be considered an important method in long term storage and germplasm conservation of species with recalcitrant seeds.



Growth of cryopreserved embryonic axis
Plate 1A: After 2 weeks of culture.
B: After 3 weeks of culture.
C: After 4 weeks of culture.
D: Seedling from cryopreserved embryonic axis (left) and seedling from control axis (right).

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