UNIVERSITI PUTRA MALAYSIA

DESICCATION AND PRECULTURE EFFECTS ON SURVIVAL OF ENCAPSULATED ZYGOTIC EMBRYOS OF RUBBER (HEVEA BRASILIENSIS MUEL. -ARG) FOLLOWING LIQUID NITROGEN EXPOSURE

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By

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ABBREVIATIONS

ABA	abscisic acid
BAP	benzylaminopurine
DMSO	dimethyl sulphoxide
DSC	differential scanning calorimetry
DTA	differential thermal analysis
GA3	gibberellic acid
HMTL	high moisture freezing limit
MC	moisture content
MS	Murashige and Skoog medium, 1962
NAA	alpha-naphthalene acetic acid
RCBD	randomised complete block design
TMC	threshold moisture content
+LN	with liquid nitrogen exposure
-LN	without liquid nitrogren exposure

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The effects of desiccation, sucrose as cryoprotectant and abscisic acid (ABA) as chemical ameliorant on the cryopreservation of *Hevea* zygotic embryos were evaluated using the alginate encapsulation method.

The first part of the study was on the effects of desiccation on the survival of alginate-encapsulated *Hevea* zygotic embryos following liquid nitrogen exposure. The embryos need to be desiccated to at least 26% moisture content to enable some survival after exposure to liquid nitrogen. Embryos desiccated to moisture content of 14% and 18% gave comparatively higher survival after cryopreservation (42.5% and 47.5% respectively). Encapsulation of the embryos enhanced desiccation tolerance and desiccation was allowed even until 10% moisture content with some survival even though *Hevea* seeds are known to be recalcitrant. Encapsulation had broaden the window for cryopreservation by allowing the embryos to survive desiccation and

cryopreservation at a broader range of moisture content compared to naked embryos done in previous work. However, a very low percentage of embryos developed into normal plantlets.

The importance of sucrose preculture for cryopreservation of encapsulated *Hevea* embryos is also proven in this study. Sucrose preculture at low concentration of 0.3 M improved viability and survival before and after cryopreservation significantly to 70% and 60% respectively. Desiccation and freezing resistance were further enhanced when the encapsulated embryos were precultured on 0.5 M sucrose with viability as high as 82% (after cryopreservation). However, after twelve weeks culture, the percentage of survival after cryopreservation was maintained as when precultured on 0.3 M sucrose, with 14% and 16% moisture levels showing better results (51% and 59% respectively). Preculture with 0.5 M sucrose improved perculture of embryos developed into normal plantlet (as high as 35% and 32% survival before and after cryopreservation).

As the concentration of sucrose preculture was increased further to 0.7 M and 0.9 M, the freezing tolerance of the embryos reduced considerably. A very low percentage of normal plantlet was obtained after cryopreservation (3 to 9%). Desiccation tolerance was also slightly reduced as indicated by lower survival before cryopreservation.

This study concluded that sucrose preculture can enhance desiccation and freezing tolerance of *Hevea* encapsulated embryos. Sucrose preculture at 0.3 M and 0.5 M may be best for cryopreservation of encapsulated *Hevea* embryos as survival was highest (60%) at these two concentrations.

Preculture with abscisic acid did not induce desiccation tolerance of encapsulated *Hevea* embryos as none survived liquid nitrogen exposure after preculture with abscisic acid. Abscisic acid is therefore not a good chemical ameliorant for cryopreservation of *Hevea* embryos.

Embryos of *Hevea* with different treatments were subjected to differential thermal analysis (DTA) to detect phase transitions of embryo moisture. A single exotherm was obtained in the DTA profile for all the treatments. The threshold moisture content (TMC) of naked embryos were 17% and further increased to 21% when encapsulated with sodium alginate. The TMC was maintained after the encapsulated embryos were precultured on MS basal medium and on 30 μM ABA. When the encapsulated embryos were precultured with 0.3 M sucrose, the freezable water was absent at a moisture content of 22%. The TMC was further elevated to 24% when the encapsulated embryos were precultured in 0.5 M sucrose. Thus, it can be concluded that sucrose preculture caused the TMC of encapsulated *Hevea* embryos to be raised, thereby broadening the window for *Hevea* cryopreservation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains Pertanian.

KESAN PENGERINGAN DAN PRAKULTUR TERHADAP KEMANDIRIAN EMBRIO ZIGOTIK TERKAPSUL GETAH (*HEVEA BRASILIENSIS* MUEL.-ARG) SELEPAS PENDEDAHAN KEPADA NITROGEN CECAIR.

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Kesan pengeringan serta prakultur sukrosa dan asid absisik (ABA) ke atas pengkrioawetan embrio *Hevea* berselaput alginat telah dikaji.

Bahagian pertama kajian ini adalah tentang kesan pengeringan ke atas kemandirian embrio *Hevea* berselaput alginat selepas pendedahan kepada nitrogen cecair. Embrio didapati memerlukan pengeringan sehingga sekurang-kurangnya 26% kandungan lembapan untuk membolehkan sebilangan embrio berjaya untuk hidup selepas didedahkan kepada nitrogen cecair. Embrio-embrio yang dikeringkan ke paras kelembapan 14% dan 18% menunjukkan peratus kemandirian yang lebih tinggi (42.5% dan 47.5% masing-masing) secara perbandingan selepas pengkrioawetan. Penyelaputan embrio telah meningkatkan toleransi pengeringan embrio dan embrio boleh dikeringkan sehingga ke paras kelembapan 10% walaupun biji *Hevea* dikenali sebagai biji jenis rekalsitran. Penyelaputan telah meluaskan

tingkap untuk pengkrioawetan dengan membenarkan embrio-embrio berjaya untuk hidup selepas pengeringan dan pengkrioawetan pada satu julat paras kelembapan yang lebih panjang berbanding dengan embrio yang terdedah yang didapati dalam kajian sebelum ini. Walau bagaimanapun, peratus embrio yang berkembang membentuk anak benih yang sempurna adalah rendah.

Kepentingan prakultur sukrosa dalam pengkrioawetan embrio *Hevea* berselaput telah dibuktikan dalam kajian ini. Prakultur sukrosa pada kepekatan 0.3 M berjaya memperbaiki peratus viabiliti dan kemandirian embrio secara bererti kepada 70% dan 60% sebelum dan selepas pengkrioawetan. Ketahanan embrio-embrio terhadap pengeringan dan pembekuan telah dipertingkatkan apabila ia diprakulturkan dalam 0.5 M sukrosa dengan peratus viabiliti (selepas pengkrioawetan)mencapai setinggi 82%. Walau bagaimanapun, peratus kemandirian (selepas 12 minggu) selepas pengkrioawetan telah dikekalkan seperti dalam 0.3 M prakultur dengan 14% dan 16% peratus kelembapan menunjukkan keputusan yang lebih baik. Prakultur dengan 0.5 M sukrosa telah memperbaiki peratusan embrio yang berkembang membentuk anak benih sempurna (35% sebelum and 32% selepas pengkrioawetan).

Apabila kepekatan sukrosa dipertingkatkan kepada 0.7 M dan 0.9 M, toleransi pembekuan embrio-embrio telah menurun. Peratusan anak benih yang sempurna yang diperolehi adalah rendah (3 ke 9%) setelah dikrioawetkan.Toleransi pengeringan juga telah dikurangkan sedikit seperti yang ditunjukkan oleh peratus kemandirian yang rendah sebelum pengkrioawetan.

Kajian ini mendapati bahawa prakultur sukrosa boleh memperbaiki rintangan pengeringan dan pembekuan embrio-embrio *Hevea* berselaput. Prakulturan sukrosa pada 0.3 M dan 0.5 M adalah yang terbaik untuk pengkrioawetan embrio-embrio *Hevea* berselaput kerana peratus kemandirian yang tertinggi (60%) untuk kedua-dua kepekatan ini.

Asid absisik didapati tidak merangsangkan toleransi pengeringan embrio-embrio *Hevea* yang berselaput. Tiada viabiliti diperhatikan selepas embrio-embrio diprakulturkan dengan asid absisik dan dibekukan dengan nitrogen cecair. Dengan itu, asid absisik bukan satu perangsang kimia yang sesuai untuk krioawetan embrio-embrio *Hevea*.

Differential Thermal Analysis (DTA) telah dijalankan ke atas embrio-embrio *Hevea* yang telah dirawat dengan pelbagai rawatan. Satu eksoterma telah diperhatikan dalam profil DTA untuk semua rawatan. Ambang kandungan kelembapan atau 'Threshold moisture content (TMC)' untuk embrio terdedah adalah 17% dan ditingkatkan kepada 21% apabila diselaputkan dengan natrium alginat. TMC dikekalkan apabila embrio diselaputkan dan diprakulturkan dengan medium MS basal dan dengan 30 μM ABA. Setelah embrio berselaput diprakulturkan dengan 0.3 M sukrosa, air bolehbeku didapati tidak hadir pada embrio yang berkelembapan 22%. TMC dipertingkatkan sekali lagi kepada 24% apabila embrio berselaput diprakulturkan dengan 0.5 M sukrosa. Dengan itu, kesimpulan boleh dibuat bahawa prakultur dengan sukrosa boleh menyebabkan peningkatan TMC embrio *Hevea*

berselaput, seterusnya memperluaskan tingkap yang selamat untuk pengkrioawetan *Hevea*.

CHAPTER 1

INTRODUCTION

Hevea brasiliensis Muell. -Arg. originated from the tropical rain forests of South America and belongs to the family Euphorbiaceae. It was introduced in Malaysia in 1877 from the original 22 Wickham seedlings received in Singapore within the same year. Though new *Hevea* materials were introduced in the subsequent years, conservation of the existing and newly collected *Hevea* germplasm is necessary to provide a viable nucleus genestock for maximum genetic diversity in future breeding programmes.

Presently, *Hevea* germplasm is conserved in field collections and seeds are stored only for short periods of four months to one year. Field conservation method is more risky as it exposes the field germplasm to natural calamities as well as pest and diseases. At the same time, it competes for limited land for development purposes and requires more labour for management and data filing which is a drawback since the country is facing a labour shortage. Seed storage provides an alternative for conservation of genetic resources but is not applicable for recalcitrant seeds such as *Hevea*.

Seeds can be divided into two major groups based on their storage physiology, that is orthodox and recalcitrant (Roberts, 1973). Storage of orthodox seeds is not as difficult as they can withstand drying to as low as 3% moisture and can survive long term storage at sub-freezing temperature. Recalcitrant seeds however, are more problematic as they are sensitive to desiccation and are killed when their moisture content is reduced below some relatively high critical value of 12 - 31% (Roberts, 1973). Storage in moist conditions could not prolong storage significantly as the seeds either germinate or deteriorate at a relatively fast rate (King and Roberts, 1980). Therefore, long term seed storage is not possible and survival was limited to around a year for *Hevea* (Normah *et al.*, 1986). Like many other recalcitrant seeds, *Hevea* seeds are also intolerable to extreme temperature and are killed when exposed to freezing temperature (Chin *et al.*, 1981).

In recent years, a more promising method of conservation namely cryopreservation was developed. Cryopreservation involves storing tissues at the temperature of liquid nitrogen (LN) at -196°C. Preliminary studies of this method on *Hevea* was carried out by Normah *et al.* (1986), but as *Hevea* seeds are relatively large, excised zygotic embryos were used in their study. The study showed 69 - 71% survival after desiccation for 2 and 3 hours, followed by the step-wise or direct plunging into liquid nitrogen. As only moderate survival was obtained, further improvement should be carried out to enhance survival after LN exposure.

There are several factors which affect cryopreservation including the use of cryoprotectants or chemical ameliorants, alginate encapsulation, desiccation, freezing rate, thawing rate and recovery medium. In this study, the effects of desiccation, sucrose as cryoprotectant and abscisic acid (ABA) as chemical ameliorant were evaluated using the alginate encapsulation method. The objectives are:

1. To evaluate the effects of desiccation on the survival of alginate-encapsulated *Hevea* embryos following LN exposure.
2. To determine the effects of sucrose pretreatment on survival of encapsulated *Hevea* embryos after exposure to LN.
3. To investigate the effects of abscisic acid on the survival of encapsulated *Hevea* embryos after exposure to LN.
4. To determine the freezing characteristics of encapsulated *Hevea* embryos caused by desiccation, sucrose and ABA exposure.

CHAPTER 2

REVIEW OF LITERATURE

Cryopreservation

Early work on cryobiology was started in 1940 by Luyet and Gehennio on animal cells but only in the last two decades had some progress been achieved. Lovelock (1953a, 1953b) showed that at very low temperatures, cells were destroyed by rapid chilling and that this was prevented by the development of cryoprotective agents, such as glycerol and DMSO (dimethyl sulphoxide). As the techniques progresses with remarkable results, incorporation of cryogenic storage into conservation of plant genetic resources commenced in mid-1970's.

Cryopreservation is a valuable method for long term preservation of plant material. It is based on the reduction and subsequent arrest of metabolic functions of biological materials, while maintaining viability at the temperature of liquid nitrogen (Bajaj, 1991). At this temperature (-196°C), almost all the metabolic activities of cells are at a standstill and they can be preserved in such a state for extended periods. Even at a higher temperature of -120°C or below, all biochemical and physical processes causing biological deterioration are slowed down or minimized (Kartha, 1985). Being different from other methods of conservation, cryopreservation requires minimum

space, low maintenance and it is non-dependence on electricity. Most cryopreserved material appears to remain genetically stable (James, 1983).

Stanwood and Bass (1981) applied cryopreservation on seeds of 120 plant species and some have been shown to be able to withstand ultra-low temperatures. Besides seeds, other parts of plants have been successfully stored cryogenically; meristems (Sakai *et al*., 1978), pollen embryos (Bajaj, 1976), callus (Sakai and Sugawara, 1973), and cell suspensions (Nag and Street, 1975; Withers, 1979). Surprisingly, works on zygotic embryos especially on recalcitrant species are relatively few.

Cryopreservation of Naked Zygotic Embryos

For a number of tropical fruits, timber trees and plantation crops, seeds are large-sized and recalcitrant in behaviour and thus cannot be preserved under conventional conditions. Experiments subjecting seeds to LN exposure showed that seeds survived cryogenic storage when they were exposed at low moisture content in the range of 2.2 to 17.5 percent (Stanwood and Bass, 1978; Stanwood, 1980; Styles *et al*., 1982).

Considerable difficulties were encountered when attempts were made to cryopreserve recalcitrant and semi-recalcitrant seeds. They are generally shed at relatively high moisture content while being sensitive towards desiccation and