UNIVERSITI PUTRA MALAYSIA

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

YAP LIP VUN

FP 1998 10
DESICCATION AND PRECULTURE EFFECTS ON SURVIVAL OF ENCAPSULATED ZYGOTIC EMBRYOS OF RUBBER (HEVEA BRASILIENSIS MUEL. -ARG) FOLLOWING LIQUID NITROGEN EXPOSURE

By

YAP LIP VUN

Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Agricultural Science in the Faculty of Agriculture Universiti Putra Malaysia

April 1998
ACKNOWLEDGEMENTS

My heartiest appreciation to my Supervisory Committee Chairman Ass. Prof. Dr. Hor Yue Luan for his supervision, constructive criticisms, advice and friendship. I am extremely grateful for the many hours he spent on stimulating discussions, advice in seminar presentations as well as the presentation of this thesis.

My sincere thanks goes to my Supervisory Committee Members Ass. Prof. Dr. Saleh b. Kadzimin, Dr. Mihdzar b. Abd. Kadir and Prof. Dr. Normah bt. Mohd. Noor for their valuable advice, attention and constructive criticisms in completing this study.

Thanks also to Mr. Ong Choon Hoe and Puan Nor Rafidah Yusoff, laboratory assistants of Seed Technoloy Research Laboratory, for their assistance in the laboratory during the study.

I also wish to convey my thanks to Prof. Chin Hoong Fong for his encouragements throughout the study; Yen Yen, Lay Yieng, Evenni, Florence, Adrian and Soon Lye for their friendship and help in various ways throughout this project.
# TABLE OF CONTENT

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF PLATES</td>
<td>x</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>xii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xiii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>xvi</td>
</tr>
</tbody>
</table>

## CHAPTER

1. **INTRODUCTION** ................................................. 1

2. **REVIEW OF LITERATURE** ........................................ 4
   - Cryopreservation ........................................... 4
   - Cryopreservation of Naked Zygotic Embryos .................. 5
   - Desiccation Step in Cryopreservation ...................... 7
   - Alginate-encapsulation and Cryopreservation ............... 9
   - Sucrose as Cryoprotectant ................................ 11
   - Abscisic Acid as Chemical Ameliorant ..................... 13
   - Alginate-encapsulation and Sucrose Preculture ........... 14
   - Differential Thermal Analysis (DTA) ...................... 16

3. **MATERIALS AND METHODS** .................................... 17
   - Seed Materials ........................................... 17
   - Experimental Procedures ................................. 18
     - Seed Treatment ......................................... 18
     - Sterilisation of Seeds and Excision of Embryonic Axes .. 18
     - Encapsulation of Embryos .............................. 18
     - Preparation of Stock Solutions and Culture Medium ...... 20
Preparation of Sucrose and Abscisic Acid
Preculture Medium........................................... 23
Desiccation of Embryos........................................ 23
Freezing and Thawing Procedure.............................. 27
Incubation of Culture.......................................... 27
Measurement and Observation.................................. 29
   Determination of Moisture Content........................ 29
   Viability and Survival on Agar Medium.................... 29
   Differential Thermal Analysis............................... 31
Experiments....................................................... 32
Part I:  Effects of Desiccation on the Survival of Alginate-encapsulated *Hevea* Embryos Following LN Exposure........... 32
Part II: Effects of Sucrose Pretreatment and Desiccation on the Survival of Alginate-encapsulated *Hevea* Embryos Following LN Exposure............... 33
Part III: Effects of ABA Pretreatment and Desiccation on the Survival of Alginate-encapsulated *Hevea* Embryos Following LN Exposure............... 35
Part IV: Effects of Desiccation on Freezing Characteristics of Encapsulated *Hevea* Embryos Using Differential Thermal Analysis (DTA)........... 36
Statistical Analysis............................................. 40

RESULTS.................................................................. 41
Part I:  Effects of Desiccation on the Survival of Alginate-encapsulated *Hevea* Embryos Following LN Exposure......................... 41
Part II: Effects of Sucrose Pretreatment and Desiccation on the Survival of Alginate-encapsulated *Hevea* Embryos Following LN Exposure............... 45
Part III: Effects of ABA Pretreatment and Desiccation on the Survival of Alginate-encapsulated *Hevea* Embryos Following LN Exposure............... 55
Part IV: Effects of Desiccation on Freezing Characteristics of Encapsulated *Hevea* Embryos Using Differential Thermal Analysis (DTA).............. 57

5 DISCUSSION......................................................................................................................... 66

Part I: Effects of Desiccation on the Survival of Alginate-encapsulated *Hevea* Embryos Following LN Exposure................................................... 66

Part II: Effects of Sucrose Pretreatment and Desiccation on the Survival of Alginate-encapsulated *Hevea* Embryos Following LN Exposure........ 68

Part III: Effects of ABA Pretreatment and Desiccation on the Survival of Alginate-encapsulated *Hevea* Embryos Following LN Exposure............. 71

Part IV: Effects of Desiccation on Freezing Characteristics of Encapsulated *Hevea* Embryos Using Differential Thermal Analysis (DTA)........... 72

6 SUMMARY AND CONCLUSION ......................................................................................... 75

REFERENCES .................................................................................................................................. 80

APPENDICES .................................................................................................................................. 87

APPENDIX A
Murashige and Skoog (1962) Inorganic Salts and Vitamins ............... 88

APPENDIX B
Additional Tables....................................................................................................................... 89

APPENDIX C
Statistical Analysis..................................................................................................................... 94

VITA ........................................................................................................................................... 104
# List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Percentage viability and survival of encapsulated <em>Hevea</em> embryos before (-LN) and after (+LN) liquid nitrogen exposure following desiccation to various moisture contents (MC)</td>
<td>41</td>
</tr>
<tr>
<td>2</td>
<td>Percentage viability and survival of encapsulated <em>Hevea</em> embryos precultured with 0 M sucrose (MS basal medium) for 24 hours, before (-LN) and after (+LN) liquid nitrogen exposure</td>
<td>46</td>
</tr>
<tr>
<td>3</td>
<td>Percentage viability and survival of encapsulated <em>Hevea</em> embryos precultured with 0.3 M sucrose for 24 hours, before (-LN) and after (+LN) liquid nitrogen exposure</td>
<td>47</td>
</tr>
<tr>
<td>4</td>
<td>Percentage viability and survival of encapsulated <em>Hevea</em> embryos precultured with 0.5 M sucrose for 24 hours, (+LN) liquid nitrogen exposure</td>
<td>49</td>
</tr>
<tr>
<td>5</td>
<td>Percentage viability and survival of encapsulated <em>Hevea</em> embryos precultured with 0.7 M sucrose for 24 hours, before (-LN) and after (+LN) liquid nitrogen exposure</td>
<td>51</td>
</tr>
<tr>
<td>6</td>
<td>Percentage viability and survival of encapsulated <em>Hevea</em> embryos precultured with 0.9 M sucrose for 24 hours, before (-LN) and after (+LN) liquid nitrogen exposure</td>
<td>53</td>
</tr>
<tr>
<td>7</td>
<td>Percentage viability and survival of encapsulated <em>Hevea</em> embryos precultured with 30 μM ABA for 3 days, before (-LN) and after (+LN) liquid nitrogen exposure</td>
<td>55</td>
</tr>
<tr>
<td>8</td>
<td>Percentage viability and survival of encapsulated <em>Hevea</em> embryos precultured with 60 μM ABA for 3 days, before (-LN) and after (+LN) liquid nitrogen exposure</td>
<td>56</td>
</tr>
<tr>
<td>9</td>
<td>Percentage viability and survival of encapsulated <em>Hevea</em> embryos precultured with 90 μM ABA for 3 days, before (-LN) and after (+LN) liquid nitrogen exposure</td>
<td>56</td>
</tr>
<tr>
<td>10</td>
<td>Morphological categorisation for viability and survival evaluation of encapsulated <em>Hevea</em> embryos before (-LN) and after (+LN) liquid nitrogen exposure</td>
<td>88</td>
</tr>
<tr>
<td>11</td>
<td>Morphological categorisation for viability and survival evaluation of encapsulated and 0M sucrose precultured <em>Hevea</em> embryos before (-LN) and after (+LN) liquid nitrogen exposure</td>
<td>89</td>
</tr>
<tr>
<td>Page</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Morphological categorisation for viability and survival evaluation of encapsulated and 0.3 M sucrose precultured <em>Hevea</em> embryos before (-LN) and after (+LN) liquid nitrogen exposure ........................................ 90</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Morphological categorisation for viability and survival evaluation of encapsulated and 0.5 M sucrose precultured <em>Hevea</em> embryos before (-LN) and after (+LN) liquid nitrogen exposure ........................................ 91</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Morphological categorisation for viability and survival evaluation of encapsulated and 0.7 M sucrose precultured <em>Hevea</em> embryos before (-LN) and after (+LN) liquid nitrogen exposure ........................................ 92</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Morphological categorisation for viability and survival evaluation of encapsulated and 0.9 M sucrose precultured <em>Hevea</em> embryos before (-LN) and after (+LN) liquid nitrogen exposure ........................................ 93</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>Illustration of desiccation apparatus</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>Differential Thermal Analysis profiles of naked rubber (<em>Hevea brasiliensis</em>) embryos dehydrated to various moisture content</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>Exothermal temperature of naked rubber (<em>Hevea brasiliensis</em>) embryos as a function of embryo moisture content. The shaded portion denote embryo moistures where no exotherm was detected.</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>Differential Thermal Analysis profiles of alginate-encapsulated rubber (<em>Hevea brasiliensis</em>) embryos dehydrated to various moisture content</td>
<td>61</td>
</tr>
<tr>
<td>5</td>
<td>Exothermal temperature of rubber (<em>Hevea brasiliensis</em>) encapsulated embryos as a function of embryo moisture content. The shaded portion denote embryo moistures where no exotherm was detected.</td>
<td>61</td>
</tr>
<tr>
<td>6</td>
<td>Differential Thermal Analysis profiles of alginate-encapsulated rubber (<em>Hevea brasiliensis</em>) embryos precultured with 0 M sucrose (MS basal) and dehydrated to various moisture content</td>
<td>62</td>
</tr>
<tr>
<td>7</td>
<td>Exothermal temperature of rubber (<em>Hevea brasiliensis</em>) encapsulated embryos precultured with 0 M sucrose (MS basal) as a function of embryo moisture content. The shaded portion denote embryo moistures where no exotherm was detected.</td>
<td>62</td>
</tr>
<tr>
<td>8</td>
<td>Differential Thermal Analysis profiles of alginate-encapsulated rubber (<em>Hevea brasiliensis</em>) embryos precultured with 0.3 M sucrose and dehydrated to various moisture content</td>
<td>63</td>
</tr>
<tr>
<td>9</td>
<td>Exothermal temperature of rubber (<em>Hevea brasiliensis</em>) encapsulated embryos precultured with 0.3 M sucrose as a function of embryo moisture content. The shaded portion denote embryo moistures where no exotherm was detected.</td>
<td>63</td>
</tr>
<tr>
<td>10</td>
<td>Differential Thermal Analysis profiles of alginate-encapsulated rubber (<em>Hevea brasiliensis</em>) embryos precultured with 0.5 M sucrose and dehydrated to various moisture content</td>
<td>64</td>
</tr>
<tr>
<td>11</td>
<td>Exothermal temperature of rubber (<em>Hevea brasiliensis</em>) encapsulated embryos precultured with 0.5 M sucrose as a function of embryo moisture content. The shaded portion denote embryo moistures where no exotherm was detected.</td>
<td>64</td>
</tr>
</tbody>
</table>
Differential Thermal Analysis profiles of alginate-encapsulated rubber (Hevea brasiliensis) embryos precultured with 30 μM ABA and dehydrated to various moisture content.

Exothermal temperature of rubber (Hevea brasiliensis) encapsulated embryos precultured with 30 μM ABA as a function of embryo moisture content. The shaded portion denote embryo moistures where no exotherm was detected.
**LIST OF PLATES**

<table>
<thead>
<tr>
<th>Plate</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Excised <em>Hevea</em> embryos (left) and <em>Hevea</em> seeds (right).</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>Excised zygotic embryos of <em>Hevea</em> with some cotyledon tissue intact. The embryos were placed on filter paper moistened with MS basal solution right after excision.</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>Aseptically excised <em>Hevea</em> embryos suspended in sterilised 3% sodium alginate solution.</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>The <em>Hevea</em> embryos were picked up and dispensed individually with modified sterile pasteur pipette into 100 Mm CaCl$_2$. Surface complexing and polymerisation begins immediately to form beads with embryo inside.</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>Alginate-encapsulated <em>Hevea</em> embryos blotted dry with sterile filter paper before use.</td>
<td>22</td>
</tr>
<tr>
<td>6</td>
<td>Encapsulated <em>Hevea</em> embryos placed on sterile aluminium boat. The embryos were evenly spread to provide uniform desiccation.</td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td>Desiccation using compressor pump, silica gel and drying chamber (from the right). The apparatus was placed in a laminar air flow cabinet.</td>
<td>26</td>
</tr>
<tr>
<td>8</td>
<td>Boats of embryos placed on perforated platform in the drying chamber.</td>
<td>26</td>
</tr>
<tr>
<td>9</td>
<td>Test materials in cryovials secured to cryocanes.</td>
<td>28</td>
</tr>
<tr>
<td>10</td>
<td>Freezing of test materials using liquid nitrogen in a cryogenic tank.</td>
<td>28</td>
</tr>
<tr>
<td>11</td>
<td>Categories of <em>Hevea</em> embryos or plantlets for evaluation of viability (6 weeks after culture) and survival (12 weeks after culture).</td>
<td>30</td>
</tr>
<tr>
<td>12</td>
<td>Encapsulated <em>Hevea</em> embryos precultured on preculture medium.</td>
<td>34</td>
</tr>
<tr>
<td>13</td>
<td>Sucrose precultured and partially desiccated <em>Hevea</em> embryos.</td>
<td>34</td>
</tr>
<tr>
<td>14</td>
<td>A thermocouple with polypropylene cryovial mounted. Aluminium cap (made from aluminium foil) was used to wrap the embryo firmly to the thermocouple before being enclosed in the cryovial.</td>
<td>38</td>
</tr>
<tr>
<td>Page</td>
<td>Text</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Probes submerged in an ethanol bath (Julabo F40-HC) for freezing process.</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Apparatus for the data acquisition for DTA. Control of the freezing protocol and data acquisition were accomplished using a high speed and resolution interface card (Omega WB-AA1-B8) connected to an IBM 386 computer.</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Fresh control of encapsulated <em>Hevea</em> embryos after 12 weeks of culture on MS medium supplemented with 1 mg/l of each NAA, BAP and GA3.</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Encapsulated <em>Hevea</em> embryos (18% MC), after exposure to liquid nitrogen and cultured on MS medium supplemented with 1 mg/l of each NAA, BAP and GA3 for 12 weeks.</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Encapsulated <em>Hevea</em> embryos (14% MC), after exposure to liquid nitrogen and cultured on MS medium supplemented with 1 mg/l of each NAA, BAP and GA3 for 12 weeks.</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Encapsulated <em>Hevea</em> embryos after precultured with 0.5 M sucrose and desiccated to 14% MC before exposure to liquid nitrogen (12 weeks after culture).</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Encapsulated <em>Hevea</em> embryos after precultured with 0.5 M sucrose, desiccated to 14% MC and exposed to liquid nitrogen (12 weeks after culture).</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Encapsulated <em>Hevea</em> embryos after precultured with 0.9 M sucrose, desiccated to 18% MC, before exposure to liquid nitrogen (12 weeks after culture).</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Encapsulated <em>Hevea</em> embryos after precultured with 0.9 M sucrose, desiccated to 18% MC, after exposure to liquid nitrogen (12 weeks after culture).</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
<td></td>
</tr>
<tr>
<td>BAP</td>
<td>benzylaminopurine</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
<td></td>
</tr>
<tr>
<td>DSC</td>
<td>differential scanning calorimetry</td>
<td></td>
</tr>
<tr>
<td>DTA</td>
<td>differential thermal analysis</td>
<td></td>
</tr>
<tr>
<td>GA3</td>
<td>gibberellic acid</td>
<td></td>
</tr>
<tr>
<td>HMTL</td>
<td>high moisture freezing limit</td>
<td></td>
</tr>
<tr>
<td>MC</td>
<td>moisture content</td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog medium, 1962</td>
<td></td>
</tr>
<tr>
<td>NAA</td>
<td>alpha-naphthalene acetic acid</td>
<td></td>
</tr>
<tr>
<td>RCBD</td>
<td>randomised complete block design</td>
<td></td>
</tr>
<tr>
<td>TMC</td>
<td>threshold moisture content</td>
<td></td>
</tr>
<tr>
<td>+LN</td>
<td>with liquid nitrogen exposure</td>
<td></td>
</tr>
<tr>
<td>-LN</td>
<td>without liquid nitrogen exposure</td>
<td></td>
</tr>
</tbody>
</table>
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Agricultural Science.

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

By

YAP LIP VUN

April 1998

Chairman : Associate Professor Hor Yue Luan, Ph. D.

Faculty : Agriculture

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 preculture 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.

Oleh

YAP LIP VUN

April 1998

Pengerusi Penyelia : Prof. Madya Hor Yue Luan, Ph. D.

Fakulti : Pertanian.

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 dikerkingkan 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 dikerkingkan sehingga ke paras kelembapan 10% walaupun biji Hevea dikenali sebagai biji jenis rekalsitran. Penyelaputan telah meluaskan

xvi
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