UNIVERSITI PUTRA MALAYSIA

MICROPROPAGATION AND STORAGE OF
Plectranthus amboinicus (Loureiro) Sprengel SHOOT APICES FOR
GERMPLASM CONSERVATION

GREETHA ARUMUGAM

FP 2018 88
MICROPROPAGATION AND STORAGE OF
Plectranthus amboinicus (Loureiro) Sprengel SHOOT APICES FOR
GERmplasm conservation

By

GREETHA ARUMUGAM

Thesis Submitted to the School of Graduate Studies,
Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree
of Doctor of Philosophy

April 2018
All material contained within the thesis, including without limitation text, logos, icons, photographs and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright © Universiti Putra Malaysia
Appa, Amma, Param, Kumutha, Aravin & Yugen,

Thank you for bringing colours into my life!
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

MICROPROPAGATION AND STORAGE OF Plectranthus amboinicus (Loureiro) Sprengel SHOOT APICES FOR GERMLASM CONSERVATION

By

GREETHA ARUMUGAM

April 2018

Chair : Professor Uma Rani Sinniah, PhD
Faculty : Agriculture

Plectranthus amboinicus is a valuable medicinal plant under threat due to indiscriminate collection. This study communicates a reproducible micropropagation method and complimentary conservation strategy for a sustainable utilisation of this herb. For micropropagation, ideal growth of apical and axillary buds of P. amboinicus under in vitro culture conditions observed on semi-solid MS media supplemented with 0.4 mg l⁻¹ BAP. Rooting of shoot cultures observed on half-strength semi-solid MS media producing 12.47 ± 0.35 roots per explant. Further, acclimatisation of rooted cultures on peat-moss moistened with sterile MS media produced 76.7 ± 5.8% survival. Subsequent, EO analysis identified carvacrol as major constituent at 43.3% in field grown and 45.1% micropropagated samples. This efficient micropropagation system permits mass propagation of P. amboinicus and ensures continuous supply of raw materials to various industries. For conservation of P. amboinicus, encapsulation conditions were optimised enabling easier germplasm exchange while providing protection towards pretreatments of short- and long-term conservation protocols. Optimisation of encapsulation conditions with 3% (w/v) sodium alginate in 100 mM CaCl₂ solution was found ideal and employed in further studies. Standardisation of in vitro shoot tips of 3-5 mm size dissected from 1st or 2nd subcultures were chosen exhibiting 100.0 ± 0.0% survival with 4.9 ± 0.2 shoots per explant desirable for conservation studies. Nodal segments exhibiting poor regeneration were not preferred for conservation. Encapsulated shoot tips exhibited superior conversion frequency when inoculated on agar (78%) followed by peat-moss (58%) and cotton (38%). While, in short-term storage conditions, synthetic seeds of P. amboinicus stored up to 30 days at 4°C retained 77% survival rate. In cryopreservation, sucrose preculture is a
prerequisite for protocol optimisation. Sucrose preculture between 0.25 to 0.5 M greatly enhanced tolerance of shoot tips towards dehydration and freezing. Succeeding, encapsulation-dehydration method establishment, in vitro grown shoot tips of *P. amboinicus* were precultured in 0.4 M sucrose for 24 hrs, encapsulated in 3% ca-alginate matrix and subjected silica gel desiccation for 12 hrs and cryopreserved produced finest post thaw recovery with 50.0 ± 5.8% survival and 36.7 ± 5.8% regrowth. Meanwhile, encapsulation-vitrification technique gave up to 57% survival and 37% regrowth when in vitro shoot tips were precultured in 0.4 M sucrose (24 hrs), followed by 3% ca-alginate matrix coating, loaded with L1 (0.4 M sucrose + 1.0 M glycerol) or L3 (0.4 M sucrose + 1.0 M glycerol + 5% (w/v) DMSO) for 40 mins, and dehydrated with PVS2 (30% (w/v) glycerol, 15% (w/v) EG, 15% (w/v) DMSO) for 20 mins and exposed to liquid nitrogen. In both techniques encapsulated shoot tips were dehydrated to an average of 35 to 36% moisture content through either direct (silica gel) or chemical (cryoprotectants) dehydration before freezing exhibited average regeneration suggesting *P. amboinicus* could be extremely cold-sensitive. Further, histological analysis on cryopreserved *P. amboinicus* shoot tips revealed appropriate pretreatments are essential to provide maximal protection and induce tolerance during exposure to ultra-low temperatures. To the best our knowledge, this is the first report on *P. amboinicus* cryopreservation by vitrification-based techniques, which would provide a wider platform for propagation and conservation of tropical herbal germplasms of Malaysia.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBIAKAN MIKRO DAN PENYIMPANAN TUNAS Plectranthus amboinicus (Loureiro) Sprengel UNTUK PEMULIHARAAN JANA PLASMA

Oleh

GREETHA ARUMUGAM

April 2018

Pengerusi : Profesor Uma Rani Sinniah, PhD
Fakulti : Pertanian

Plectranthus amboinicus merupakan tumbuhan herba bernilai yang terancam pupus kerana penggumpulan berleluasa untuk pelbagai produk. Kajian ini menyampaikan kaedah pembiakan mikro dan strategi pemuliharaan jana plasma alternatif bagi penggunaan mampun herba ini. Dalam pengandaan tunas, nilai tertinggi tunas-tunas apikal dan aksilar diperhatikan atas media MS ditambah dengan 0.4 mg l⁻¹ BAP. Untuk proses perakaran, kultur pucuk dipindahkan ke atas media MS sepuruh-kekuatan dengan penghasilan 12.47 ± 0.35 akar per eksplan. Seterusnya, aklimatisasi atas tanah-gambut yang dilembabkan dengan media MS yang steril menghasilkan kadar hidup sebanyak 76.7 ± 5.8%. Analisis minyak pati seterusnya, mengenai pasti carvacrol sebagai konstituen utama dalam pokok liar (43.3%) hampir sama dengan kultur pucuk (45.1%). Oleh itu, kajian ini mengizinkan penggandaan tunas yang banyak sambil memastikan bekalan bahan mentah yang berterusan kepada pelbagai industri. Seterusnya, untuk pemuliharaan P. amboinicus, kaedah pengkapsulan yang ideal dikenalpasti bagi memudahkan pengedaran jana plasma dan melindungi eksplan semasa penubuhan sistem penyimpanan jangka-pendek dan panjang. Kapsul dihasilkan daripada 3% (w/v) natrium alginate dalam 100 mM larutan CaCl₂ diputuskan sebagai berkualiti unggul dan digunakan untuk pengeluaran biji benih sintetik. Bagi penyeragaman eksplan, tunas pucuk bersaiz 3-5 mm diasingkan daripada subkultur pertama dan kedua yang memberikan kadar hidup tinggi sebanyak 100.0 ± 0.0% dengan bilangan pucuk 4.9 ± 0.2 per eksplan dipilih. Prestasi tunas pucuk lebih baik berbanding dengan segmen nod. Apabila, tunas pucuk dikapsulkan dengan kaedah optimal dan diinokulasi pertumbuhan semula yang tinggi direkod atas agar (78%), diikuti oleh tanah-gambut (58%) and kapas (38%). Ini mencerminkan potensi
kapsul tunas pucuk untuk digunakan sebagai biji benih sintetik. Biji benih sintetik *P. amboinicus*, juga berkemampuan untuk disimpan sehingga 30 hari dalam suhu 4°C dengan penghasilan kadar hidup 77%, menunjukkan strategi penyimpanan jangka-pendek. Bagi krioawetan, toleransi tunas pucuk terhadap dehidrasi dan pembekuan dapat dipertingkatkan melalui prakultur dalam sukrosa 0.25 – 0.5 M. Prakultur merupakan prasyarat yang penting bagi pengoptimuman protokol krioawetan. Dalam teknik krioawetan pengkapsulan-dehidrasi kadar hidup (50.0 ± 5.8%) and pertumbuhan semula (36.7 ± 5.8%) terbaik diperolehi apabila, tunas pucuk diprakulturkan dalam sukrosa 0.4 M selama 24 jam, dikapsulkan dengan 3% Ca-alginat dan dihidrasi selama 12 jam dengan jel silica dan disimpan dalam cecair nitrogen. Sebaliknya, teknik pengkapsulan-vitrifikasi menghasilkan kadar hidup 57% and pertumbuhan semula 37% selepas krioawetan bila tunas pucuk diprakulturkan dalam sukrosa 0.4 M selama 24 jam, dikapsulkan dengan 3% Ca-alginat, dirawat dengan larutan muatan (LS) antara L1 (sukrosa 0.4 M + glicerol 1.0 M) atau L3 (sukrosa 0.4 M + glicerol 1.0 M + DMSO 5%) untuk 40 minit diikuti hidrasi dalam PVS2 (glicerol 30%, EG 15%, DMSO 15%). Kedua-dua teknik ini dapat menghidrasi kapsul tunas pucuk dengan purata kelembapan antara 35 hingga 36% melalui hidrasi terus (gel silica) atau hidrasi kimia (pelindungkrio). Kadar regenerasi sederhana pucuk tunas menunjukkan pokok *P. amboinicus* sangat sensitive terhadap pembekuan. Analisis histologi selanjutnya, mencadangkan sel-sel tunas pucuk yang dikrioawet selepas prarawatan sesuai memberikan perlindungan maksima semasa penyimpanan dalam suhu yang sangat rendah. Pada pengetahuan kami, ini merupakan laporan pertama mengenai teknik krioawetan berasaskan vitrifikasi bagi *P. amboinicus*. Laporan ini menyediakan platform yang lebih luas untuk pembiakan mikro dan pemuliharaan jana plasma tumbuhan herba tropika di Malaysia.
ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my advisor Professor Uma Rani Sinniah and the members of my supervisory committee Professor Paul T. Lynch and Assoc. Prof. Dr Saleh Kadzimin for the continuous support during my Ph.D. study and related research. Their patience, guidance, motivation, and immense knowledge helped me pull through the research and writing of my thesis. Besides my supervisory committee, I would like to thank Dr Kumara Swamy Mallapa for his insightful comments, encouragement, and for the complex questions which pushed me to widen my research from various perspectives.

Next, I would like to thank my fellow labmates for the stimulating discussions, the sleepless nights before deadlines, and for all the fun we have had in the last years.

Finally, I must express my very profound gratitude to my parents (Mr & Mrs Arumugam-Sarojini), my partner (Param), and beloved family (Kumutha, Aravinth Ram and Yugendran) for providing me with unfailing support and continuous inspiration throughout my years of study. This accomplishment would not have been possible without them.

Thank you very much, everyone
I certify that a Thesis Examination Committee has met on 19 April 2018 to conduct the final examination of Greetha a/p Arumugam on her thesis entitled "Micropropagation and Storage of Plectranthus ambonicus (Loureiro) Sprengel Shoot Apices for Germplasm Conservation" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

**Hawa binti Jaafar, PhD**
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

**Parameswari a/p Namasivayam, PhD**
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

**Adam bin Puteh, PhD**
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

**Prakash P. Kumar, PhD**
Associate Professor
National University of Singapore
Singapore
(External Examiner)

[Signature]

**NOR AIMLAB. SHUKOR, PhD**
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 28 June 2018
This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

**Uma Rani Sinniah, PhD**  
Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Chairman)

**Saleh Kadzimin, PhD**  
Associate Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Member)

**Paul T. Lynch, PhD**  
Professor  
University of Derby  
United Kingdom  
(Member)

---

**ROBIAH BINTI YUNUS, PhD**  
Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date:
Declaration by graduate student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: _____________________________ Date: _____________________

Name and Matric No.: Greetha Arumugam, GS28614
Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: ____________________________
Name of Chairman of Supervisory Committee: Professor Uma Rani Sinniah

Signature: ____________________________
Name of Member of Supervisory Committee: Associate Professor Saleh Kadzimin

Signature: ____________________________
Name of Member of Supervisory Committee: Professor Paul T. Lynch, PhD
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ABSTRACT</th>
<th>i</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRAK</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>vi</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xvii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xxi</td>
</tr>
<tr>
<td><strong>CHAPTER</strong></td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>2 LITERATURE REVIEW</td>
<td></td>
</tr>
<tr>
<td>2.1 Botanical description</td>
<td>4</td>
</tr>
<tr>
<td>2.2.1 Taxonomy</td>
<td>4</td>
</tr>
<tr>
<td>2.2.2 Morphological features</td>
<td>4</td>
</tr>
<tr>
<td>2.2.3 Origin, wild relatives and geographical distribution</td>
<td>6</td>
</tr>
<tr>
<td>2.2 Commercial value (medicinal, nutritional and ornamental)</td>
<td>9</td>
</tr>
<tr>
<td>2.3 Propagation and conservation of <em>P. amboinicus</em></td>
<td>9</td>
</tr>
<tr>
<td>2.4 Micropropagation</td>
<td>10</td>
</tr>
<tr>
<td>2.4.1 Culture initiation and multiplication</td>
<td>13</td>
</tr>
<tr>
<td>2.4.2 <em>In vitro</em> rooting and acclimatisation</td>
<td>15</td>
</tr>
<tr>
<td>2.5 Encapsulation technology</td>
<td>16</td>
</tr>
<tr>
<td>2.5.1 Concept and application</td>
<td>16</td>
</tr>
<tr>
<td>2.5.2 Choice of explant</td>
<td>16</td>
</tr>
<tr>
<td>2.5.3 Encapsulating materials</td>
<td>17</td>
</tr>
<tr>
<td>2.5.4 Planting substrates</td>
<td>17</td>
</tr>
<tr>
<td>2.5.5 Synthetic seed as short-term storage strategy for medicinal plant germplasms</td>
<td>18</td>
</tr>
<tr>
<td>2.6 Cryopreservation</td>
<td>23</td>
</tr>
<tr>
<td>2.6.1 Cryopreservation method development</td>
<td>23</td>
</tr>
<tr>
<td>2.6.2 Cryopreservation of shoot tips and meristems</td>
<td>25</td>
</tr>
<tr>
<td>3 MICROPROPAGATION OF <em>Plectranthus amboinicus</em> (Lour.)</td>
<td>34</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>34</td>
</tr>
<tr>
<td>3.2 Materials and methods</td>
<td>35</td>
</tr>
<tr>
<td>3.2.1 Surface sterilisation</td>
<td>35</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>3.2.2 Media and culture conditions</td>
<td>36</td>
</tr>
<tr>
<td>3.2.3 Shoot initiation and proliferation</td>
<td>36</td>
</tr>
<tr>
<td>3.2.4 Rooting</td>
<td>36</td>
</tr>
<tr>
<td>3.2.5 Acclimatisation</td>
<td>37</td>
</tr>
<tr>
<td>3.2.6 Experimental design and statistical analysis</td>
<td>37</td>
</tr>
<tr>
<td>3.2.7 Essential oil extraction</td>
<td>37</td>
</tr>
<tr>
<td>3.2.8 Gas chromatography–mass spectrometry (GC-MS) Analysis</td>
<td>38</td>
</tr>
<tr>
<td>3.3 Results and discussion</td>
<td>38</td>
</tr>
<tr>
<td>3.3.1 Optimisation of surface sterilisation protocol</td>
<td>38</td>
</tr>
<tr>
<td>3.3.2 Influence of plant growth regulators on shoot initiation and multiplication of P. amboinicus apical and axillary buds</td>
<td>40</td>
</tr>
<tr>
<td>3.3.3 Shoot development of P. amboinicus shoot buds derived from different position under in vitro conditions supplemented with 0.4 mg/L BAP</td>
<td>45</td>
</tr>
<tr>
<td>3.3.4 Effects of different MS media strength on in vitro rooting of P. amboinicus plantlets</td>
<td>49</td>
</tr>
<tr>
<td>3.3.5 Acclimatisation of in vitro P. amboinicus plantlets</td>
<td>51</td>
</tr>
<tr>
<td>3.3.6 Essential oil characterisation</td>
<td>54</td>
</tr>
<tr>
<td>3.4 Conclusion</td>
<td>57</td>
</tr>
</tbody>
</table>

4 ENCAPSULATION OF P. amboinicus (Lour.) IN VITRO SHOOT TIPS AND SHORT-TERM STORAGE 58
4.1 Introduction                                                        | 58   |
4.2 Materials and methods                                               | 59   |
4.2.1 Plant material                                                   | 59   |
4.2.2 Calcium alginate (Ca-alginate) bead preparation                   | 59   |
4.2.3 Shoot regeneration and multiplication                            | 60   |
4.2.4 Shoot apices standardisation                                     | 60   |
4.2.5 Encapsulation and plantlet development in Ca-alginate matrix      | 60   |
4.2.6 Storage and recovery assessment of encapsulated shoot apices     | 61   |
4.2.7 Establishment of encapsulated shoot apices in various planting substrates | 61   |
4.2.8 Experimental design and statistical analysis                     | 62   |
4.3 Results and discussion                                             | 62   |
4.3.1 Effect of alginate percentages and CaCl₂ concentrations on gel complexation | 62   |
4.3.2 Effect of successive subculture on the shoot development          | 65   |
4.3.3 Effect of shoot apices size and type                              | 66   |
4.3.4 Effect of gel concentration on regeneration of encapsulated shoot tips
4.3.5 Effects of storage duration and temperature on regeneration of encapsulated shoot tips
4.3.6 Effects of different substrate as growth media for regeneration of encapsulated shoot tips

4.4 Conclusion

5 CRYOPRESERVATION OF IN VITRO GROWN P. amboinicus (Lour.) SHOOT TIPS USING ENCAPSULATION-DEHYDRATION TECHNIQUE
5.1 Introduction
5.2 Materials and methods
5.2.1 Plant material
5.2.2 Sucrose preculture
5.2.3 Moisture content determination
5.2.4 Encapsulation-dehydration
5.4.5 Liquid nitrogen storage, thawing and regeneration
5.4.6 Experimental design and statistical analysis
5.4.7 Histological studies on cryopreserved shoot tips

5.3 Results and discussion
5.3.1 Effects of silica gel desiccation on moisture content and shoot recovery of encapsulated and non-encapsulated in vitro grown P. amboinicus shoot tips
5.3.2 Effects of sucrose preculture and subsequent silica gel desiccation on in vitro grown P. amboinicus shoot tips
5.3.3 Effects of liquid nitrogen exposure on in vitro grown encapsulated P. amboinicus shoot tips
5.3.4 Histological analysis on P. amboinicus shoot tips cryopreserved using optimised encapsulation-dehydration technique

5.4 Conclusion

6 CRYOPRESERVATION OF IN VITRO GROWN P. amboinicus (Lour.) SHOOT TIPS USING ENCAPSULATION-VITRIFICATION TECHNIQUE
6.1 Introduction
6.2 Materials and methods
6.2.1 Plant material
6.2.2 Encapsulation and loading treatment
6.2.3 Sucrose preculture and loading treatment
6.2.4 Vitrification 100
6.2.5 Liquid nitrogen storage, thawing and regeneration 101
6.2.6 Experimental design and statistical analysis 101
6.2.7 Histological studies on cryopreserved shoot tips 102
6.3 Results and discussion 102
6.3.1 Effects of components in loading solution and exposure duration on the recovery of in vitro grown P. amboinicus encapsulated shoot tips 102
6.3.2 Effects of sucrose preculture and loading treatment on the shoot recovery of in vitro grown P. amboinicus encapsulated shoot tips 105
6.3.3 Effects of PVS2 exposure duration on the recovery of in vitro grown P. amboinicus encapsulated shoot tips 107
6.3.4 Effects of cryopreservation on the recovery of in vitro grown P. amboinicus encapsulated shoot tips 110
6.3.5 Histological analysis on P. amboinicus shoot tips cryopreserved using optimised encapsulation-vitrification technique 114
6.4 Conclusion 116

7 GENERAL DISCUSSION, CONCLUSION AND FUTURE PROSPECTS 117
7.1 General discussion, recommendations and conclusion 117
7.2 Future prospects 121

BIBLIOGRAPHY 123
APPENDICES 154
BIODATA OF STUDENT 169
LIST OF PUBLICATIONS 170
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Vernacular names and traditional uses of <em>Plectranthus amboinicus</em> commonly used by locals in their respective countries</td>
<td>7</td>
</tr>
<tr>
<td>2.2 Effect of explants and PGRs on <em>in vitro</em> morphogenesis of selected species of genus <em>Plectranthus</em> and <em>Coleus</em> spp.</td>
<td>11</td>
</tr>
<tr>
<td>2.3 Optimum conditions of synthetic seed production for vegetative propagules of selected medicinal plant species</td>
<td>20</td>
</tr>
<tr>
<td>2.4 Examples of vitrification-based methods employed on cryopreservation of shoot apices from different plant species</td>
<td>30</td>
</tr>
<tr>
<td>3.1 Effect of mechanical and chemical sterilant on endurance of <em>P. amboinicus</em> shoot apices for <em>in vitro</em> culture initiation</td>
<td>39</td>
</tr>
<tr>
<td>3.2 Influences of BAP and GA₃ concentrations on shoot regrowth of <em>P. amboinicus</em> apical buds after 20-25 days of culture initiation</td>
<td>42</td>
</tr>
<tr>
<td>3.3 Influences of BAP and GA₃ concentrations on shoot regrowth of <em>P. amboinicus</em> axillary buds after 20-25 days of culture initiation</td>
<td>43</td>
</tr>
<tr>
<td>3.4 Influence of different positions of <em>P. amboinicus</em> shoot buds on shoot length cultured on MS media supplemented with 0.4 mg/L BAP</td>
<td>47</td>
</tr>
<tr>
<td>3.5 Influence of different positions of <em>P. amboinicus</em> shoot buds on number of shoots cultured on MS media supplemented with 0.4 mg/L BAP</td>
<td>48</td>
</tr>
<tr>
<td>3.6 Influences of different Murashige &amp; Skoog (MS) media strength on shoot and root development of <em>P. amboinicus</em> shoot cultures at day 30</td>
<td>50</td>
</tr>
<tr>
<td>3.7 Acclimatisation of <em>in vitro</em> derived <em>P. amboinicus</em> plantlets</td>
<td>52</td>
</tr>
</tbody>
</table>
3.8 Essential oil composition (%) of *P. amboinicus* isolated from field-growing plants and *in vitro* shoot cultures

4.1 Physical characteristics of Ca-alginate beads and its complexation duration in various combinations of alginate (1.0 - 4.0 % w/v) and CaCl₂ (50 - 100 mM) solutions

4.2 Effect of successive subcultures on the shoot development of *in vitro* cultures of *P. amboinicus* cultured in MS medium supplemented with 0.4 mg/L BAP

4.3 Influence of type and size of *in vitro* derived *P. amboinicus* shoot apices on the shoot development.

4.4 Influence of Na-alginate concentration (%) and shoot tips size on the survival and regrowth frequency of *P. amboinicus* (Data 30 days after inoculation)

4.5 Effects of storage condition (temperature and duration) on the survival and regeneration of *P. amboinicus* in *vitro* derived encapsulated shoot tips.

4.6 Influence of planting substrates agar, cotton and peat-moss on the shoot and root development of encapsulated *P. amboinicus* shoot tips.

5.1 Effects of sucrose preculture concentrations (0-1.0 M) followed by silica gel desiccation (0-16 hrs) on the survival (%) of encapsulated *P. amboinicus* shoot tips at 25 days after inoculation.

5.2 Effects of sucrose preculture concentrations (0-1.0 M) followed by silica gel desiccation (0-16 hrs) on the regrowth frequency (%) of encapsulated *P. amboinicus* shoot tips at 25 days after inoculation.

5.3 Effects of sucrose preculture concentrations ranging between 0 to 1.0 M for 24 hrs followed by silica gel desiccation (0-16 hrs) on the number of shoots per bud produced from encapsulated of *P. amboinicus* shoot tips 25 days after inoculation.
6.1 Effects of sucrose preculture (24 hrs) followed by loading treatment (40 min) on the percentage of survival rate, regrowth frequency and moisture content of encapsulated *P. amboinicus* shoot tips at day 25 after inoculation.

6.2 Influence of sucrose concentrations (0-0.5 M), loading solution (L1 and L3) and PVS2 dehydration duration (10-20 min) on the shoot recovery of *in vitro* grown *P. amboinicus* encapsulated shoot tips with and without LN exposure.

6.3 Influence of sucrose concentrations (0-0.5 M), loading solution (L1 and L3) and PVS2 dehydration duration (10-20 min) on moisture content percentages of encapsulated *in vitro* grown *P. amboinicus* shoot tips at the time of freezing.
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Botanical morphology of <em>Plectranthus amboinicus</em> (Lour.) Sprengel, illustrated as <em>Coleus amboinicus</em> Lour. Description of morphology; 1. Flower spikes, 2. Leafy branch, 3. Calyx and pistil, and 4. Corolla laid open</td>
</tr>
<tr>
<td>2.2</td>
<td><em>P. amboinicus</em> and <em>P. amboinicus</em> 'varigatus' used as ornamental plants in compound surrounding, Komplex AgroBio, University Putra Malaysia, Serdang. a) <em>P. amboinicus</em> grown as shrub in surrounding gardens, and; b) <em>P. amboinicus</em> ‘varigatus’ planted as cover crop along walkways</td>
</tr>
<tr>
<td>3.1</td>
<td>Response of apical and axillary buds of <em>P. amboinicus</em> initiated on semi-solid MS media supplemented with BAP. a) i. Apical bud at day 3; ii. Apical bud at day 15 and; b) i. Axillary buds at day 5; ii. Axillary buds at day 20 (Bar = 1 cm)</td>
</tr>
<tr>
<td>3.2</td>
<td>Influence of BAP and GA3 concentrations on <em>P. amboinicus</em> shoot apices. a) A normally regenerated plantlet from an apical bud on MS media supplemented with 0.3 mg/L BAP at day 20 (bar = 1 cm); b) Abnormal plantlets with spindle shaped leaves and cabbage-like growth on higher concentration of BAP and; c) Abnormal plantlets with elongated stem on higher concentration of GA3</td>
</tr>
<tr>
<td>3.3</td>
<td>Healthy field grown <em>P. amboinicus</em> cutting, a) arrow indicates the position of shoot apices in order on a stem cutting, and; b) sizes of shoot apices in order after aseptic excision; i. AP= Apical Bud, ii. AX1= Axillary Bud 1, iii. AX2= Axillary Bud 2, iv. AX3= Axillary Bud 3 and; v. AX4= Axillary Bud 4 (Bar = 1 cm)</td>
</tr>
<tr>
<td>3.4</td>
<td>Rooting of <em>in vitro</em> grown <em>P. amboinicus</em> shoot cultures. a) arrow indicates adventitious roots emerging form stem at day 10 in H-MS shoot culture, and; b) rooting from shoot cultures in H-MS and Q-MS media at day 30 (Bar = 1 cm)</td>
</tr>
</tbody>
</table>
3.5 An acclimatised *P. amboinicus* plantlet, 1 year 5 months after transfer to field condition.

4.1 Encapsulation technology employed on *in vitro* grown shoot tips of *P. amboinicus* a) Ca-alginate encapsulation of shoot tips; and, b) Storage of synthetic seeds in bridged vials to avoid water logging.

4.2 Gel matrix formation with a range of alginate percentages (1 - 4%) and its combinations at various CaCl₂ concentrations (50mM, 75mM and 100mM; from top to bottom). i) 1% (w/v) alginate, ii) 2% (w/v) alginate, iii) 3% (w/v) alginate and, iv) 4% (w/v) alginate (Bar = 1cm).

4.3 Shoot apices from *in vitro* shoot cultures of *P. amboinicus*. a) shoot tips aseptically derived for storage studies; b) nodal segments aseptically derived for storage studies; c) shoot tips of >3 to <5 mm growing new shoots (*indicated by arrow*) at day 7; d) nodal segments of >3 to <5 mm growing new shoots (*indicated by arrow*) day 7 and; e) a non-surviving nodal segment (Bar = 1cm).

4.4 Effect of planting substrates (agar, cotton and peat-moss) on the shoot and root development of *P. amboinicus* a) Shoot and root development of a synthetic seed in agar at week 5; b) A fully developed synthetic seed in agar at week 8; and, c) profuse rooting of synthetic seed in peat-moss at week 10 (*indicated by arrow*).

4.5 Survival and conversion percentages of encapsulated *P. amboinicus* shoot tips in planting substrates agar, cotton and peat-moss 8 weeks after inoculation.

5.1 Optimisation of encapsulation-dehydration protocol for cryopreservation of *in vitro* grown *P. amboinicus* shoot tips. (a) Shoot tips excised aseptically from 4-week-old *in vitro* shoot cultures; (b) Shoot tips with 2 primodial leaves cultured in sucrose preculture medium (0-1.0 M) for 24 hrs; and, (c) Precultured shoot tips encapsulated with ca-alginate matrices and desiccated (0-16 hrs) in chambers with 80gms of baked silica gel under LAF.
5.2 Effects of silica gel desiccation on percentage of moisture content of encapsulated (♦) and non-encapsulated (♦) in vitro derived *P. amboinicus* shoot tips.

5.3 Effects of silica gel desiccation on survival rate (%) and shoot regrowth frequency (%) of (A) encapsulated and (B) non-encapsulated in vitro derived *P. amboinicus* shoot tips 30 days after inoculation.

5.4 Effects of sucrose preculture concentrations ranging between 0 to 1.0 M for 24 hrs followed by silica gel desiccation (0-16 hrs) on the number of shoots per bud produced from encapsulated of *P. amboinicus* shoot tips 25 days after inoculation.

5.5 Encapsulation-dehydration protocol development for cryopreservation of in vitro grown *P. amboinicus* shoot tips. (A) Encapsulated shoot tips after 0.4 M sucrose preculture and 12 hrs desiccation in silica gel; and, (B) Shoot tip recovery after LN exposure after 0.4 M sucrose preculture and 14 hrs desiccation in silica gel (arrow indicates new shoot regrowth from frozen shoot bud).

5.6 Longitudinal histological sections of shoot tips of *P. amboinicus* before and after cryopreservation via encapsulation-dehydration. (A) and (B) A positive control untreated shoot tip, safranin ‘O’ stained, well preserved cytoplasm with visible nucleus; (C) and (D) A cryopreserved, surviving shoot-tip that had been precultured in 0.4 M sucrose for 24 hrs and silica gel desiccated for 12 hrs; (E) and (F) A negative control non-surviving shoot-tip that had been precultured in 0.4 M sucrose for 24 hrs and silica gel desiccated for 8 hrs.

6.1 Effects of different components in loading solutions (L1-L4) and the exposure duration (0-60 min) on the recovery of encapsulated *P. amboinicus* shoot tips. (A) **L1**: 0.4 M sucrose + 1.0 M glycerol (w/v), (B) **L2**: 0.4 M sucrose + 2.0 M glycerol (w/v); (C) **L3**: 0.4 M sucrose + 1.0 M glycerol (w/v) + 5% DMSO (w/v), and (D) **L4**: 60% of PVS2 concentration.
6.2 Effects of PVS2 dehydration exposure duration (0 to 40 mins) at 4°C on the on the survival (%) and regeneration frequency (%) of encapsulated *P. amboinicus* shoot tips.

6.3 Longitudinal histological sections of shoot tips of *P. amboinicus* before and after cryopreservation via encapsulation-vitrification. (A) and (B) A positive control untreated shoot tip, safranin ‘O’ stained, well preserved cytoplasm with visible nucleus; (C) and (D) Surviving shoot tip -LN; 0.4 M sucrose precultured (24 hrs), osmoprotected in L1 (40 min) and PVS dehydrated (20 min); (E) and (F) Surviving shoot tip +LN; 0.4 M sucrose precultured (24 hrs), osmoprotected in L1 (40 min) and PVS dehydrated (20 min).
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BAP</td>
<td>6-Benzylaminopurine</td>
</tr>
<tr>
<td>Ca-alginate</td>
<td>Calcium alginate</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distil water</td>
</tr>
<tr>
<td>DMRT</td>
<td>Duncan’s Multiple Range Test</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EO</td>
<td>Essential oil</td>
</tr>
<tr>
<td><em>et al.</em></td>
<td><em>et alia</em></td>
</tr>
<tr>
<td><em>etc.</em></td>
<td><em>et cetera</em></td>
</tr>
<tr>
<td>FAA</td>
<td>Formaldehyde acetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g/L</td>
<td>gram per litre</td>
</tr>
<tr>
<td>GA₃</td>
<td>Gibberellic acid</td>
</tr>
<tr>
<td><em>i.e.</em></td>
<td><em>id est</em> (that is)</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole 3-acetic acid</td>
</tr>
<tr>
<td>IBA</td>
<td>Indole butyric acid</td>
</tr>
<tr>
<td>hrs</td>
<td>hour</td>
</tr>
<tr>
<td>Kn</td>
<td>Kinetin</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LAF</td>
<td>Laminar Air Flow</td>
</tr>
<tr>
<td>LN</td>
<td>Liquid Nitrogen</td>
</tr>
<tr>
<td>LS</td>
<td>Loading Solution</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mg/L</td>
<td>milligram per litre</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog (1962) inorganic salt</td>
</tr>
<tr>
<td>NAA</td>
<td>Naphthaleneacetic acid</td>
</tr>
<tr>
<td>NaOCl</td>
<td>Sodium hypochlorite</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>ns</td>
<td>non-significant</td>
</tr>
<tr>
<td>NS</td>
<td>Nodal segments</td>
</tr>
<tr>
<td>P&gt;0.05</td>
<td>Probability at 95%</td>
</tr>
<tr>
<td>PGR</td>
<td>Plant growth regulators</td>
</tr>
<tr>
<td>PPM</td>
<td>Plant Preservative Mixtures</td>
</tr>
<tr>
<td>PVS</td>
<td>Plant Vitrification Solution</td>
</tr>
<tr>
<td>RCBD</td>
<td>Randomized Complete Block Design</td>
</tr>
<tr>
<td>SAS</td>
<td>Statistical Analysis System</td>
</tr>
<tr>
<td>ST</td>
<td>Shoot tips</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>°C</td>
<td>Degree centigrade</td>
</tr>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

_Plectranthus amboinicus_ (Loureiro) Sprengel is a perennial herb under the family Lamiaceae, containing about 300 species (Lukhoba _et al._, 2006), well distributed throughout the tropics and warm regions of the world (Retief, 2000). It is fleshy, succulent and famous for its distinct oregano-like flavour and odour. _P. amboinicus_ is mostly cited in literature for its medicinal properties accounting for about 68% of all traditional uses of Lamiaceae (Lukhoba _et al._, 2006). This is mainly due to the natural production of essential oil with high amounts of carvacrol (Castillo and Gonzalez, 1999), thymol (Singh _et al._, 2002), β-selinene, α-humulene, p-cymene, α-terpineol, γ-terpinene, and β-caryophyllene found in the oil fraction of this herb (Murthy _et al._, 2009; Senthilkumar and Venkatesalu, 2010). Biochemical components of _P. amboinicus_ are reported to have anti-microbial, lavicidal, anti-inflammatory, anti-tumorigenic properties much valued in the drug discovery (Goncalves _et al._, 2012; Rice _et al._, 2011). In addition, it is also added in food (Sahaykhare _et al._, 2001) and planted in home gardens as decorative (Lukhoba _et al._, 2006).

The raising global demand for _P. amboinicus_ in various biotechnologies has led to its habitat exploitation through uncontrolled harvest from wild. Similarly, another species under the same genus, _P. barbatus_ has become extinct in India due to indiscriminate collection (Gupta, 1988) whilst, _P. amboinicus_ is the next highly sorted medicinal herb around the globe (Lukhoba _et al._, 2006). Hence, a feasible storage system is necessary to conserve this herb.

Conservation of _P. amboinicus_ in seed bank is challenging as the plant rarely flowers or set seeds and the resultant plants are not true-to-type. Moreover, the hybridisation potential and seed sterility is a common phenomenon amongst _Plectranthus_ species (Lukhoba _et al._, 2006, Brits _et al._, 2001). Presently, vegetative propagation and _ex situ_ conservation in field gene banks are the most practiced and preferred methods for _P. amboinicus_ since conservation through seed banks is not a viable option. _Ex situ_ conservation of germplasm in field gene banks and botanical gardens are costly, labour and land intensive, and exposed to natural calamities (O’Brien _et al._, 2016). This exemplifies the urgent need for an alternate conservation method for _P. amboinicus_.

Cryopreservation is a feasible, complimentary way to conserve genetic resources of vegetatively propagated plants for a long-term avoiding effects of climate changes, diseases and pest incursions (Abdelnour-Esquivel and
Engelmann, 2002). Cryopreservation is defined as conservation of biological specimens at ultra-low temperatures, normally in liquid nitrogen (-196°C) (Withers and Engelmann, 1997), when most biochemical and physical processes are substantially arrested (Panis and Lambardi, 2005). Cryopreservation of biological materials is achievable only if the harmful intracellular ice crystal formation during freezing is avoided, as it destroys the semi-permeability causing irreversible harm to the cell membranes (Mazur, 2004; Sharma et al., 2013). Various approaches of cryopreservation such as, two-step freezing, preculture, desiccation, encapsulation-dehydration, vitrification and encapsulation-vitrification have been employed on different plant tissues (Matsumoto et al., 1995). Amongst them, encapsulation-dehydration (Fabre and Dereuddre, 1990) and vitrification are the simple and inexpensive techniques of long-term conservation while, encapsulation-vitrification is a combination of these two techniques. Encapsulation-vitrification minimises any potential injury of toxic vitrification solutions (Moges et al., 2004). Encapsulation-dehydration and encapsulation-vitrification techniques could be more suitable for cryopreservation of P. amboinicus vegetative propagules i.e., shoot tips, nodal segments and axillary buds, while retaining its clonal property.

Pretreatment of vegetative propagules in cryoprotectants is a prerequisite in cryopreservation protocol development (Matsumoto and Sakai, 1995; Sakai et al., 2000). For instance, vegetative propagules are often precultured with sugars i.e., sucrose, sorbitol and mannitol to increase tolerance towards dehydration and freezing. In such conditions, sugar accumulation helps to maintain the liquid crystalline state of the membrane bilayers and stabilise proteins (Crowe et al., 1987; Kendall et al., 1993; Rajasekharan, 2006). Besides that, propagules are treated with viscous cryoprotectants such as dimethylsulfoxide, glycerol, and ethylene glycol to increase the cell intracellular concentration to achieve a non-crystalline metastable glass state avoiding ice crystal formation (Yap et al., 2011). Generally, pretreatments are extremely toxic to cells especially delicate vegetative tissues. For that reason, encapsulation technology becomes a crucial step in cryopreservation protocol development, protecting plant material during osmotic-, chemical-dehydration, vitrification and cooling (Sharma et al., 2013, Rai et al., 2009). Encapsulation matrices protects cell from osmotic shocks from viscous cryoprotectants allowing gradual and effectual treatments to take place.

Encapsulated propagules are also known as synthetic seed can be used in direct sowing and as germplasm conservation strategy (Sharma et al., 2013, Rai et al., 2009). Synthetic seeds of vegetative propagules can offer great advantage for plant which has seasonal limitation, rarely produce seed, heterozygosity, and low germination (Saiprasad, 2001). Minute size of the synthetic seeds is also convenient mode of microbial-free germplasm exchange between laboratories. Synthetic seeds can also be subjected to germplasm storage from 30 up to 180 days if stored at ideal conditions, and considered as short-term storage strategy.
Establishment of storage protocol highly depends on efficient plant micropropagation system. Micropropagation allows rapid and large scale production of genetically and biochemically identical plants using relatively small amounts of space, supplies and time (Odutayo et al., 2004). This provides continuous uniformed propagules for storage studies. Moreover, standardisation of explant for short-term and long-term storage, encapsulation strategy and revival after storage are usually done in vitro, under controlled environment. In addition to this, micropropagation can be a desirable alternative to efficiently produce beneficial secondary metabolites (Ruffoni et al., 2010) greatly facilitating industries in providing uniformed and contamination free cultures and biochemical compounds (Hole et al., 2009).

Considering these facts, the potential of micropropagation and germplasm conservation of *P. amboinicus* was investigated with the following objectives:-

i. To establish a micropropagation protocol for *P. amboinicus*.
ii. To standardise explant, encapsulation conditions and establish short-term storage strategy for *P. amboinicus*.
iii. To develop a long-term storage protocol for *P. amboinicus* shoot apices using encapsulation-dehydration and encapsulation-vitrification techniques
ABBIBIBLIOGRAPHY


vitrification and replacement of cold hardening with preculture on medium enriched with sucrose and/or glycerol. Sci Hort 148: 104–108.


Sorensen, M. (1937). *Plectranthus amboinicus* (Lour.) Sprengel [as *Coleus amboinicus* Lour.] In: Coloured illustrations and popular descriptions of


