UNIVERSITI PUTRA MALAYSIA

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

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By

GREETHA ARUMUGAM

Thesis Submitted to the School of Graduate Studies,
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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 GERMPLASM CONSERVATION

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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 $CaCl_2$ 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

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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 mampan 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 separuh-kekuatan dengan penghasilkan 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, mengenalpasti *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 alginat dalam 100 mM larutan $CaCl_2$ 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 dihidrasi 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 + gliserol 1.0 M) atau L3 (sukrosa 0.4 M + gliserol 1.0 M + DMSO 5%) untuk 40 minit diikuti hidrasi dalam PVS2 (gliserol 30%, EG 15%, DMSO 15%). Kedua-dua teknik ini dapat menghidrasikan kapsul tunas pucuk dengan purata kelembapan antara 35 hingga 36% melalui hidrasi terus (gel silika) 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.

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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:

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BAP	6-Benzylaminopurine
Ca-alginate	Calcium alginate
$CaCl_2$	Calcium Chloride
dH_2O	Distil water
DMRT	Duncan's Multiple Range Test
DMSO	Dimethyl sulfoxide
EO	Essential oil
et al.	*et alia*
etc.	*et cetera*
FAA	Formaldehyde acetic acid
g	gram
g/L	gram per litre
GA_3	Gibberellic acid
i.e.	*id est* (that is)
IAA	Indole 3-acetic acid
IBA	Indole butyric acid
hrs	hour
Kn	Kinetin
L	Litre
LAF	Laminar Air Flow
LN	Liquid Nitrogen
LS	Loading Solution
M	Molar
mg/L	milligram per litre
mM	millimolar
min	minute
MS	Murashige and Skoog (1962) inorganic salt
NAA	Naphthaleneacetic acid
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
ns	non-significant
NS	Nodal segments
P>0.05	Probability at 95%
PGR	Plant growth regulators
PPM	Plant Preservative Mixtures
PVS	Plant Vitrification Solution
RCBD	Randomized Complete Block Design
SAS	Statistical Analysis System
ST	Shoot tips
v/v	volume/volume
w/v	weight/volume
°C	Degree centigrade
%	Percentage

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, ρ-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-tumorogenic 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 emcapsulation-vitrification techniques

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