IN VITRO PROPAGATION OF SABAH'S ENDANGERED JEWEL ORCHID, *Macodes limii*

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UNIVERSITI MALAYSIA SABAH
2018

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THESIS SUBMITTED IN FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE

FACULTY OF SCIENCE AND NATURAL RESOURCES
UNIVERSITI MALAYSIA SABAH
2018

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ACKNOWLEDGEMENT

Foremost, I would like to express my sincere gratitude to my thesis supervisors, Assoc. Prof. Dr. Jualang Azlan Gansau for this opportunity, for his continuous support of my MSc. study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance had helped me in all the time of research and writing of this thesis. Not forgotten, my appreciation to my co-supervisor, Assoc. Prof. Dr. Zaleha Abdul Aziz for all her advices and comments regarding this research.

My sincere thanks also go to the Unit for Orchid Studies, ITBC, UMS for allowing me to use the laboratory and greenhouse and staff especially Mdm. Jumatiah (laboratory assistant) for her cooperation during my research.

I thank my fellow friends; Alan, Amelia, Asnawi, Clarice, Emanuel, Florina, Usran, Vickly and Zaidey, for the stimulating discussions, for the sleepless nights we have worked together before deadlines, and for all the fun we have had in the last four years. Also, I thank PSKK team for the consistent support.

Last but not the least; I would like to thank my family, especially my parents for providing me with unfailing support and continuous encouragement throughout my years of study.

Lastly, big appreciation to the Ministry of Higher Education (MOHE) and UMS for providing me with financial supports through MyBrain and Teaching assistance Scheme respectively. This research was also supported by a research grant (SBK-0096-SG-2013).

Halyena Indan
14th February 2018

ABSTRACT

Macodes limii is a terrestrial jewel orchid of Sabah that grows in the deep shade and moist forest floor. It is highly valued for its unique leaves with sparkling golden yellow veins on blackish to purplish background of leaves. However, this species is recognised as an endangered species by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) due to over-collection and the loss of its suitable habitats. Through *in vitro* propagation of orchids, the problems can be overcome and protect this orchid from extinction by maintaining them in tissue culture or acclimatised as *ex situ* germplasm. In this study, protocols for *in vitro* propagation of *Macodes limii* using seeds and vegetative explants (shoot tip and nodal explants) were developed. The study on flowers, capsules and seeds biology (development and morphology), photoperiods (24-hour light, 16-hour light, 24-hour dark), complex additives (peptone, coconut water, banana homogenate) and plant growth hormones (BAP, Kin, TDZ, NAA, IAA, IBA) on asymbiotic seed germination, protocorm development and *in vitro* axillary shoot formation of *Macodes limii* were investigated. Immature capsules from artificial hand-pollinated of two to three-day aged flowers were harvested between days 21 to 23 for asymbiotic seeds germination. Seeds incubated under 24-hour dark produced the best germination response (29.6±3.57%) followed by 16-hour light (1.7±0.50%). No seeds were germinated under 24-hour light and thus, the beneficial effect of continuous darkness in asymbiotic seeds germination of *Macodes limii* was used for subsequent study. Higher seed germination percentage was observed on ½ strength MS medium with addition of 5% (w/v) banana homogenate (14.5±3.50%). The presence of 0.5 mg/L BAP in the medium also enhanced seed germination by 8.5±3.77%. Protocorms from seeds germinated under 24-hour dark condition were successfully regenerated and developed under the presence of light (16-hour light). Decontamination of apical shoot tip explant from *ex vitro* resulted in lower percentage of contamination (20±0.45%) and higher percentage of survival (60±0.55%) when immersed in 2% (v/v) Clorox™ for 15 minutes, as compared to nodal explant. The highest number of axillary shoots (four shoots per explant) and the longest axillary shoot (4.67±0.54 cm) were obtained on 0.5 mg/L TDZ using shoot tip explants. The well-developed shoots were further cultured on 1.0 mg/L IAA to initiate two to three roots with the length of 2.12±0.41 cm. The rooted plantlets were successfully acclimatised in a greenhouse after three months. The data presented in this study delivered new information for a better understanding of the propagation and conservation of this Sabah's orchid.

Keywords: Axillary shoot, asymbiotic seed germination, complex additives, jewel orchid, *Macodes limii*, micropropagation, plant growth hormones

ABSTRAK

Propagasi In Vitro Orkid Permata Terancam Sabah, Macodes limii.

Macodes limii adalah orkid permata terestrial Sabah yang tumbuh di lantai hutan yang lembap. Ia sangat dihargai kerana daunnya yang unik dengan urat-urat kuning emas berkilauan pada permukaan daun yang kehitaman dan keungguan. Walau bagaimanapun, spesies ini dikenalpasti sebagai spesies terancam oleh Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) berpunca daripada pengumpulan orkid tidak terkawal dan kehilangan habitat sesuai. Melalui propagasi orkid secara in vitro, masalah ini dapat diatasi dan melindungi orkid ini daripada kepupusan dengan mengekalkan spesies ini secara kultur tisu atau diaklimatasi sebagai germplasma ex situ. Dalam kajian ini, protokol propagasi in vitro Macodes limii dengan menggunakan biji benih dan eksplan vegetatif (hujung pujuk dan nod) telah dibangunkan. Kajian tentang biologi bunga, kapsul dan biji benih (perkembangan dan morfologi), fotokala (24 jam cahaya, 16 jam cahaya, 24 jam gelap), aditif kompleks (pepton, air kelapa, homogenat pisang) dan hormon pertumbuhan tumbuhan (BAP, Kin, TDZ, NAA, IAA, IBA) terhadap percambahan biji benih asymbiotik, perkembangan protokom dan regenerasi pucuk aksil in vitro Macodes limii dikaji. Kapsul tidak matang yang terhasil daripada pendebungaan tiruan pada bunga yang berumur dua hingga tiga hari dituai pada hari ke-21 ke hari ke-23 untuk percambahan biji benih asymbiotik. Kajian terhadap fotokala menunjukkan bahawa biji benih yang diinkubasi di bawah 24 jam gelap menghasilkan tindak balas percambahan biji benih terbaik (29.6±3.57%) diikuti oleh 16 jam cahaya (1.7±0.50%) manakala tiada benih bercambah di bawah 24 jam cahaya dan oleh itu, kebaikan kesan gelap ini dalam percambahan biji benih asymbiotik Macodes limii digunakan untuk eksperimen seterusnya. Peratusan percambahan biji benih yang tinggi telah diperhatikan pada medium ½ kekuatan MS dengan penambahan 5% (w/v) homogenat pisang. Kehadiran 0.5 mg/L dalam medium juga meningkatkan percambahan biji benih (8.5±3.77%). Protokom daripada kultur 24-jam gelap berjaya berkembang menjadi anak pokok di bawah kehadiran cahaya (16 jam cahaya). Dikontaminasi hujung pucuk apikal ex vitro menghasilkan peratusan kontaminasi yang lebih rendah (20±0.45%) dan peratusan eksplan yang hidup lebih tinggi (60±0.55%) apabila direndam dengan 2% (v/v) Clorox™ selama 15 minit mempunyai berbanding eksplan nod. Bilangan maksimum pucuk aksil (empat pucuk/pucuk) dengan panjang pucuk aksil terpanjang (4.67±0.54 cm) didapati pada medium 0.5 mg/L TDZ dengan menggunakan eksplan hujung pucuk. Untuk pengakaran in vitro, pucuk yang dikultur pada 1.0 mg/L IAA mengaruh pembentukan dua hingga tiga akar dengan panjang akar terpanjang (2.12±0.41 cm). Selepas tiga bulan, anak pokok in vitro yang mempunyai akar yang baik berjaya diaklimatisasi di rumah hijau. Dapatan kajian dalam penyelidikan ini menyalurkan infomasi baru mengenai pemahaman terhadap propagasi dan konservasi orkid Sabah.

Kata kunci: Pucuk aksil, percambahan biji benih asymbiotik, aditif kompleks, orkid permata, Macodes limii, mikropropagasi, hormon pertumbuhan tumbuhan

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2,4-D	→	2, 4-dichlrophenoxyacetic acid
˚C	→	degree celsius
AC	→	activated charcoal
BAP	→	6-benzylaminopurine
EDTA	→	ethylenediaminetetraacetic acid
g	→	gram
HCl	→	hydrochloric acid
IAA	→	indole-3-acetic acid
IBA	→	indole-3-butyric acid
Kin	→	kinetin
L	→	litre
M	→	Morality
MS	→	Murashige and Skoog (1962) medium
mg/L	→	milligram per litter
mg	→	milligram
ml	→	millilitre
mm	→	millimetre
NAA	→	α-naphthaleneacetic acid
N	→	normal solution
NaOH	→	sodium hydroxide
PFD	→	photon flux densities
pH	→	a measure of hydrogen ion concentration [H], and hence of the acidity or alkalinity of an aqueous solution.
psi	→	vound per square inch
TDZ	→	Thidiazuron
v/v	→	polume per volume (concentration)
w/v	→	weight per volume (concentration)

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CHAPTER 1

INTRODUCTION

For most people, the word "orchid" brings to mind exotic flowers with tall stems with sweet fragrances. However, some orchids are popular for its leaves rather than its flowers which are popularly known as jewel orchids (Ong *et al.*, 2012). It is a grouping based on their unique leaf morphology and does not represent a specific genus of orchids (Bhattacharjee and Chowdhery, 2012) but comes from different subfamilies. Some common genera in jewel orchids are *Anoectochilus*, *Corybas*, *Dossinia*, *Goodyera*, *Ludisia*, *Macodes* and *Malaxis* (Wood *et al.*, 2011). Some jewel orchids such as *Ludisia discolor* and *Anoectochilus* spp. are not only limited as an ornament but also have potential in the pharmaceutical industry (Lee *et al.*, 2015b; Budluang *et al.*, 2016).

The genus of *Macodes* has around 11 species which are currently accepted based on The Plant List website. Among the *Macodes* genus, *Macodes limii* has beautiful leaves with ten primary nerves with distinctive sparkling golden-yellow vein under the light that uniformly distributed on blackish and purplish green background and pinkish red beneath the leaves (Wood *et al.*, 2011). Like other vulnerable orchids, *Macodes limii* also faced declining population in the wild due to regular collecting to satisfy the market demand. In addition, prolonged drought and forest fires from Hempuen Hill to Telupid also affected the orchid population especially *Macodes* spp. (Chan *et al.*, 1994). Habitat fragmentation can also interfere with the natural biological process (Wong and Chan, 1997). Germination of seed in the wild is difficult without the dependence of specific fungi because essential food cannot be supplied to seed via the outer layer of root cells (Roberts and Dixon, 2008). *Macodes* spp. is now recognised as an endangered species by the Convention on International Trade in Endangered Species of Wild Fauna and

Flora (CITES). Orchids can be propagated vegetatively to produce new plants but these methods resulted in slow growth (Luan *et al.*, 2006). The conventional propagation is insufficient for large-scale cultivation. Therefore, an efficient strategy needs to be developed not only to save orchid from disappearing but also to meet the increasing demand for orchids (Ong *et al.*, 2012). The application of tissue culture through various methods can serve as a substitute method to propagate *Macodes limii*. Many successful reports on *in vitro* propagation of rare and threatened orchid have been reported (Park *et al.*, 2002; Decruse *et al.*, 2003; Dutra *et al.*, 2008; Pant and Pradhan, 2010; Suzuki *et al.*, 2012; Zeng *et al.*, 2012) but in those reports, *in vitro* propagation of orchids were significantly influenced by medium composition, types of explants, capsule maturity and culture conditions. These media compositions and culture conditions are often modified because the responses and requirement of orchids are often species-specific and have its unique requirements (Arditti, 1967; Lauzer *et al.*, 2007). Therefore, this brings the interest to study the factors affecting *in vitro* propagation of *Macodes limii* due to such inconsistency in the tissue culture response.

Asymbiotic seed germination serves as the most reliable and effective method for conservation and mass multiplication of endangered orchid species (Parthibhan *et al.*, 2012) since a large number of healthy heterozygous seedling can be produced in a single capsule (Rittershausen and Rittershausen, 2011). The success of *in vitro* seed germination, however, is inadequate by only describing an efficient *in vitro* seed germination culture method without studying orchid biology such as the development and morphology of flowers, capsules and seeds because these provide the basic knowledge for better understanding of the propagation and conservation of orchids (Cribb *et al.*, 2003). *In vitro* seed germination of orchids is also significantly affected by medium composition such as complex additives, plant growth hormones and culture condition. These germination media and culture conditions are often modified because the responses and requirement of orchids often have its unique requirements (Arditti, 1967; Lauzer *et al.*, 2007). For example, the influence of coconut water in improving seed germination has been reported in several orchid species such as *Paphiopedilum wardii* (Zeng *et al.*, 2012) and *Coelogyne nervosa* (Abraham *et al.*, 2012) but others orchid species such as *Anoectochilus formosanus* (Shiau *et al.*, 2002) reported banana homogenate has

promoted seed germination significantly. Plant growth hormones such as 6-benzylaminopurine (BAP) was found to help and promote seed germination of *Calanthe tricarinata* (Godo *et al.*, 2010) and *Epidendrum ibaguense* (Hossain, 2008) but Bektaş *et al.* (2013) found that the addition of 1 mg/L IAA provided the best *in vitro* germination and protocorm formation of *Orchis coriophora*. Light also significantly affect the seed germination and protocorm development in *Anoectochilus formosanus*, *Geodorum densiflorum* and *Haemaria discolour* (Roy and Banerjee, 2001; Shiau *et al.*, 2002; Shiau *et al.*, 2005) while in certain species of orchids, light condition sometimes have little or no effect at all on seed germination although the development of seedling might be affected (Vasudevan and Van Staden, 2010).

In vitro clonal micropropagation is another substitute method for orchids that difficult to propagate through seed which produce similar ornamental characteristics and for medicinal purposes (Chugh *et al.*, 2009). The challenges arise from this method are the initiation of aseptic culture and subsequent optimisation of their growth and propagation by manipulating factors that affect the successful outcome of this method. The use of shoot tip and nodal explants have become very popular due to its high efficiency in producing rapid mass propagation of some orchids (Pant and Swar, 2011) and reliable method of uniformity and true-to-type regenerated plantlet (George and Debergh, 2008). However, specific plant growth hormones are important to be tested to enhance the multiplication and regeneration of explants (Ket *et al.*, 2004; Sherif *et al.*, 2012; Gangaprasad *et al.*, 2000; Thi *et al.*, 1998; Zhang *et al.*, 2015). An effective *in vitro* rooting of the plantlets by having a greater number of roots is the fundamental element for acclimatisation and survival from the natural environment of micropropagated plants (George and Debergh, 2008) through the addition of IAA and IBA in medium (Malabadi *et al.*, 2004; Mohanty *et al.*, 2012; Dewir *et al.*, 2015). This investigation was initiated to establish protocols for *in vitro* propagation of *Macodes limii* by determining the appropriate types and concentration of complex additives and plant growth hormones, types of explants, capsule maturity and culture conditions for *Macodes limii*. The data presented in this study delivered new information for a better understanding of the propagation and conservation of this Sabah orchid.

This study focused on the following objectives:

1. To assess the morphology and growth development of the *Macodes limii*'s flowers, capsules, and seeds.
2. To determine the effect of photoperiods, complex additives and plant growth hormones on asymbiotic seed germination and protocorm development of *Macodes limii*.
3. To determine optimum exposure time of Clorox™ on initiation of aseptic culture, and the effect of explants types and plant growth hormones on *in vitro* axillary shoots and roots formation of *Macodes limii*.