MASS SPECTROMETRY-BASED METABOLITE PROFILING OF WILD AND *IN VITRO* PROPAGATED SABAH JEWEL ORCHID *Macodes limii* J.J. Wood & A.L. Lamb

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DECLARATION

I hereby declare that the material in this thesis is my own except quotations, accepts, equations, summaries, and references, which have been duly acknowledged.

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ABSTRACT

Jewel orchids are commonly known for their beautiful foliage venations and some of them are appreciated for their remarkable medicinal properties. *Macodes limii* is a terrestrial jewel orchid endemic to Sabah, which can only be found in specific areas with ultramafic soil. The plant is vulnerable to extinction due to its specific growth requirements, besides unsustainable collection by orchid collectors for trading purposes. Despite its high ornamental value, less effort was given to protect this native species as well as to explore its chemical composition. Therefore, the current study was conducted to establish an *in vitro* regeneration protocol for *M. limii*, as well as to decipher the chemical composition of this endemic plant. The results revealed that *M. limii* can be regenerated via shoot tip and nodal cultures. However, to promote shoot multiplication, nodal explant was superior to shoot tip when cultured on half-MS basal media containing 0.5 mg/L of kinetin or 1.0 mg/L TDZ, which has promoted up to three shoots after 90 days of culture. Meanwhile, the total phenolic and flavonoid contents in the wild and *in vitro* cultivated *M. limii* plants revealed a strong positive correlation to the antioxidant potential by DDPH and FRAP assays. The integration of gas chromatography (GC) and liquid chromatography (LC) coupled with mass spectrometry (MS) has profiled the metabolites in the wild and *in vitro* cultivated *M. limii* plants that mostly consist of carbohydrates and their derivatives, organic acids, amino acids, fatty acids as well as flavonoids and phenolics compounds. Interestingly, this approach also has detected one potential compound, putatively annotated as 3-hydroxy-4-butanolide, a synonym to kinsenoside, which was previously reported to be the main bioactive compound in other medicinal jewel orchids. In combination with the multivariate statistical analysis, it was revealed that growing environments (wild and *in vitro* cultivation), as well as the age of plants (9, 18, and 27 months of culture), influenced the chemical composition of *M. limii*. The obtained results were the first ever reported on the micropropagation protocol as well as the metabolite profiling in *M. limii* and for the *Macodes* genus. The establishment of this *in vitro* propagation protocol is significant in reducing the risk of exploitation of this plant from the wild, and information on the plant metabolite profiles might serve as fundamental in understanding the metabolite variations in jewel orchids.

ABSTRAK

PEMPROFILAN METABOLIT MENGGUNAKAN SPEKTROMETRI JISIM KE ATAS Macodes limii J.J. Wood & A.L. Lamb DARIPADA LIAR DAN PENANAMAN SECARA IN VITRO

Orkid jewel secara umumnya terkenal dengan urat yang cantik pada dedaunnya dan beberapa jewel orkid dihargai atas sifat perubatannya yang menakjubkan. Macodes limii adalah orkid tanah yang endemik kepada Sabah, dan tumbuhan ini hanya boleh ditemui di kawasan yang spesifik dengan tanah ultramafik. Tumbuhan ini menghadapi ancaman kepupusan disebabkan keperluan habitatnya yang spesifik disamping pengambilan orkid daripada habitat asal secara tidak lestari oleh pengumpul orkid bagi tujuan perdagangan. Walaupun orkid ini mempunyai nilai ornamental yang tinggi, kurang inisiatif diberikan untuk memulihara tumbuhan ini termasuk mengkaji komposisi kimia tumbuhan tersebut. Oleh itu, kajian ini dijalankan untuk menghasilkan satu protokol regenerasi M. limii secara in vitro, dan juga merungkai komposisi kimia pada tumbuhan endemik ini. Keputusan menunjukkan M. limii boleh dipropagasi melalui kultur pucuk dan keratan nod. Tetapi, untuk menggalakkan penggandaan pucuk, penggunaaan eksplan nodal didapati lebih baik daripada pucuk apabila dikultur pada media ½ MS yang mengandungi 0.5 mg/L kinetin atau 1.0 mg/L TDZ, di mana ia telah menggalakkan pertumbuhan tiga pucuk selepas 90 hari pengkulturan. Sementara itu, kandungan fenolik dan flavonoid dan telah menunjukkan korelasi positif yang kuat terhadap potensi antioksidan pada M. limii daripada hutan dan kultivasi in vitro melalui ujian DPPH dan FRAP. Integrasi kromatografi gas dan kromatograpi cecair dengan spektrometri jisim telah memprofilkan metabolit M. limii yang diambil daripada liar ataupun melalui penanaman in vitro, di mana ia terdiri daripada karbohidrat dan sebatiannya, asid organik, asid amino, asid lemak serta flavonoid dan asid fenolik. Yang menariknya, kaedah ini telah menemui satu kompoun yang berpotensi dianggap sebagai 3-hydroxy-4-butanolide, merupakan sinonim kepada kinsenoside, yang mana telah dilapor merupakan kompoun bioaktif dalam orkid jewel berubat yang lain. Dengan kombinasi analisis statistik multivariat, telah menunjukkan bahawa persekitaran pertumbuhan (hutan dan in vitro) serta umur anak pokok (9, 18 dan 27 bulan pengkulturan) mempengaruhi komposisi kimia M. limii. Hasil dapatan kajian ini merupakan kali pertama ia dilaporkan terhadap protokol mikropropagasi dan komposisi M. limii seterusnya untuk genus Macodes. Protokol propagasi M. limii secara in vitro adalah signifikan untuk mengurangkan risiko eksploitasi tumbuhan ini daripada hutan, dan maklumat terhadap jujukan metabolit akan menjadi asas kepada pemahaman terhadap kepelbagaian metabolit pada orkid jewel.

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2,4-D	-	2,4-Dichlorophenoxy acetic acid
µL	-	Microlitre
µg	-	microgram
°C	-	Degree Celsius
BAP	-	6-benzylaminopurine
cm	-	centimetre
DAC	-	Days after culture
DNA	-	Deoxyribonucleic acid
GC-MS	-	Gas chromatography-mass spectrometry
g	-	Gram
IAA	-	Indole acetic acid
L	-	Litre
LC-MS	-	Liquid chromatography-mass spectrometry
Kin	-	Kinetin
min	-	minute
mg	-	Milligram
mM	-	Millimolar
MS	-	Murashige and Skoog medium
m/z	-	Mass-to-charge ratio
mL	-	Millilitre
NAA	-	α-naphthalene acetic acid
nm	-	nanometer
Pic	-	Picloram
PCR	-	Polymerase chain reaction
PGRs	-	Plant growth regulators
rpm	-	Revolutions per minute
s	-	seconds
TDZ	-	Thidiazuron
% v/v	-	Percent volume/volume
% w/v	-	Percent weight/volume

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Orchidaceae is among the largest family of flowering plants, comprising more than 25,000 species distributed throughout the world (Chase *et al.,* 2015). Orchids are commonly known for their beautiful and distinctive types of flowers, and some of them are appreciated for their beautiful foliage which is known as "jewel orchids". Jewel orchids comprise orchids from several genera such as *Anoectochilus*, *Dossinia*, *Goodyera*, *Ludisia,* and *Macodes* (Bhattacharjee and Chowdhery, 2012; Bhattacharjee, 2013).

The *Macodes* genus consists of 12 species according to the World Checklist of Selected Plant Families (WCSP, 2022), with four species reported to be distributed in Sabah (Wood *et al.,* 2011). Among them, *Macodes limii* J.J. Wood & A.L. Lamb is endemic to Sabah and this orchid can be found distributed in hill forests and lower montane ridge forests which are restricted to ultramafic soil (Wood *et al.,* 2011). Like other jewel orchids, *M. limii* also has a distinctive foliage characteristic with high ornamental value. The uniqueness and rarity of many Sabah wild orchids have contributed to the unsustainable collection from their natural habitat, hence letting many orchid species vulnerable to extinction (Besi *et al.*, 2020, 2021). Moreover, orchid conservation has become a challenge practically under the current climate change scenario, added with other increasing threats including habitat destruction caused by deforestation for development or agricultural purposes (Fay, 2018; Gale *et al.,* 2018; Juiling *et al.*, 2020). In fact, the Orchidaceae family is now included in the Appendix-II of Convention on International Trade in Endangered Species of Wild

Fauna and Flora (CITES), where international trade is strictly controlled and monitored (https://cites.org/eng).

Plant tissue culture is by far the most promising approach not only for large-scale propagation but also for orchid conservation. This method promised a high opportunity for *ex situ* conservation and reintroduction of many endangered orchid species back to their natural habitat. As for jewel orchids, the *in vitro* seed germination protocol has been successfully applied to *A. formosanus* (Shiau *et al.,* 2002; Chou & Chang, 2004). However, due to slow plant growth and seasonal flowering of jewel orchids, the *in vitro* seed germination protocol was not favorable for mass propagation (Han *et al.*, 2020). Therefore, micropropagation has become a reliable approach for mass propagation for both conservation and commercialization. This approach has maintained the genetic stability of many jewel orchids cultures including *A. formosanus* (Ket *et al.,* 2004); *A. elatus* (Sherif *et al.,* 2016); *A. roxburghii* (Li *et al.,* 2018), and *Ludisia discolor* (Poobathy *et al.,* 2019).

Apart from the ornamental features, jewel orchids are also recognized as medicinal orchids due to their various functions in traditional medicines. Among them, *A. formosanus and A. roxburghii* have been recognized as the "King of Medicine" as they were reported beneficial to protect the liver, preventing cancer and diabetes, and treating cardiovascular diseases (Wang *et al.*, 2020; Ye *et al.*, 2020a; Wu *et al.*, 2021), and *Ludisia discolor* was reported to exhibit anti-fatique property (Shi *et al.*, 2016). The medicinal properties of jewel orchids are due to the presence of secondary metabolites such as flavonoids, polysaccharides, kinsenoside, alkaloids, sterols, organic acids, amino acids, and other active ingredients (He *et al.,* 2006; Huang *et al.,* 2007; Han *et al.,* 2008).

Metabolite profiling or metabolomics is a bioanalytical tool that can provide an essential unbiased, comprehensive qualitative, and quantitative overview of the metabolites present in a biological sample at a specific time under specific conditions (Hong *et al.*, 2016). Analytical technologies such as gas chromatography (GC) and liquid chromatography (LC) coupled with mass spectrometry (MS) are commonly used in plant metabolomics studies (Jorge *et al.,* 2016), as a powerful tool for

improving understanding of the physiology and biochemistry of plants in many aspects including growth and development (Qin *et al.*, 2020), response to external stress (Wong *et al.*, 2020), as well as the nutritional requirements (Magangana *et al.*, 2018). Interestingly, this approach could be extended to natural product drug discovery by studying the relationship between the whole metabolome of natural-derived remedies and their biological effects (Carvalho *et al.*, 2021). In orchid studies, the metabolomics approach has been successfully implemented to profile the metabolites of the medicinal *Dendrobium* species (Jin *et al.,* 2016), to compare the vanillin biosynthesis between vanilla pods and beans (Gu *et al.*, 2017), and also to uncover the flavonoid accumulation in two *Cymbidium tortisepalum* var. *longibracteatum* cultivars (Jiang *et al.,* 2022).

1.2 Justification of Study

Micropropagation of jewel orchids not only supports the horticulture industry but also acts as a source of bioactive compounds such as polysaccharides and kinsenosides for medicinal purposes (Refish & Fu, 2018; Jin *et al.,* 2018). The ornamental and medicinal characteristics of the jewel orchids as described previously have attracted our attention to explore the potential of the Sabah jewel orchid, *M. limii*. So far, no available comprehensive data or research is available concerning the *in vitro* propagation protocol as well as the metabolite profiling of *M. limii*. The only scientific information on *M. limii* available so far was on the plant morphology characterization that has been well described by Wood *et al.* (2011), and also the flower and capsule development of *M. limii*, as well as its preliminary *in vitro* seed germination that was reported by Indan *et al.* (2021).

Considering the limited information on this endemic jewel orchid *M. limii*, along with the risk of extinction, the current study was proposed to establish an efficient propagation protocol, as well as to decipher the metabolite profiles of this plant.

1.3 Research Questions

The justification of the study has generated several research questions as follows:

i. How to propagate *M. limii* plants by using the plant tissue culture technique?

ii. What are the metabolite compositions in the wild plant of *M. limii*?

iii. Are the wildly grown *M. limii* plants different from the *in vitro* derived plants in terms of their phytochemical contents, antioxidant activity, and metabolic profiles?

iv. Do the metabolite profiles of the *in vitro M. limii* plants varies at different ages?

1.4 Objectives

To answer the questions, this study was carried out with four objectives as follows:

i. To determine the effects of explant types and plant growth regulators on *in vitro* regeneration of *M. limii.*

ii. To profile the metabolites composition in wild *M. limii* by using the mass spectrometry-based approaches.

iii. To compare the phytochemical contents, antioxidant activities, as well as metabolite profiles between the wild and *in vitro* cultivated *M. limii.*

iv. To investigate the metabolite profiles of the *in vitro M. limii* plants at different ages.

1.5 Hypothesis

i. The type of explants and PGRs influenced the *in vitro* regeneration of *M. limii.*

ii. Different growing environments (wildly grown and *in vitro* cultivation) influenced the metabolite compositions, phytochemical contents, and antioxidant activity of *M. limii*.

iii. Different ages of plants influenced the metabolite profiles of the *M. limii*.

1.6 Significance of the Study

Findings from this study were the first ever reported on *M. limii* as well as for the *Macodes* genus. The establishment of the *in vitro* regeneration protocol will serve as an alternative to the unsustainable collection of this plant from the wild. Meanwhile, the exploitation of mass spectrometry-based metabolite profiling analysis will decipher the metabolite variation in *M. limii* plants. In addition, the preliminary study on the phytochemical contents and antioxidant activity in the wild and *in vitro* propagated *M. limii* will provide a reliable theoretical basis for future bioactive compound discovery from this native plant.

CHAPTER 2

LITERATURE REVIEW

2.1 Orchidaceae

Orchidaceae is among the largest family of flowering plants, estimates range from 25,000 to 35,000 species from 736 currently recognized genera distributed throughout the world (Chase *et al.,* 2015). Orchids are valued mainly for their long shelf life and their exotic beauty of flowers with incredible range of colours, shapes, and fragrances. Some orchids are also prized for their beautiful veined foliage and are known as "jewel orchids" (Sumathi *et al.,* 2003; Bhattacharjee & Chowdhery, 2012). Meanwhile, two *Vanilla* orchids such as *V. planifolia* and *V. tahitensis* are cultivated for their pods, as a source of vanilla extract that is widely used as spice and fragrance (Khoyratty *et al.,* 2018; Singletary 2020).

Orchids grow as terrestrial, epiphytic, or lithophytic and are distributed varies widely within the tropics. Orchid-rich areas include the northern Andes of South America, Madagascar, Sumatra, and Borneo for mostly epiphytic species, Indochina for both epiphytic and terrestrial species, and southwestern Western Australia as a center of terrestrial orchid richness (Cribb *et al.,* 2003). In Borneo, more than 2,500 to 3,000 species of orchids were documented which represent approximately 10% of the total number of orchids in the world (Chan *et al.,* 1994). While in Sabah, the high richness of orchid species can be found concentrated in the Mount Kinabalu area. The mountain supports a diverse range of unique orchid species with over 720 species of orchids from 121 genera including rare and endemic orchids (Wood *et al.,* 2011).

2.2 Jewel Orchids and Their Benefits

Orchids from the genera *Anoectochilus*, *Goodyera*, *Ludisia,* and *Macodes* belong to the subtribe Goodyerinae of the Cranichideae tribe (Orchidaceae: Orchidoideae) are widely distributed in tropical Asia (Smidt *et al.*, 2021) and also found in Argentina, New Zealand and South Africa (Pridgeon *et al.,* 2003). Orchid species from these genera are termed "jewel orchids" due to their attractive foliar venations on the upper surface of their leaves. Jewel orchids are terrestrial plants with creeping rhizomes and are mostly found on the shady forest floor with cool, humid conditions, with good drainage (Pridgeon *et al.,* 2003).

Jewel orchids from the genus *Anoectochilus* including *A. formosanus*, *A. roxburghii*, and *A. koshunensis* are considered high-value medicinal orchids used in Traditional Chinese Medicines in China, Taiwan, South Korea, and Japan (Tseng *et al.,* 2006; Du *et al.,* 2008; Luo *et al.,* 2018). The fresh or dried whole plant of these orchids which are referred to as the "King of Medicines", have been primarily used to cure heart disease, lung and liver diseases, cancer, and hypertension (Zhang *et al.*, 2015b; Zeng *et al.*, 2016; Ye *et al.*, 2017; Yang *et al.*, 2019; Gao *et al.*, 2021; Chac *et al.*, 2022). Meanwhile, a native Borneo jewel orchid *Anoectochilus reinwardtii* Blume, was reported used to treat infertility in the Iban and Kelabit tribes (Teoh, 2016). According to this tribe, this orchid is believed to possess magical properties that might help the infertile woman to conceive if the leaves of a single plant are placed under their sleeping mat (Teoh, 2016). *Ludisia discolor* is another jewel orchid that is distributed from southern China through Indochina, Thailand, Malaysia and Indonesia is also used in traditional Chinese medicine to nourishes the lungs, regulates body fluids, purifies the bloods and act as anti-inflammatory (Teoh, 2016).

2.3 *Macodes limii* J.J. Wood & A.L. Lamb

The genus Macodes (Blume) Lindl. (Subtribe Goodyerinae; tribe Cranichideae; subfamily Orchidoideae) comprises terrestrial herbs with creeping and fleshy rhizomes and erect stems with few rosulate leaves (Wood *et al.,* 2011). *Macodes* are distributed from Peninsular Malaysia to Borneo, Indonesia, Philippines, Papua New Guinea, and the Solomon Islands (MyBIS, 2023). This genus holds 12 species according to the World Checklist of Selected Plant Families (WCSP, 2022) of which four species including *M. limii*, *M. angustilabris*, *M. petola* and *M. aff. angustilabris* were found in Sabah (Wood *et al.,* 2011).

Macodes limii J.J Wood & A.L Lamb is notable for its attractive foliage which consists of sparkling golden-yellow venations, uniformly distributed on its leaves. The orchid exhibits a monopodial growth pattern with a rhizomatous stem and is grown on leaf litter and humus or mossy rocks. *Macodes limii* is distributed in the area of hill forest, lower montane ridge forest, restricted to ultramafic soil at elevations 250m to 1000m specifically in Mt. Kinabalu, Kota Belud district (600 m), Telupid area, and Maliau Range in Labuk and Sugut districts (Wood *et al.,* 2011). *Macodes limii* is characterized by its robust plant at 30 – 40 cm tall, which leaves has oblong-elliptic (9.5 x 7 cm) when mature, an upper surface of leaves is blackish to purplish green with 10 golden to yellow primary nerves, and the flower is small only at 1.8 cm across, non-resupinate and asymmetric because the lip and the column are twisted with an outer surface covered in white hairs (Wood *et al.,* 2011). The plant was named in honour of Mr. Herbert Lim a senior agriculturist in Sabah (Wood *et al.,* 2011).

The development of flowers and capsules in *M. limii* had been successfully documented previously (Indan *et al.,* 2021). The flowering stage began with bud initiation at the shoot apex, followed by inflorescence formation and later differentiate into floral meristem which took around 54 – 60 days until the first flower start to bloom (Figure 2.1). The full bloom of *M. limii* flower was pollinated manually, and capsule formation was visible after 4 days of pollination. The capsule reaches its maturity and dehisced after 25 days of pollination (Indan *et al.,* 2021).