ESTABLISHMENT OF ADVENTITIOUS ROOT CULTURES IN *Labisia pumila* (var. *alata*, var. *pumila,* var. *lanceolata*) FOR THE PRODUCTION OF SECONDARY METABOLITES WITH ANTIOXIDATIVE PROPERTIES

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FACULTY OF SCIENCE AND NATURAL RESOURCES
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THESIS SUBMITTED IN FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE

FACULTY OF SCIENCE AND NATURAL RESOURCES
UNIVERSITI MALAYSIA SABAH
2017

DECLARATION

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

20 AUGUST 2017

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DEGREE : **MASTER OF SCIENCE (BIOTECHNOLOGY)**

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ABSTRACT

Labisia pumila (Kacip Fatimah) is a herbaceous plant traditionally used for facilitating childbirth and post-natal recovery. *L. pumila* are highly demanded for commercial production, thus there is a need to establish a new *in vitro* cultivation system that produce large amounts of biomass with increased accumulation of secondary metabolites. Adventitious root culture is a promising technique to be applied in *L. pumila* for the rapid production of its secondary products. Therefore, this study was carried out to establish adventitious root cultures for biomass and secondary metabolite production from three varieties of *L. pumila* namely as var. *alata*, var. *pumila* and var. *lanceolata*. The root culture was initiated by selecting *in vitro* source materials from each variety. Optimisation of plant growth regulators, MS medium strength, sugars and elicitors were subsequently carried out to enhance root biomass and metabolites production. Shoot buds induction from adventitious root explants of *L. pumila* was also investigated. Out of 50 clones for each variety; var. *alata* line 30 (LPA30), var. *pumila* line 28 (LPP28) and var. *lanceolata* line 32 (LPL32) were selected as superior *in vitro* plantlets that had the highest DPPH radical scavenging activity (35.01±2.56 mg Trolox/g DW, 34.17±1.83 mg Trolox/g DW and 33.22±1.41 mg Trolox/g DW, respectively). LPA30 also had the highest ferric reducing ability (5.84±0.06 mg Trolox/g DW) and total phenolics (8.32±0.22 mg gallic acid/g DW) and flavonoids (3.99±0.07 mg quercetin/g DW) content as compared to LPP28 (4.71±0.11 mg Trolox/g DW, 6.77±0.22 mg gallic acid/g DW, 3.54±0.02 mg quercetin/g DW) and LPL32 (2.39±0.02 mg Trolox/g DW, 4.45±0.08 mg gallic acid/d DW, 2.35±0.08 mg quercetin/g DW), respectively. Optimisation of plant growth regulators revealed that 1 mg/L NAA was the best auxin for biomass production with the highest yield of phenolics and flavonoids in LPA30, LPP28 and LPL32 root cultures. The combination of 1 mg/L NAA with cytokinin (BAP and KN at 0.1, 0.5, 1 mg/L) failed to produced roots with high biomass and metabolite accumulation. Furthermore, full strength MS medium and 3% (w/v) sucrose provided the highest metabolite yields and biomass production in all root cultures. The elicitation of chitosan and salicylic acid in LPA30 root cultures failed to enhance phenolics and flavonoids production. In contrast, metabolite accumulation was enhanced in both LPP28 and LPL32 roots using 10 mg/L chitosan and 5 mg/L salicylic acid, respectively, although biomass formation was hindered. LPP28 and LPL32 roots elicited with 10 mg/L chitosan enhanced up to 1.5 and 2.2 folds phenolics, and 1.2 and 1.9 folds flavonoids content, respectively. Meanwhile, LPP28 and LPL32 roots elicited with 5 mg/L salicylic acid enhanced up to 1.9 and 2.5 folds phenolics, and 1.5 and 2.2 folds total flavonoids content, respectively. The HPLC analysis showed gallic acid and myricetin were detected in adventitious roots and *in vitro* plantlets. Quercetin was only detected in *in vitro* plantlets. Positive correlations were observed between TPC and TFC with DPPH and FRAP assay in *L. pumila* samples. Treatment of 3 mg/L TDZ successfully induced shoot buds formation in LPA30 adventitious root explants (14.81±6.24%), as compared to 5 mg/L TDZ for LPP28 (40.74±6.42%) and LPL32 (85.19±12.83%). Those explants with shoot buds were transferred into MS media without hormone for preliminary study. From this study, LPA30, LPP28 and LPL32 were selected as plantlets with high antioxidative properties. Adventitious root cultures of the plantlets were established in liquid shake culture system for phenolics and flavonoids production. Shoot regeneration from adventitious root explants of *L. pumila* can be further studied in the future.

Keywords: *Labisia pumila*, *in vitro* selection, adventitious root, shoot bud induction

ABSTRAK

PEMBANGUNAN KULTUR AKAR ADVENTITIUS Labisia pumila (var. alata, var. pumila, var. lanceolata) UNTUK PENGHASILAN METABOLIT SEKUNDER DENGAN CIRI ANTIOKSIDATIF

Labisia pumila (Kacip Fatimah) merupakan sejenis tumbuhan herba yang sering digunakan dalam perubatan tradisional di Malaysia. Oleh kerana herba ini mendapat permintaan tinggi di pasaran, terdapat keperluan untuk membangunkan satu sistem baru dalam propagasi spesies ini secara in vitro bagi meningkatkan penghasilan biojisim dan kandungan metabolit sekunder. Kultur akar adventitius merupakan satu teknik yang berpotensi untuk diaplikasikan pada L. pumila bagi penghasilan metabolit sekunder dalam jangka masa yang singkat. Oleh itu, kajian ini dijalankan untuk membangunkan kultur akar adventitius daripada tiga varieti L. pumila (var. alata, var. pumila dan var. lanceolata). Kajian dimulakan dengan memilih anak tumbuhan in vitro daripada ketiga-tiga varieti yang mempunyai kapasiti antioksidan yang tertinggi. Pengoptimuman jenis pengawalatur tumbuhan, kekuatan medium MS, gula dan pengelisit terhadap pertumbuhan dan penghasilan metabolit sekunder dalam kultur akar adventitius L. pumila turut dikaji. Selain itu, kajian pengaruhan tunas pucuk daripada eksplan akar adventitius turut dijalankan. Hasil dapatan kajian pemilihan daripada 50 anak tumbuhan in vitro menunjukkan LPA30, LPP28 dan LPL32 mempunyai kapasiti antioksidan tertinggi mengikut varieti. LPA30 mempunyai kapasiti antioksidan tertinggi (35.01±2.56 mg Trolox/g DW), diikuti oleh LPP28 (34.17±1.8 3mg Trolox/g DW) dan LPL32 (33.22±1.41 mg Trolox/g DW). LPA30 turut mempunyai nilai tertinggi untuk asai FRAP (5.84±0.06 mg Trolox/g DW) dan kandungan fenolik (8.32±0.22 mg asid galik/g DW) dan flavonoid (3.99±0.07 mg quercetin/g DW) berbanding LPP28 (4.71±0.11 mg Trolox/g DW, 6.77±0.22 mg asid galik/g DW, 3.54±0.02 mg quercetin/g DW) dan LPL32 (2.39±0.02 mg Trolox/g DW, 4.45±0.08 mg asid galik/g DW, 2.35±0.08 mg quercetin/g DW). Hasil kajian mendapati 1 mg/L NAA adalah rawatan terbaik untuk menghasilkan kandungan metabolit dan berat akar tertinggi dalam kultur akar LPA30, LPP28 dan LPL32. Gabungan 1 mg/L NAA dengan sitokinin (BAP dan KN; 0.1, 0.5, 1 mg/L) tidak menyumbang kepada peningkatan berat dan kandungan metabolit pada kultur akar. Selain itu, medium MS pada kekuatan penuh (1X) dan 3% (w/v) sukrosa menghasilkan berat akar dan kandungan metabolit tertinggi bagi kultur LPA30, LPP28 dan LPL32. Pengelisitan menggunakan kitosan dan asid salisilik tidak menyumbang kepada peningkatan berat akar dan kandungan metabolit akar LPA30. Namun begitu, pengelisitan kitosan dan asid salisilik berjaya meningkatkan penghasilan metabolit sekunder tetapi menghalang proliferasi akar LPP28 dan LPL32. Pengelisitan 10 mg/L kitosan pada akar LPP28 dan LPL32 telah meningkatkan kandungan fenolik masing-masing sebanyak 1.5 dan 2.2 kali ganda, dan flavonoid sebanyak 1.2 dan 1.9 kali ganda. Selain itu, pengelisitan 5 mg/L asid salisilik pada akar LPP28 dan LPL32 juga meningkatkan kandungan fenolik masing-masing sebanyak 1.9 dan 2.5 kali ganda, dan flavonoid sebanyak 1.5 dan 2.2 kali ganda. Analisis HPLC menunjukkan asid galik dan myricetin dikesan dalam akar adventitius dan anak tumbuhan in vitro, manakala quercetin hanya dijumpai pada anak tumbuhan in vitro. Terdapat korelasi positif di antara kandungan fenolik dan flavonoid dengan aktiviti antioksidan sampel L. pumila. Dalam kajian pengaruhan tunas pucuk, respon tertinggi untuk eksplan akar LPA30 direkodkan pada rawatan 3 mg/L TDZ (14.81±6.42%) manakala untuk LPP28 (40.74±6.42%) dan LPL32

(85.19±12.83%) pula diperolehi menggunakan rawatan 5 mg/L TDZ. Eksplan akar LPA30, LPP28 dan LPL32 yang telah menghasilkan tunas pucuk kemudiannya dipindahkan ke medium MS tanpa hormon untuk kajian awal regenerasi. Melalui kajian ini, LPA30, LPP28 dan LPL32 telah dipilih sebagai anak tumbuhan in vitro dengan kapasiti antioksidan tertinggi. Kultur akar L. pumila telah berjaya dibangunkan di dalam sistem kultur cecair goncang untuk penghasilan fenolik dan flavonoid. Kajian lanjut bagi menambahbaik protokol regenerasi pucuk daripada eksplan akar adventitius L. pumila boleh dijalankan pada masa akan datang.

Kata kunci: Labisia pumila, pemilihan in vitro, akar adventitius, pengaruhan tunas pucuk

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LIST OF ABBREVIATIONS AND SYMBOLS

±	Plus minus
%	Percentage
µM	Micromolar
µm	Micrometre
µg	Microgram
cm	Centimetre
g	Gram
L	Litre
M	Molar
mAu	Milli absorbance unit
mg	Milligram
mL	Millilitre
mm	Millimetre
mM	Millimolar
N	Normal
nm	Nanometre
rpm	Revolutions per minute
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
FW	Fresh weight
FRAP	Ferric reducing antioxidant power
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KN	Kinetin
LPA	*Labisia pumila* var. *alata*
LPL	*Labisia pumila* var. *lanceolata*
LPP	*Labisia pumila* var. *pumila*
MS	Murashige and Skoog
NAA	α-Naphtaleneacetic acid
NaOH	Sodium hydroxide
SEM	Scanning electron microscope
SPSS	Statistical Package for Social Science
TDZ	Thidiazuron
TPC	Total phenolics content
TFC	Total flavonoids content

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CHAPTER 1

INTRODUCTION

Labisia pumila or commonly known as Kacip Fatimah in Malaysia is a herbaceous plant widely used in folk medicine for facilitating childbirth and post-partum recovery (Bodeker, 2009). The phytochemical constituents of this herb have been well documented with phenolics and flavonoids being the main compounds (Norhanisah *et al.*, 2013). Several scientific studies reported that *L. pumila* possesses biological activities such as antioxidant (Norhaiza *et al.*, 2009; Karimi *et al.*, 2011), anti-carcinogenic (Pihie *et al.*, 2011), anti-microbial (Karimi *et al.*, 2011), antifungal and anti-inflammatory activities (Karimi *et al.*, 2013).

Considering the interesting pharmacological values that *L. pumila* has to offer, raw materials of this herb is highly demanded for commercial production. However, the propagation and growth rate of wild *L. pumila* is rather slow and time consuming (Mohd. Noh *et al.*, 2002; Jaafar *et al.*, 2009). Hence, a propagation system that can supply *L. pumila* continuously must be established to accommodate the demand of bioactive compounds synthesised by this herb.

Plant cell culture is an ideal biotechnological approach for secondary metabolites production as it produce continuous and reliable source of plant-based pharmaceutical products (Rao & Ravishankar, 2002; Yue *et al.*, 2016). Research to date has successfully produces high yielding cultures from various medicinal plants in either undifferentiated or differentiated cultures (Yue *et al.*, 2016). Undifferentiated cell suspension cultures lack stability and uniformity (Habibi *et al.*, 2017) which resulted in lower production of high value natural products (Yue *et al.*, 2016). In contrast, organ culture, especially adventitious root culture is more

favourable due to its fast growth and stable production of secondary metabolites (Murthy *et al.*, 2008; Habibi *et al.*, 2017).

Establishment of organ cultures that produce large amounts of biomass with increased accumulation of secondary metabolites is possible through specific strategies (Murthy *et al.*, 2014a). These includes the selection of high-yielding clones, optimisation of medium composition such as type of basal medium, carbon source and plant growth regulators; and physical factors such as temperature, medium pH, agitation and aeration. Other approaches such as elicitation, precursor feeding, permeabilisation and immobilisation could also assist with the accumulation of metabolites (Abouzid, 2014; Malik *et al.*, 2014; Murthy *et al.*, 2014a; Ali *et al.*, 2016; Yue *et al.,* 2016; Andrews & Robert, 2017).

Through optimisation of *in vitro* culture conditions of adventitious root culture, high product concentration and efficacy can be achieved from the continuous source of secondary metabolites of root cultures (Murthy & Praveen, 2012). This study will highlight some of the strategies undertaken to increase *L. pumila* adventitious root metabolites yield including selection of clones, optimisation of plant growth regulators, MS medium strength and carbon source; and also elicitation. Initiation of organ cultures began with selecting parent plants that showed higher contents of the desired secondary product for organ induction (Murthy *et al.*, 2014a). The selection of a specific organ for the induction of *in vitro* adventitious roots is essential as the accumulation of metabolites varies in different organs of the same species. Following selection of high performing organ lines, another key consideration is to establish optimum media and culture composition (Ochoa-Villarreal *et al.*, 2016). Typical modifications to the adventitious root culture medium include the addition of phytohormones (Wu *et al.*, 2006; Baque *et al.*, 2010a; Fazal *et al.*, 2014), modification of the salt strength (Baque *et al.*, 2010b; Li *et al.*, 2015; Deepthi & Satheeshkumar, 2017) and sugar concentration (Baque *et al.*, 2012; Yin *et al.*, 2013; Li *et al.*, 2015). In addition, metabolite production in organ cultures can be stimulated *in vitro* by adding elicitors into the culture medium as metabolites are produced by plants in response to the imposed stresses (Naik & Al-Khayri, 2016; Andrews & Robert, 2017).

Apart from producing secondary metabolites, adventitious root can also serve as a reliable micropropagation method in tissue culture especially when numerous small shoots arise rapidly from each explant, hence leading to high rate of propagation. Previous studies on shoot regeneration of *L. pumila* only focused on leaf and stem explants (Hartinie, 2007; Ling *et al.*, 2013; Ozayanna, 2015; Syafiqah *et al.*, 2016). No attempt was done to explore the potential of adventitious roots explants of *L. pumila* for shoot regeneration purpose.

Therefore, the present study has focused on the aforementioned strategies to produce bioactives from adventitious root cultures of *L. pumila* with antioxidative properties. In addition, the potential of adventitious root explants of *L. pumila* for producing new shoots will also be investigated. The objectives of the study are;

i) To select superior *in vitro* source materials from each variety of *L. pumila* (var. *alata*, var. *pumila* and var. *lanceolata*) for high antioxidative properties
ii) To evaluate the effects of exogenous hormones, MS medium strength, sugar and elicitors on the biomass and secondary metabolites production from adventitious roots of *L. pumila* selected clones
iii) To regenerate shoots from adventitious root explants of *L. pumila*

CHAPTER 2

LITERATURE REVIEW

2.1 *Labisia pumila* (Bl.) Fern. Vill

2.1.1 Origin, distribution and taxonomy of *Labisia pumila*

Labisia pumila (Bl.) Fern. Vill is herbaceous plant which grows wildly in the rain forest of Malaysia, Indochina, Thailand and Papua New Guinea (Sunarno, 2005). The distribution of *L. pumila* is shown in Figure 2.1. In Malaysia, this herb is usually known as Kacip Fatimah. Other local names of *L. pumila* include Selusoh Fatimah, Kacit Fatimah, Tadah Matahari and Mata Pelanduk Rimba (Sunarno, 2005; Jamal, 2006).

Figure 2.1 : Distribution of *L. pumila*

Source : Global Biodiversity Information Facility (GBIF) Secretariat (2016)

According to Sunarno (2005), there are eight varieties of *L. pumila* namely var. *alata*, var. *discoplacenta*, var. *gladiata*, var. *lanceolata*, var. *pumila*, var. *malintangensis*, var. *neriifolia* and var. *sessilifolia*. Among these eight varieties, only var. *alata,* var. *pumila* and var. *lanceolata* are well-known in Malaysia (Stone, 1990). These three varieties can be distinguished from each other via their petiole and leaf physical appearances (Sunarno, 2005).

The taxonomy of *L. pumila* is shown in Table 2.1. *Marantodes pumilum* (Blume) Kuntze is a heterotypic synonym of *L. pumila* that has been accepted by The Plant List (2013). This name was originally found in Post and Kuntze (1903) as accepted taxon in the genus Marantodes (family Primulaceae). Myrsinaceae and Primulaceae are two best known families in Ericales. The taxon limits of Myrsinaceae and Primulaceae have been substantially changed, therefore the limits of Primulaceae was extended based on numerous synapomorphies within the group (Mabberly, 2008; Bremer *et al*., 2009).

Table 2.1: Taxonomy of *L. pumila*

Taxonomy	
Domain	Eukaryota
Kingdom	Plantae
Phylum	Magnoliophyta
Class	Magnoliopsida
Order	Ericales
Family	Myrsinaceae
Genus	Labisia
Species	*Labisia pumila*

Source: Global Biodiversity Information Facility (GBIF) Secretariat (2016)