PROLIFERATION AND ELICITATION OF PROTOCORM-LIKE BODIES OF *Dendrobium* SABIN BLUE FOR *IN VITRO* PRODUCTION OF ANTHOCYANIN

by

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LIST OF ABBREVIATIONS

µl	Microlitre
µg/mL	Microgram per mililitre
µL	Microlitre
µmol m-2 s-1	Micromole per metre square per second
ºC	Degree Celcius
2iP	6-(γ,γ-Dimethylallylamino)purine
ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
DAMD	Directed amplification of minisatellite DNA
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
Fe	Iron
FAA	Formaldehyde - acetic acid - ethanol
FW	Fresh weight
g	Gram
g/L	Gram per liter
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
JSP	Jasad seperti protokom
lTCL	Longitudinal TCL
mg	Miligram
mg/L	Milligram per litre
MgCl2	Magnesium chloride

mL	Millilitre
mM	MiliMolar
mm	Millimeters
MS	Murashige and Skoog
NAA	Naphthaleneacetic acid
nm	Nanometre
PCR	Polymerase chain reaction
PGR	Plant growth regulator
PLBs	Protocorm-like bodies
SEM	Scanning electron microscope
SPSS	Statistical package for the social sciences
TBA	Tertiary-butyl alcohol
TBE	Tris-Borate-EDTA
TCL	Thin cell layer
tTCL	Transverse TCL
UV	Ultraviolet

PROLIFERASI DAN ELISITASI JASAD SEPERTI PROTOKOM *Dendrobium* SABIN BLUE UNTUK PENGHASILAN ANTOSAININ SECARA *IN VITRO*

ABSTRAK

Permintaan tinggi bunga kuntum, serta penghasilan metabolit sekunder dari *Dendrobium* orkid, menyebabkan terlalu banyak eksploitasi orkid dari habitat semula jadi. Penggunaan orkid hibrid secara *in vitro* adalah salah satu strategi yang baik untuk memenuhi permintaan. Potensi *Dendrobium* Sabin Blue (spesies hibrid antara *Dendrobium* Blue Angel dan *Dendrobium* Sanan Blue) untuk menghasilkan antosainin tidak pernah diterokai. Tujuan kajian ini adalah untuk meningkatkan pembiakan *in vitro* jasad seperti protokom (JSP) *Dendrobium* Sabin Blue dan meningkatkan kandungan antosainin dengan menggunakan elisitor. Untuk percambahan JSP, empat jenis hormon pertumbuhan tumbuhan (asid 1-Naphthaleneacetic, 6-Benzylaminopurine, kinetin dan picloram) dengan kepekatan yang berbeza telah diuji. Keputusan menunjukkan bahawa 10 µM NAA menghasilkan indeks pertumbuhan tertinggi JSP dengan kadar 10.947 ± 0.825 berbanding dengan kawalan 1.67 ± 0.111. Di samping itu, analisis histologi menunjukkan sel meristematik yang padat dan kehadiran butiran kanji yang membuktikan sel aktif membahagi. Di samping itu, teknik yang teliti telah digunakan untuk menggandakan JSP melalui kultur *in vitro* lapisan sel nipis (TCL). Selepas empat minggu kultur, keputusan menunjukkan bahawa TCL melintang (tTCL) menghasilkan bilangan JSP yang paling tinggi dengan sejumlah 59 JSP dihasilkan dari satu potong lapisan tipis berbanding TCL membujur (lTCL). tTCL juga menghasilkan jumlah pucuk yang paling tinggi dengan sebanyak 15 pucuk.

Pemerhatian histologi mengesahkan bahawa JSP muncul dari lapisan epidermis yang berhampiran atau di kawasan yang dipotong. Pengimbasan mikroskop elektron mendedahkan asal JSP yang baru terbentuk dari bahagian tepi JSP. Tekanan biotik dan abiotik adalah batasan untuk pertumbuhan dan penghasilan sistem kultur tisu. Elisitor yang digunakan untuk induksi antosianin adalah metil jasmonat, asid salisilik, dan melatonin. Berdasarkan keputusan yang dicapai, ketiga-tiga elisitor telah merangsang pengeluaran antosainin. Kandungan antosainin dalam JSP yang dirawat dengan asid salisilat meningkat sebanyak 39.6% pada kepekatan 25 μM tetapi menurun dalam jisim segar dan kering. Elisitasi dengan 10 μM MeJA kandungan antosainin bertambah baik dengan meningkat sebanyak 27.62% walaupun kadar penurunan massa segar dan kering dalam semua kepekatan MeJA. Melatonin pada kepekatan 10 μM menghasilkan peningkatan pengeluaran 20.4% kandungan anthocyanin dan meningkat dengan ketara dalam jisim JSP segar dan kering pada 0.5 μM. Walaupun kandungan antosainin tidak memberi kesan atau rendah, JSP *in vitro* kultur boleh dianggap sebagai strategik dan lebih ekonomik daripada menggunakan pokok induk yang memerlukan masa selama tiga tahun untuk mendapat penghasilan antosainin.

PROLIFERATION AND ELICITATION OF PROTOCORM-LIKE BODIES OF *Dendrobium* SABIN BLUE FOR *IN VITRO* PRODUCTION OF ANTHOCYANIN

ABSTRACT

High demands for *Dendrobium* cut flowers as well as the secondary metabolite products lead to the over-exploitation of the orchid from its natural habitat. The use of *in vitro* hybrid orchid is one of a prominent strategy to meet the demand. *Dendrobium* Sabin Blue (a hybrid species between *Dendrobium* Blue Angel and *Dendrobium* Sanan Blue) orchid was used in this study to produce anthocyanin. The aim of this study is to improve *in vitro* proliferation of protocorm-like bodies (PLBs) of *Dendrobium* Sabin Blue and enhancing anthocyanin content by using elicitors. For PLBs proliferation, four different types of plant growth regulators (1-Naphthaleneacetic acid, 6-Benzylaminopurine, kinetin and picloram) with different concentrations were tested. The results revealed that 10 μM of NAA produced the highest growth index of PLBs with 10.947 ± 0.825 as compared to the control with only 1.67 ± 0.111. In addition, histological analysis showed a dense meristematic cell and presence of starch grains which indicates the actively growing of cell. In addition, a delicate technique was utilized to further promote a rapid and mass propagation of PLBs through *in vitro* culture of thin cell layer (TCL). After four weeks of culture, results showed that transverse TCL (tTCL) produced the highest number of PLBs with a total of 59 PLBs were produced from a single thin layer cut as compared to longitudinal TCL (lTCL). The tTCL also produced significantly higher number of shoots with 15 shoots as the highest record. Histological observation confirmed that the PLBs emerged from the epidermal layer of near/on

the cut area. Scanning electron microscopy revealed the origin of newly formed PLB from the peripheral region of PLBs. Biotic and abiotic stress are the main limitation to plant growth and yield in tissue culture system. Elicitors used for the induction of anthocyanin were methyl jasmonate, salicylic acid, melatonin. All three elicitors have stimulated the production of anthocyanin. Anthocyanin content in PLBs treated with salicylic acid was notably increased by 39.60 % at concentration 25 µM but declined in fresh and dry mass. Elicitation with 10 µM of MeJA insignificantly improved anthocyanin content by 27.62 % despite significantly decreased in fresh and dry mass in all concentration of MeJA. Melatonin at concentration of 10 µM yield to 20.4% increment production of anthocyanin content and significantly increase in fresh and dry mass of PLBs at 0.5 µM. Despite the insignificant or low anthocyanin content achieved in elicited PLBs as compared to the mother plant, *in vitro* culture of PLBs can be considered as a more economic strategy than using the three-years old mother plant.

CHAPTER ONE

INTRODUCTION

1.1 General introduction

Dendrobium is the largest orchid genus in the family of Orchidaceae. It is widely distributed across the continents of Asia, Europe and Australia (Cheng et al., 2018) comprising of more than 1500 species worldwide (Xu et al., 2015). The enthusiastic demand for *Dendrobium* is due to their ornamental and aesthetic values as the flowers are naturally eye catching in terms of their colour's variation ranging from soothing to bright or dark purple colour (Abu et al., 2017).

Dendrobium flowers are flat, round and elongated in shape and mostly purple in colour (Mudalige et al., 2003). The rostellum of this genus is hollow yet swollen and when touched release opaque, glue-like liquid (Xiang et al., 2013). As a country known for its innovative exploitation of native natural resources, China has been largely utilizing *Dendrobium* as an alternative medicine. The dried roots of *Dendrobium* are processed into tonic drink to nourish the stomach, prevent cataract development and boost up body immunity (Bhattacharyya et al., 2016). The leaves extracts are used as ointment (Tsering et al., 2017). In India and Nepal, *Dendrobium* are used to treat various ailments such as diabetes, fractured bones and pain reliever (Singh & Duggal, 2009). Malaysia is one of the top exporting countries in floriculture products, made up 85% of the total tropical orchid trade market (Sheela, 2008).

1.2 Multiplication of protocorm-like bodies (PLBs)

The overexploitation from the continuous and uncontrolled collection of *Dendrobium* orchids from the wild has put this genus under the danger of extinction (Sheelavantmath et al., 2000). In addition, the low rates of natural regeneration of *Dendrobium* does not seem to offer a promising future for the plant sustenance (Zheng et al., 2012). These drawbacks have led to the development of *in vitro* cultures for fast mass propagation of desired and disease-free clones in a controlled condition (Pola et al., 2009).

The addition of plant growth regulators (PGR) has been reported to enhance multiplication and regeneration rate of protocorm-like bodies (PLBs) of *Dendrobium lowii*, *Candidum* and *Cymbidium and Phalaenopsis gigantea* sp. (Murdad et al., 2006; Zhao et al., 2008; Da Silva, 2014; Gansau et al., 2016). Plant growth regulators (PGR) function as chemical messengers to promote the growth rate or cell division in the plant. In *Dendrobium* studies, the most commonly used PGR to enhance the propagation are 1-Naphthaleneacetic acid, 6-Benzylaminopurine, indole-3-acetic acid, picloram, kinetin and thidiazuron, which function as plant hormone (Da Silva et al., 2015). According to Kozlowski & Pallardy (1997), plant responses towards PGR are not only dependent on the type of growth regulators and dosages applied but also on the plant species and their genotype, stage of plant development, and environmental conditions.

Due to the possible variation in the responses, the present study aimed to investigate the effects of PGR on the growth proliferation of *Dendrobium* Sabin Blue.

1.3 Production of secondary metabolites using elicitor

Secondary metabolites are phytochemical that are produced by plant as an adaptive mechanism to the environment. Their role as antibiotic, antifungal and antiviral properties help to protect plants from pathogens (Bourgaud et al., 2001). In recent years, the investigations of secondary metabolites produced by *Dendrobium* have been increased. Several studies have revealed that *Dendrobium* possess various secondary metabolites from the class alkaloids, terpenes, flavonoids, phenols and polysaccharides (Fan et al., 2001; Bulpitt, 2005; Xu et al., 2013). These compounds represent important source of active pharmaceuticals as possible anti-tumor, anti-oxidation or immunity boosting and many more (Pan et al., 2012; Tian et al., 2015).

Dendrobium specimens obtained in wild have shown low in concentrations and produce inconsistent production of secondary metabolite compounds (Jiang et al., 2014). Different strategies to improve the production of secondary metabolites have been studied extensively. One of them is by introducing elicitor, a compound that is known to induce stress in plant that triggers the production of secondary metabolite. Methyl jasmonate, salicylic acid and melatonin are elicitors that have been described to be successfully enhancing the production of anthocyanin, a secondary metabolite that is beneficial in pharmaceutical industries (Akula & Ravishankar, 2011).

However, there is no literature reported on the application of methyl jasmonate, salicylic acid and melatonin on protocorm-like bodies of *Dendrobium* species for production of anthocyanin.

1.4 Rationale of the study

Plant tissue culture is one of the promising ways to minimize the pressure on natural populations of medicinal orchids, thus ensuring continued existence of the plants. Therefore, this research was carried out on protocorm-like bodies (PLBs) to induced mass propagation of clones using various exogenous auxin and cytokinin. While PLBs have been useful in the micropropagation of *Dendrobium*, secondary metabolites have been overlooked as potential valuable sources. This research was carried out to establish a protocorm culture system to produce the desired compounds to overcome the inconsistent yield from cultivated *Dendrobium* orchids. To date, quantification of anthocyanins has not been reported in PLBs of *Dendrobium.*

1.5 Objectives

The objectives of this study were

i. To enhance the multiplication rate of protocorm-like bodies (PLBs) of *Dendrobium* Sabin Blue using plant growth regulators (PGRs) such as benzylaminopurine (BAP), 1- Naphthaleneacetic acid (NAA), picloram, and kinetin,

ii. To investigate different protocols of thin cell layer culture on the development of protocorm-like bodies of *Dendrobium* Sabin Blue,

iii. To study the effect of three abiotic elicitors (methyl jasmonate, salicylic acid, melatonin) on the yielded amount of anthocyanin content in protocorm-like bodies.

CHAPTER TWO

LITERATURE REVIEW

2.1 Orchidaceae

Orchidaceae is the second largest family after Asteraceae, consisting of 880 genera and more than 25,000 species globally (Niu et al., 2017). The term Orchids originated from a Greek word (órkhis) defined as testicle due to the shape of the twin tubers in some species (Ramírez et al., 2007). Orchids are often grown as an ornamental plant and popular for cut flower industry owing to the unique design and exquisite nature. Orchid flowers can last up to one month minimum, depending on their species. A number of orchid species tend to produce aromatic smell meanwhile there are counterparts which emit unpleasant smell or odourless. The size of orchid flowers may range from as small as an inch to more than a foot. Their incredible diversity in terms of shape, colours, and sizes on top of long shelf life made orchids a unique family.

Earlier orchids were predominantly found in Asia, Southern and Central America, northwest South America, and countries that lie along the Andes Mountains (Jukofsky, 2002) nowadays wet tropics around the world became their largest habitat. Orchids are able to grow in almost all climates but not in polar or arid regions although they exhibit adaptations to these surrounding (Chase, 2005). Orchids are pantropical yet endemic as they are distributed in specific habitats because of certain factor such as pollinator specialization, mycorrhizal specificity and low viability or germination rates of the seeds (Zhang et al., 2015). Thus, compared to plants from other families' orchids are extremely susceptible to habitat disturbance, which may limit their survival

rate.

2.2 The genus *Dendrobium*

Dendrobium is one of the largest genus of Orchidaceae comprised of 1500 - 2000 species (Cheng et al., 2018). The name *Dendrobium* stemmed from Greek words *dendron* and *bios* defined as tree and life indicating on the epiphytic nature of *Dendrobium* (da Silva et al., 2014).

Dendrobium is the most diverse and evolved genus distributed from Southeast Asia to New Guinea and Australia (Bhattacharyya et al., 2016). *Dendrobium* is one of the leading cut flower candidates due to the demand driven by variation in regard to colour, shape and appearance and longer shelf life characteristics. Furthermore, *Dendrobium* exhibits anticancer potentials, immune stimulatory effects and antioxidant activities (Luo et al., 2010). In recent years, scientists are interested to study medicinal plants traditionally used by old folks because of their healing abilities while being non-toxic (Johnson & Janakiraman, 2013).

2.2.1 Botany

Dendrobium is epiphytic and sympodial. *Dendrobium* propagate vegetatively via pseudobulb, hence, individual sympodials are connected on a single rhizome. *Dendrobiums* survive in a wide variety of habitats ranging from high altitudes 1400 - 1600 m of Himalayan Mountains to the dry climate of the Australian desert. *Dendrobium* flowers consist of three sepals and three petals whereby one of the latter is known as lips. The lips function as the landing site for pollinators meanwhile the size and shape may vary for each species. On average four to ten blooms per stem of mainly purple, yellow or blue colours which may stand for up to two months as cut

flowers are produced by the *Dendrobium* plants. High number of flowers per inflorescence and recurrent flowering makes *Dendrobium* the high-ranking ornamental orchid in the cut flower industry (Puchooa, 2004).

Dendrobium grows well at high altitude of 1400 to 1600 m mountainous area in a humid environment with mild temperature (Jukofsky, 2002). Due to broad geographical distribution in Asia, Australia and Europe, *Dendrobium* species have become highly diversified and produce high frequency of inter-specific hybrids with distinctive morphological characteristics (Wood, 2006). Indo-Asia and the Pacifics have one of the most diverse and taxonomically challenging orchid groups (Clements, 2003).

2.2.2 Ethnobotany

Recognised beyond a hobby, orchids are widely cultivated to cater 8% of the world ornamental and floriculture trade (Chugh et al., 2009). Apart from Singapore and Malaysia, Thailand for example tops among the Asian exporters to supply 50 million dollars worth cut flowers (Goh & Kavaljian, 1989). However, orchids are lesser known for medicinal property despite of the economic importance. According to Tsering et al. (2017), Himalayans emphasize on the economic value but ignore to advance on the phytochemical and pharmacological study on orchids. On the other hand, Bulpitt (2005) highlighted on the medicinal and flavouring properties of orchids. For example, *Vanilla planifolia*, is cultivated for the production of natural vanilla flavour and fragrance from the seed pods (Gallage & Møller, 2015). Bulpitt (2005) proclaimed the Chinese to be the first to describe on the medicinal properties of orchids. For instance, *Bletilla striata* is used traditionally to treat haemoptysis and

haemorrhage from gastric ulcer and to decrease the coagulation time which promotes the closure of the wound (He et al., 2017).

Dendrobium is a well-known horticultural plant and traditionally used as herbal medicine for the past thousands of years (Feng et al., 2014). Chinese folks were the first to identify the medicinal properties of *Dendrodium* and they have used 30 out of the 75 species of *Dendrobium* found in China as herbal medicine (Wang et al., 2011). *Dendrobium* mixture known as '*Shi-hu*' was derived from the stems of *D. chrysanthum, D. fimbriatum, D. loddigesii, D. nobile,* and *D. candidum* (Shiau et al., 2005) to be taken to replenish '*yin*' tonic for stomach nourishment, promoting secretion of body fluid and increase the immunity level of the body (Bao et al., 2001; Xu et al., 2006). Furthermore, according to Luo et al. (2010) the fluid extract from *Dendrobium*'s stem has been used to treat cataracts and digestive system disorders.

India is the habitat to more than 2000 species from 167 genera of orchids (Tsering et al., 2017) and Orchidaceae groups used extensively in Ayurvedic treatments. For example, *D. aphyllum* was used to treat wounds, earache, epilepsy, paralysis, abdominal pain, diabetes, and menstruation related issues (Akhter et al., 2017). An ayurvedic drug called 'Jivanti' which is inclusive of *D. macraei* (Singh & Duggal, 2009; Tsering et al., 2017) was used to cure fever, improve vision and to treat open wounds. Similarly, extract endemic *D. longicornu* of Northeast India is used to treat fever and coughs (Dohling et al., 2012). Alternatively, poultry industry adds pseudobulb of *Dendrobium* orchid to the feed as roughage to enhance milk production (Yonzone et al., 2012). Europeans consume the tuber of the orchids to treat men's reproductive problems such as to stimulate lust and helps in increasing men's fertility (Bulpitt, 2005). As described by Tsering et al. (2017) six different species of *D.*, namely *D. amoenum* Wall. ex Lindl., *D. crepidatum* Lindl. & Paxton, *D. densiflorum*

Lindl., *D. eriiflorum* Griff., *D. moschatum, D. transparens* Wall. ex Lindl. were used to treat fracture and dislocate of bones by using its pseudobulb.

2.2.3 Phytochemistry

Orchids are resource of phytochecmicals such as alkaloid, flavonoid, cyanogenic glycoside, carotenoids, polyphenols, bibenzyl derivatives, flavonoids, sesquiterpenes, and phenanthrenes (Johnson & Janakiraman, 2013; Bhattacharyya et al., 2016). Among them, alkaloids, polyphenols, flavonoid, and bibenzyl derivatives play significant role in age related diseases (Bhattacharyya et al., 2015).

Alkaloids are organic compounds consist of nitrogenous heterocyclic compounds and amino acid base. Alkaloids of *Dendrobium* are excellent antioxidants that fight against cancer and demonstrate neuroprotective activities (Ng et al., 2012). Dendrobine is one such alkaloids found in *Dendrobium* species (Hossain, 2009; Yang et al., 2018). Alkaloid dendrobine is a tertiary base with a methyl group and a lactone ring (Sut et al., 2017) and has been identified in *D. nobile* Lindl., *D. linawianum* Rchb.f. and *D. findlayanum* (Bullpit et al., 2007). Dendrobine exhibits weak anti-pyretic and analgesic effect (Bullpit et al., 2007) but improves immunity, slows down aging process and demonstrates anti-cancer activity. However, effects and dosage of dendrobine require further study.

Flavonoids are present in most of the *Dendrobium* orchids (Maridass et al., 2008) and responsible for the pigmentation of flowers. For example, anthocyanin and delphinidin compounds develop blue colour *Dendrobium* orchids (Harborne & Williams, 2000). Flavonoids are phenolics with 15-carbon skeleton consisting of two phenyl rings and a heterocyclic ring. They are ubiquitous in plant especially in

photosynthetic plant cells. Flavonoids play a major role in combating oxidative stress caused by reactive oxygen species (ROS) as they are excellent antioxidants that prevent free radicals' formations (Heim et al., 2002). Flavonoids suppress ROS generation by absorbing the high-energy UV-A and UV-B solar wavelengths to prevent stress responses in plants which lead to the increase ROS production (Agati et al., 2012). Flavonoids found in the chloroplast scavenge on singlet oxygen in preventing free radical formation to stabilize the outer membrane of the chloroplast (Yang et al., 2007; Pérez-Gregorio et al., 2011). Yang et al. (2007) confirmed stems of *D. aurantiacum* extract contain cismelilotoside, dihydromelilotoside, and trans-melilotoside which exhibit a potent antioxidant activity.

Polyphenols are another class of phytochemicals found in orchids. South Asian origin *D. moniliforme* (synonym *D. candidum*) is known to synthesize polyphenols (Paudel et al., 2018). Polyphenols received much attention due to its ability in the treatment of various degenerative and aging related disorders (Bhattacharyya et al., 2016). Loddigesiinols and crepidatuol polyphenols were isolated from the stems of *D. loddigesii* by the Chinese folks to treat gastrosis, fever and type 2 diabetes (Lu et al., 2014).

Bibenzyl is another active compound found in most of *Dendrobium* (Xu et al., 2013). This aromatic compound exhibited anti-tumor and anti-asthmatic properties. Two common bibenzyls that can be found in more than twenty species of *Dendrobium* are moscatilin and gigantol (Xu et al., 2010). Gigantol extracted from *D. draconis* exhibited significant cytotoxic activity against several cancer cell lines (Charoenrungruang et al., 2014). The diverse forms of bibenzyl are divided based on the number and position of side chains which are mainly comprised of hydroxyl and methoxyl group.

2.2.4 *Dendrobium* Sabin Blue

Dendrobium Sabin Blue (Figure 2.1) is a hybrid species crossed between *Dendrobium* Blue Angel and *Dendrobium* Sanan Blue. This hybrid has a tall stalk with compact arrangement of round type purplish blue flowers. It has long lasting beautiful flowers which can stay bloomed up to one month.

Anthocyanin in *Dendrobium* is being investigated due to its involvement in pigmentation of flowers and plant. Anthocyanins are flavonoid glycosides accumulating in plant vacuole to emit red, purple or blue colour to many fruits, vegetables and flowers (Mudalige-Jayawickrama et al., 2005). There are numerous studies regarding the benefits of anthocyanin such as to prevent diabetic retinopathy, anti-inflammatory, anti-viral, anti-mutagenic, anti-microbial, anti-carcinogenic, and anti-diabetic effects (Ghosh & Konishi, 2007). According to Kuehnle et al. (1997), the major anthocyanin in *Dendrobium* genus is 3-hydroxylate cyanidin (reddish to magenta) while pelargonidin (dark red to scarlet) and peonidin (purplish red) tend to present low in some species. For yellow and green *Dendrobium* flowers, it may due to the combination of anthocyanins, carotenoids, and chlorophylls pigmentation in the plant genotype (Kuehnle et al., 1997). The present study investigated the presence of anthocyanin in the PLBs of *Dendrobium* Sabin Blue.

Figure 2.1: *Dendrobium* Sabin Blue flower. Scale bar represents 2 cm

2.3 Plant tissue culture system

2.3.1 The importance of *in vitro* orchids

Orchidaceae once subjected to extinction due to the mass collection from its wild habitat which exceed its natural regeneration (Rojas-Mèndez et al., 2017) and face decline of species due to excessive logging (Sheelavantmath et al., 2000). The combination of high commercial value, increasing demand in potted and cut flowers industries, and its valuable medicinal properties has led to the adulteration of many orchid products. Orchids have a long reproduction cycles which might consume several years and yet having slow seed maturation (Yang et al., 1999). Cultivated *Dendrobium* in wild habitat has been reported to produce inconsistent yield of compounds subjected to the cultivar, harvesting season and age of plant (Jiang et al., 2014). Collection from the wild *Dendrobium* had been listed in Convention on International Trade in Endangered Species of Wild Fauna and Flora in which limit its cross-border trades.

In nature, orchid's seed will germinate only with the cooperation of the mycorrhizal fungi which helps in providing minerals, water vitamins and carbohydrates for the developing embryo. The earliest person to germinate orchid seeds *in vitro* was Lewis Knudson in year 1922 in which he sowed the seeds on sterile nutrient medium amended with sucrose (Kauth et al., 2008). He successfully germinated several epiphytic orchids from the seeds. Thus, plant tissue culture technique is a brilliant way to germinate the orchid seeds to ensure the continuity and survival of the orchid's species.

Plant tissue culture may offer a large quantity of healthy, diseases and virus free plantlets production since it has been cultured under controlled condition. This

controlled environment provides a suitable condition without seasonal, weather or geographical constraints for growth and multiplication of plant tissue (Akin-Idowu et al., 2009; Smith, 2013). Micropropagation via plant tissue culture technique is widely used to produce desired clones in large scale. Moreover *in vitro* cultures of orchids could be reservoirs to induce secondary metabolite compounds production which can be used as medicines, aromatic oils, and coloring or flavoring agents (Murthy et al., 2014). Cell suspension culture was developed to provide a source of plant cells which were ideally suited for molecular and biochemical studies (Gamborg, 2002). More than 80 enzymes of alkaloid biosynthesis were developed by using cell suspension culture (Smith, 2013). The use of precursor, elicitor and inhibitor either from biotic or abiotic compounds may help in inducing the secondary metabolite of plant. With plant tissue culture, desired and important plants traits can be cloned and made.

2.3.2 Protocorm-like bodies (PLBs)

The term PLB was first coined by Morel (1960) and it was described as a structure that resemble protocorms emerged from orchid tissue explants and/or callus under *in vitro* conditions (da Silva & Tanaka, 2006). Protocorms can be obtained from the orchid seeds which appear during the early stage of germination. Protocorm-like body is the organ that resembles protocorm which is having the same function and morphology as protocorm (Teixeira & Silva, 2014).

PLBs are used for the micropropagation of orchids as morphologically they are classified as somatic embryo (Ishii et al., 1998; Huan et al., 2004). Zhao et al. (2008) found that the early formation of PLBs is achieved by somatic embryogenesis. Later, Lee et al. (2013) confirmed that PLBs were somatic embryos through the study

histochemical and immunofluorescence. Histological and histochemical studies by Julkifle et al. (2012) revealed that globular masses emerged from the epidermal cell layers of the PLBs generate secondary or tertiary PLBs. PLBs could be directly induced from leaves, shoot tips, stem nodes, flower stalk and root tips (Zhao et al., 2008).

2.4 Thin cell layer (TCL) culture

Thin cell layer (TCL) was coined by Tran Thanh Van in 1973 by culturing thin sections of explants with variable proportions of length and diameter (Tripathi et al., 2018). In Tran Thanh Van's study, TCL was demonstrated by excising thin and transverse slices of tissues from pedicels of flowering *Nicotiana tabacum* (tobacco). Various tissues or plant parts can be sliced either longitudinal TCL (lTCL) or transversal TCL (tTCL), ranging from 1-5 mm in thickness. In lTCL, there is only one type of cell or tissue present such as monolayer of epidermal cell. Whereas tTCL produces multiple cells or tissue types such as epidermal, cortical, cambium, perivascular and medullar as well as parenchyma cells (da Silva, 2003).

Notably, TCL culture is touted as an efficient alternative to obtain rapid plant regeneration with a high frequency (Van et al., 1999). The successful development and application of TCL in a span of almost 40 years has contributed to the advancement of clonal micropropagation of orchids. A considerable amount of literature has been published on PLBs formations from TCL explants of orchid cultivars or hybrids, namely *Aranda*, *Coelogyne cristata*, *Cymbidium* spp., *Dendrobium* spp., *Doritaenopsis*, *Paphiopedilum*, *Renanthera*, *Rhynchostylis*, *Spathoglottis*, and *Xenikophyton* (da Silva, 2013). According to da Silva (2013), there are three protocols

for TCL on PLBs. The first protocol involves culture of a whole single PLBs in media. Second protocol outlines the culturing of half-cut PLBs by removing apical meristem and basal part. Meanwhile, the final protocol requires a horizontal cut of the PLBs producing thin layers which are cultured on media. TCL of PLBs is an advantage because it leads to optimal induction of several centres of meristematic activity on the PLB slices similar to the protocorm.

TCL culture has been shown to be more efficient due to rapid regeneration of plantlets compared to other conventional *in vitro* cultures of some orchid species such as *Dendrobium candidum*, *Rhynchostylis gigantea* and *Aranda* Deborah, including other plant species namely *Lilium* spp., *Sorghum bicolor* and *Heliconia psittacorum* (Pereira Gomes et al., 2015). TCL of actively growing tissues such as shoots, stem nodes or PLBs of orchid has been successfully cultured for plantlet multiplication and regeneration to meet commercial demands. Furthermore, TCL helps in direct diffusion of nutrients and growth regulator between explant and supplemented media.

Nayak et al. (2002) highlighted on the advantage of TCL as cost efficient protocol because it only utilizes a very small explant as the starting material and the reduction of time interval required producing a large number of regenerants. Evidently, more than 80,000 plantlets could be produced in a year by using TCL method as compared to about 11 000 plantlets produced by the conventional shoot tip method (Lakshmanan et al., 1995). As for *Cymbidium* Sleeping Nymph orchid, tTCL can overcome the slow growth of hybrid PLBs in which secondary PLBs were fully formed within 30 days of culture (Vyas et al., 2010). TCL employed on *Panax ginseng* seedlings produced fast somatic embryos at week 6 with high percentage of embryogenesis (62 %) (Ahn et al., 1996).

2.5 Elicitation for enhancement of secondary metabolite

2.5.1 Relationship between elicitor and secondary metabolite

Secondary metabolites are present in small amount which is usually less than 1 % of dried weight and is dependent on its physiological and developmental stage (Namdeo, 2007). Secondary metabolites do not influence the growth, development or reproduction of a plant. However, interaction of plant with the environment is influenced by the presence of secondary metabolites as the latter interferes with colouring, flavouring, and scent (Verpoorte et al., 2002). Furthermore, secondary metabolites are of pharmaceutical interest due to the medicinal properties of the natural products which have been used as remedies in traditional medicine. For example, bio-production of taxol identified in *Taxus* trees was approved by the Food and Drug administration (FDA) for the treatment of ovarian and breast cancer (Angelova et al., 2006).

Elicitor is a chemical substance introduced in small concentrations to *in vitro* living cell system to initiate or improve the biosynthesis of specific compounds (Namdeo, 2007). Most secondary metabolites are associated with defence against pathogen or environmental factors. Elicitors applied to *in vitro* cultures will mimic such biotic stress to activate an array of mechanism producing secondary metabolites (Zhong, 2002) including the production of phytoalexins (Angelova et al., 2006). Secondary metabolites are biosynthesized by various mechanisms catalysed by enzymatic reactions using simple building blocks which will come in contact with plant cell receptor to overcome stress causing agents. Correspondingly, elicitor will bind to the active site of the receptor of plant cell membrane to trigger stress alert

(Angelova et al., 2006) leading to an array of biosynthesis pathway to secrete secondary metabolites to form defence.

2.5.2 Biotic elicitors

Plants produce a wide variety of secondary metabolites in response towards their environment and to withstand various abiotic and biotic elicitors (Chodisetti et al., 2015). Biotic elicitors are comprised of biological origin including cell wall-based polysaccharides such as chitin, pectin and cellulose or microorganisms (Naik & Al-Khayri, 2016) to induce the plant defence response. Pathogenic microorganisms are effective agents to improve the production of secondary metabolites of plant cell cultures (Xu et al., 2011). For instance, Satdive et al. (2007) found that *Penicillium fellutanum* and *Aspergillus tenuis* led to a 5–fold increase of the production of azadirachtin in *Azadirachta indica* hairy root cultures.

2.5.3 Abiotic elicitors

Abiotic elicitors can be derived from substances that are of nonbiological origin such as physical and chemical factors. Physical factors such as UV radiation, osmotic stress, salinity, drought and thermal stress stimulate the biosynthesis of secondary metabolite. Sandermann et al. (1998) reported that the two days exposure of ambient ozone to tobacco Bel W3 showed a 4-fold increases of β-1,3-glucanase activity. Heavy metal is one of the chemical elicitors used in agrotechnology and bioaccumulation (Cai et al., 2013). Similarly, silver nitrate and cadmium chloride have shown to elicit the production of alkaloids and tanshinone in hairy root cultures of

Brugmansia candida and root culture of *Perovskia abrotanoides*, respectively (Sandermann et al., 1998).

Methyl jasmonate (MeJA) is a volatile organic compound used to regulate plant development and response towards environmental stress thus affecting biochemical and physiological reactions in plant (Sivanandhan et al., 2013). MeJA have been proposed to be key signal compound in response towards stress (wounding, environmental stress, or pathogen) leading to the accumulation of various secondary metabolites (Ogata et al., 2004). Patil et al. (2014) reported that addition of MeJA into *Taxus* cell suspension culture accumulate high concentration of paclixatel. Kim et al. (2005) reported increase in secondary metabolite upon MeJA elicitation was accompanied by a concomitant decrease in cell growth. The finding is consistent with past finding by Sivakumar & Paek (2005) and Thiruvengadam et al. (2016). To reduce the sensitivity of plant towards MeJA by excessive subculture of cell were suggested. However excess elicitation in every cycle of long-term cell suspension culture is not advisable (Sanchez-Sampedro et al., 2009).

Salicylic acid is also one of the stress-signalling molecules that has a role in influencing plant resistance to pathogens and other stress factors. Study conducted by Khosroushahi et al. (2006) showed that salicylic acid increased the production of taxol in cell suspension culture of *T. baccata* L. Another study by Sarrou et al. (2015) reported that 1 mM of salicylic acid increased the total phenolic and flavonoid content of bitter orange leaves, as well as their antioxidant activity. Wang et al. (2007) suggested the concentration of salicylic acid at 20 mg/L become toxic to the cell culture of *T. chinensis*. However, Sakhanokho et al. (2009) did not observe any toxic effect or necrosis at high levels in *Hedychium bousigonianum* (14 and 21 mg/L). Thus,

Ahmadi et al. (2014) concluded that the exact toxic level of SA may be genotype-dependant.

Melatonin (MEL) is one of important growth regulators and effective antioxidant which directly scavenge toxic free radicals, protect enzymes and protein from oxidative stress and minimize the generation of toxic free radical. This indoleamine act as plant growth regulator with a similar role to that of indole-3-acetic acid (IAA), promoting rooting, vegetative growth and the differentiation of cells, tissues and organs. Coskun et al. (2019) identified 200 μM MEL increased the amount of rosmarinic acid on *Rosmarinus officinalis* L. callus culture.

2.6 Microscopy analyses

2.6.1 Histology analysis

Histology is the study of microscopic anatomy of cells, tissues and organs in plants and animals through staining, blocking and sectioning process prior to examining the sample via light microscope. Histological study will allow the understanding of the cellular processes and provide clues for the proposal of hypotheses for further experimentation (Yeung, 1999). Structural analysis is important to study the cell arrangement or any morphogenetic changes. According to Wicart et al. (1984), histological analysis of *Cyclamen persicum* callus culture proved on the differentiation to structures such as shoot-buds, roots, unipolar tubers, bipolar tubers and embryo. Varieties of histological methods have contributed to the understanding of *in vitro* culture systems to trace any changes caused by the experimental variables.

The first step of histological analysis is fixation whereby samples are maintained in a most possible natural state to allow subsequent procedures. Fixation

prevents cells or tissues from decaying due to post mortem changes such as autolysis or putrefaction (Ganjali & Ganjali, 2013). There are five major fixatives which are grouped according to the mechanism of actions such as aldehydes, mercurial, alcohol, oxidizing agents and picric acid. Plants are preferably fixed in FAA (Formaldehyde-Acetic acid) (Feder & O'brien, 1968), an organic compound that can easily penetrate biological tissues to form cross-linking with the proteins, primarily the residues of the basic amino acid lysine to allow preservation (Culling et al., 2014).

Next is the dehydration process which allows removal of fixative and water from the explant tissues to be replaced by dehydrating agent. Fixed samples cannot be directly infiltrated with paraffin, thus dehydration has to be done with a series of gradually increasing concentrations of alcohol, starting from 50, 70, 80, 90, 95 to 100 %. According to Culling et al. (2014), duration of dehydration depends on the size and types of the samples. For example, thick samples will be dehydrated from 24 to 48 hours, while for delicate samples the time is advised to be reduced to 2 to 4 hours. During the clearance stage, alcohol will be replaced by an intermediate clearing solution which is miscible with both alcohol and embedding medium (paraffin). Xylene is the most commonly used clearing agent (Culling et al., 2014) because it removes alcohol rapidly, renders tissues transparent, and assists in paraffin infiltration (Alwahaibi et al., 2018).

Next, xylene in the tissues will be replaced by infiltration of molten paraffin wax. A good wax should embed the sample the fastest, highly soluble in xylene, compatible with the infiltrated sample and microtome-friendly. Paraffin wax is chosen because it has low melting point at approximately 37 °C and easily solidifies at room temperature. According to Bancroft & Gamble (2008), low melting point and easily solidifying properties of wax promote a good ribboning during microtome cutting.

Embedding medium must fill the spaces between tissue and external surface to ensure full support to the specimen during sectioning.

Specific dyes are used to stain important features of the sample as well as to enhance tissue contrast for a clear understanding. Safranin and fast green are extensively used by researchers compared to any other stain (Johansen, 1940). For anatomical work, safranin is performs best in staining nuclei, lignified, suberized, or cutinized elements of plant vascular system which appeared as bright red colour (Chamberlain, 1905). Safranin is a cationic dye that binds specifically and stoichiometrically to polyanions as an orthochromatic dye (Martin et al., 1999). The safranin will be counterstained by fast green which appears as brilliant green in cytoplasm and cellulosic cell walls.

2.6.2 Scanning electron microscopy

Scanning electron microscopy (SEM) is a technique used to examine the surface of a sample by focussing beam of electrons. SEM provides an image with a great resolution and depth of field, with a three-dimensional (3D) quality that offers a visual perspective familiar to most users (Suzuki, 2002).

To withstand the vacuum condition and high energy beam of electrons while viewing, samples need to be fixed through several steps. According to Damsky et al. (1969), stabilization of the macromolecular structure to harden the sample is achieved by chemical crosslinking of proteins using aldehyde and osmium tetroxide (OsO4) to fix lipids. OsO4 is excellent in staining lipids of membranous structures and vesicles, while glutaraldehyde provides a good preservation of cell surface topography for SEM (Shelton & Mowczko, 1978). Samples must be electrically conductive and electrically

grounded before viewing under EM to prevent the accumulation of electrostatic charge. Thus, it must be coated with a conductive material to prevent build-up of high voltage charge on the specimen and minimize the accumulation of negative charge from the electron beam (Drobne et al., 2005). Samples are coated with an ultrathin coating (20 – 30 nm) of electrically conductive material (gold, platinum or high purity silver) using low-vacuum sputter coater or by high-vacuum evaporation.

2.7 Biochemical analyses

2.7.1 Anthocyanin

As mentioned earlier, anthocyanins are responsible for the plant pigmentation. Identification of anthocyanin is challenging due to the complex chemical structure and lack of reference compounds for comparison. Extraction and quantification of anthocyanin among *in vitro* cultures have been widely reported. Anthocyanin is extracted using 1% (v/v) acidified methanol at 4°C for 12 to 24 hours, followed by centrifugation at 1000g for 10 minutes (Simões et al., 2009). The extracts will be filtered with 0.45 μm membrane filter prior to quantification of anthocyanin.

There are various procedures available to identify and quantify anthocyanins. One of the most preferred methods is via high performance liquid chromatography (HPLC) which can separate individual anthocyanins according to their polarity and different elution time (Lee et al., 2008). HPLC generates peaks that can be identified by comparing retention time with reference compounds and matching their mass spectrum. Individual anthocyanins can be quantified using an external standard by calculating the peak area at a certain wavelength. Total anthocyanin content can be calculated using extinction coefficient of the compound of interest. In particular,

Abdel-Aal et al. (2006) used extinction coefficient of cyanidin-3-glucoside in the determination of total anthocyanin in wheat extracts and Lao & Giusti (2016) used the average extinction coefficient of cranberry anthocyanins to measure anthocyanin content in purple corn. Another simpler method used to measure the anthocyanin is by determining the optical density at 530 nm using UV/ visible spectrophotometer and compute colour value index (CV) for quantification. This approach of quantification has been used to determine anthocyanin content in *Phaseolus vulgaris* L. (Takeoka et al., 1997), sweet potato (Yoshinaga et al., 1999), grapevine leaf and fruit skin (Kennedy et al., 2002).

2.7.2 Chlorophyll and carotenoid

Chlorophyll is the green pigment present in green plants and in cyanobacteria which helps in absorption of light for photosynthesis. There are two types of pigments, named chlorophyll *a* and chlorophyll *b* which are involved in conversion of light energy to chemical energy. Chlorophyll *a* is the main photosynthetic pigment of aerobic organisms which absorbs energy from wavelengths of violet-blue and orange-red light to allow photosynthesis to take place (Ritchie, 2006). Chlorophyll *b* acts indirectly in photosynthesis by transferring the light captured by chlorophyll *a*. Stress caused by abiotic factors may affect the photosynthetic activities in the plant leaves thus altering the chlorophyll *a* fluorescence kinetics (Oukarroum et al., 2007).

Carotenoids are the accessory light-harvesting pigments which play two key roles in plants and algae in which they trap light energy for photosynthesis and protect chlorophyll from photodamage and harmful environmental factors (Hirschberg, 2001). Carotenoid plays an important role in quenching singlet molecular