DEVELOPMENT OF AN EFFICIENT PROTOCOL FOR THE PRODUCTION OF *Phalaenopsis* HYBRID ORCHID PLANTLETS

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by

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LIST OF ACRONYMS AND ABBREVIATIONS

PLBs	Protocorm-like bodies
MS	Murashige and Skoog
KC	Knudson C
TCLs	Thin cell layers
tTCL	Transverse thin cell layer
PGRs	Plant growth regulators
BAP	Benzylaminopurine
NAA	Naphthaleneacetic acid
IBA	Indole-3-butyric acid
2,4-D	2,4-Dichlorophenoxyacetic acid
LED	Light emitting diode
DAMD	Directed amplification minisatellite DNA
SCoT	Start Codon Targeted
GCMS	Gas chromatography-mass spectrometry
GCF	Growth correction factor
ROS	Reactive oxygen species
PCR	Polymerase chain reaction
PAR	Photosynthetic active reaction
ANOVA	Analysis of variance
SE	Standard error
SI	Similarity index
1˚ PLB	Primary protocorm-like bodies
2˚ PLB	Secondary protocorm-like bodies
w/v	Weight over volume
v/v	Volume over volume
SPD	Spectral power distribution

PEMBANGUNAN PROTOKOL YANG BEREFISIEN UNTUK PENGHASILAN ANAK POKOK HIBRID *Phalaenopsis*

ABSTRAK

Sebagai tumbuhan monopodial, *Phalaenopsis* secara tradisinya mempropagasi melalui kaedah pemotongan atau pembahagian di luar pucuk. Namun, kaedah tersebut menghasilkan kadar pertumbuhan yang rendah dan kurang berkesan untuk pengeluaran berskala besar. Mikropropagasi merupakan teknologi alternatif yang berharga untuk pengeluaran berskala besar dalam tempoh yang singkat bagi tumbuhan orkid. Kajian ini bertujuan untuk meningkatkan pertumbuhan jasad seperti protokorm (JSP) *Phalaenopsis* Fuller's Light × *Phalaenopsis* Purple Heart melalui teknik mikropropagasi. Kaedah Teknik sel lapisan nipis (TCL) menjana kadar pembentukan neo-PLB 2.16 kali lebih tinggi berbanding dengan eksplan separuh PLB konvensional. Komposisi medium MS mengandungi sukrosa 3%, ekstrak pisang 2%, dan air kelapa 15% telah meningkatkan jisim segar JSP (2.499 g) secara ketara setelah 4 minggu. Pertumbuhan pucuk dan akar dari JSP *Phalaenopsis* telah dicapai dengan penambahan 1.0 mg/L BAP dan NAA mg/L. Penggabungan 1.5 mg / L glutamin menunjukkan pertumbuhan pucuk sebanyak 70% manakala 1.5 mg / L arginine meningkatkan pembentukan akar sebanyak 87%, di mana kultur tersebut tumbuh menjadi plantlet yang lengkap. Anak pokok *Phalaenopsis* berjaya diaklimatasikan dalam campuran substrat tanah sphagnum dan arang dengan kadar kemandirian 66.67% setelah 9 bulan. Hasil korelasi pearson menunjukkan hubungan yang signifikan antara pigmen fotosintetik dengan pucuk dan akar

orkid *Phalaenopsis*. Secara anatomi, orkid *Phalaenopsis* mempunyai lebih banyak butiran kanji dan pembentukan embrio globular sekunder dapat dilihat melalui mikroskop elektron. Plantlet orkid yang dijana semula telah dinilai dengan penanda DNA seperti Directed Amplified of Minisatellite DNA (DAMD) dan Start Codon Targeted marker (SCoT), yang mengenalpatikan peratusan persamaan genetik sebanyak 87% dan 96% masing-masing. Penemuan sebatian bioaktif seperti 2-propenamide dan 4-Methyl-2,5-dimethoxybenzaldehyde telah dikesan melalui kromatografi gas-spektrometri jisim (GCMS). Sebatian tersebut mempunyai ciri-ciri perubatan seperti sifat antioksidan, perantaraan farmaseutikal, antispasmodik, anti-asma dan agen antitusif di orkid *Phalaenopsis*. Gabungan diod pemancar cahaya (DPC) dan elicitor di medium kultur menggalakkan pertumbuhan JSP dan meningkatkan kandungan metabolit sekunder di JSP. Keseluruhan kandungan flavonoid dan fenolik dicatatkan tertinggi dengan penggunaan pepton dan kitosan di bawah spektrum DPC biru: merah (20.10 µmol/s). DPC biru (15.7 µmol/s) meningkatkan jumlah aktiviti antioksidan kultur JSP. Penanda DNA SCoT menunjukkan persamaan genetik sebanyak 82.64%. Kajian ini menunjukkan JSP *Phalaenopsis* regenerasi dicapai dengan komposisi nutrien yang optimum dengan variasi somaklonal yang minimum. Selain itu, DPC dan elisitor juga bertindak sebagai sumber yang sangat baik dalam merangsang pengumpulan metabolit sekunder dalam kalangan JSP kultur *in vitro.*

DEVELOPMENT OF AN EFFICIENT *Phalaenopsis* HYBRID ORCHID PLANTLETS PRODUCTION

ABSTRACT

As a monopodial plant, *Phalaenopsis* is traditionally propagated by off-shoot cutting or division. However, this resulted in a low multiplication rate and hinders the growth of the mother plant and therefore inefficient for large-scale production. Micropropagation has provided a valuable alternative method for the large-scale production of identical orchid plantlets within a short period. The study aimed to improve the proliferation of protocorm-like bodies (PLBs) of *Phalaenopsis* Fuller's Light × *Phalaenopsis* Purple Heart through the micropropagation method. The thin cell layer culture (TCL) approach generated a 2.16 times higher neo-PLB formation rate than the conventional half-PLB explants. The medium composition of full MS medium supplemented with 3% sucrose, 2% banana extract, and 15% coconutwater significantly produced the highest PLB fresh weight (2.499 g) after 4 weeks of culture. The *Phalaenopsis* PLBs further developed into shoot and root using 1.0 mg/L BAP and NAA mg/L. The incorporation of 1.5 mg/L of glutamine resulted shoot formation with 70% while 1.5 mg/L of arginine increased root formation by 87%, wherein the cultures were differentiated into complete plantlets. The *Phalaenopsis* plantlets were successfully acclimatized in the sphagnum and charcoal soil substrate mixture with a survival rate of 66.67%. The Pearson's coefficient results displayed the significant relationship between shoot, root length with photosynthetic pigments. Anatomically, more accumulated starch grains and secondary globular embryos formation were found in PLBs under electron microscopy. Regenerated orchid plantlets were genetically evaluated

using Directed Amplified of Minisatellite DNA (DAMD) dan Start Codon Targeted marker(SCoT), which identified a genetic similarity percentage of 87 % and 96 % respectively. The presence of bioactive compounds like 2-propenamide and 4-Methyl-2,5-dimethoxybenzaldehyde were detected by gas chromatography-mass spectrometry (GCMS). These compounds carry medicinal properties like antioxidant properties, pharmaceutical intermediate, antispasmodics, and anti-asthmatics in *Phalaenopsis* orchid. The combination of light-emitting diode (LED) and elicitors in the culture medium aids in promoting the growth and development of *in vitro* cultures with high secondary metabolite content in PLBs. The total flavonoid and phenolic content were recorded the highest in peptone and chitosan elicitors under blue: red (20.10 µmol/s) LED spectrum. The blue LEDs (15.7 µmol/s s) improved the total antioxidant activity of the PLB cultures. SCoT DNA markers gave rise to genetic similarities of 82.64%. The yield of regenerated plantlets successfully achieved with optimal nutrient medium composition with minimal effects on somaclonal variations. Moreover, LEDs and elicitorsalso act as an excellent source in stimulating secondary metabolites accumulation among the *in vitro* PLB cultures.

CHAPTER 1

INTRODUCTION

The Orchidaceae is the most diverse and widespread flowering plant family, including 30 000 different species, with approximately 100 new species being introduced yearly (Hinsley et al., 2018; Zhou et al., 2021). Orchids are the best-selling plant in the global potted plant trade, which comprised 10% of all the traded cut flower (De, 2015; Hinsley et al., 2018). The countries with the largest potted and cut flowers are manufacturing in countries like Thailand, the Netherlands, Japan, and Taiwan (Hanks, 2015). The domestic and regional trade of the cultivated orchids is also important; for example, half of the orchids produced in Thailand were traded in the national market in the year 2014 (Thammasiri, 2014).

In Malaysia, the floriculture industry is growing and flourishing gradually. Malaysian orchid exports grew to US$ 2,638.24 and the import price for orchids in 2019 was US$3.37 per kg (Ariff et al., 2021). An estimated RM 150 million of export value is expected in Malaysia every year, where most orchids are *Dendrobium* (Ahmad et al., 2010). The most significant production of *Phalaenopsis* orchid is in Selangor, and Genting Highland Pahang. *Phalaenopsis* orchid has the second most economic demand as potted plants and cut flowers in the global market (Tokuhara and Mii, 2003; Gow et al., 2010). The breeding technology of *the Phalaenopsis* orchid is an essential factor in improving the vitality of the plants. Plant tissue culture of *Phalaenopsis* is desirable to overcome inconsistent propagation through seedling propagation. Various types of explant parts of *Phalaenopsis* orchid were used to establish tissue culture propagation using axillary bud, internodal segments, meristems (Park et al., 2002), shoot, and roots (Park et al., 2003).

Generally, the production of orchids is restricted by several factors. For instance, orchid seeds are naturally tiny and lack the endoplasm to propagate conventionally. Therefore, the rate of seed germination is significantly affected. Additionally, asymbiotic association must be formed between orchid seeds and mycorrhizal fungi to improve the germination rate and the symbiotic relationship is scarce. Alternatively, vegetative propagation of orchids can be done by explant excising and isolating clumps (Utami et al., 2017). These approaches have various difficulties since it takes quite a while to establish suitable tillers. The excision process increases the probability of pathogenic infections as the wounded surface area is exposed to the peripheral condition. Furthermore, the over-harvesting or overuse of wild orchids endangers their populations as their growth is restricted in nature, owing to their specific needs to be fully grown into complete plants (Hinsley et al., 2018).

Transverse thin cell layer (tTCL) *in vitro* micropropagation, which uses a very small proportion of cells or tissues, has now emerged as a potent technique in plant tissue differentiation and regeneration (Bhattacharyya et al., 2018). Light has a significant impact on plant growth and development, depending on intensity, wavelength, and time duration. Because red and blue light spectrums are the primary energy sources for photosynthetic carbon dioxide assimilation in plants, they have the greatest impact on plant growth (Billore et al., 2019). The cell culture method also provides an approach to depress cellular activities that favour secondary metabolite production, thus increasing yield (Ahmad et al., 2013). As an alternative, plant tissue culture increases the production of secondary metabolites in plantlets and protects

wild orchids. Plant tissue culture is a sustainable way of producing different biochemical products for many applications such as medicines, colouring, and perfumes (Hussain et al., 2012). In the present study, protocorm-like bodies (PLBs) of a *Phalaenopsis* hybrid is cultured, bioactive compounds with pharmaceutical properties were extracted and the plantlets were acclimatized. The minimal somaclonal variation was developed as the culture was maintained for more than two years compared to the acclimatized mother explant.

Somaclonal variation indicates the genetic instability among plantlets and cells culturedunder *in vitro* environments. Such genetic variations could be due to the regeneration system, explant source, crop medium, subculture frequency and duration,and genotypic factors (Leva et al., 2012). The genetic variation among plantlets could be noticed by morphological structures of explants, biochemical analyses, and DNA molecular markers. Although advanced equipment is not required, morphological identification that depends primarily on vegetative and floral features is limited by morphological changes due to various developments and environmental factors (Kaliaet al., 2011). The use of protein and isozyme-driven biochemical markers continues to be hindered for the same reasons. The application of biochemical markers was also restricted to limited protein and isozyme markers available for a particular species (Sharbati et al., 2013). Therefore, DNA molecular markers can be used to determine the somaclonal variation between treated samples and control explant. Start Codon Targeted polymorphism (SCoT) marker technique uses single 18-mer primers in a single primer polymerase chain reaction (PCR) targeting different regions of

the conserved region flanking the ATG start codon. Another marker called directed amplification of minisatellite region DNA (DAMD) is applied to detect the variations in the number of repeat units. Minisatellite DNAs, which are tandemly repeated portions of genomes, frequently display high degrees of length variances.

1.1 Rationale of study

As a monopodial *Phalaenopsis* orchid, it is propagated by the vegetative method, resulting in low multiplication rates and impairing its growth as well as making it ineffective for large-scale production (Khatun et al., 2020). Thus, such vegetative propagation is challenging and produces not uniform seedling characteristics. It requires around 3 years for flowering in the greenhouse environment and producing an insufficient amount of plantlets to meet the market for *Phalaenopsis* orchid. The plant tissue culture technique is alternatively an efficient tool to overcome the propagation of this orchid species (Park et al., 2002). *Phalaenopsis* orchid can be propagated rapidly through protocorm-like bodies (PLB) regeneration from any explants (Khatun et al., 2020).

The current study aims to develop a mass propagation of PLBs multiplied by thincell layer culture. It provides an ideal concept of totipotency mechanism and differentiation in cells, tissue, and plant organ (Nhut et al., 2006). However, it is challenging to get a stable regeneration method for *Phalaenopsis* due to the formation of phenolic compounds in wounded areas, which causes somaclonal variation in the culture and affects the PLB regeneration rate. Therefore, the medium composition of culture plays a vital role in maintaining the regeneration rate by adding organic supplements, carbon sources, and

amino acids. This is also important for the industrialization and acclimatization of the *Phalaenopsis* orchid in this study.

The notable role of semiconductor diodes, LED, in enhancing the growth of *in vitro* cultures is highly predictable. Furthermore, LED is also an excellent choice as an illumination source due to its changeable light wavebands and intensity, longer lifespan, and also relatively lower heat radiation (Nhut et al., 2003b). It radiates over a narrow light spectrum that induces both physiological and biochemical modification

within the medium cultures. The accumulation of several phytochemicals, like secondary metabolites, also will be studied within the *in vitro* cultures. Plants naturally developed an antioxidant defense mechanism that produced various secondary metabolites to counteract oxidative stress and free radicals (Hashim et al., 2021).

The current study focuses on the interaction of different LED spectra on secondary metabolites and the antioxidant activity of elicitors-treated PLBs. An improved valuable secondary metabolite that is produced through light elicitation can unlock new research areas and economic benefits which can apply in the pharmaceutical andnutraceutical industry (Hashim et al., 2021).

1.2 Research objectives

The study was carried out to accomplish the following objectives as statedbelow:

i. To establish the efficiency of thin cell layer (TCL) in increasing protocorm- like bodies (PLBs) proliferation capacity,

ii. To determine the proliferation rates of protocorm-like bodies (PLBs) of *Phalaenopsis* orchid by using different medium compositions,

organic additives, and carbon sources,

iii. To study the effect of plant growth regulators and amino acids on the development of PLB, shooting and rooting capacity along with morphologicalstructures, biochemical and molecular analyses,

iv. To identify the presence of bioactive compounds in PLBs and evaluation of secondary metabolites production through different types of elicitors under light-emitting diode (LED) irradiation.

CHAPTER 2

LITERATURE REVIEW

2.1 Orchid

The flowering plant Orchidaceae is known as one of the two largest plant families, which consists of 27,800 species (Cardoso et al., 2020*)* with 899 genera (Chase et al., 2015) and 100,000 hybrids made by artificial pollination (The International Orchid Register 2019). As well as their unquestionable botanical and ecological importance, orchids participate in the current systems of high-technological horticulture, which grow in environments where good climatic control is required, particularly in temperatures that permit orchids to be initiated irrespective of the time of year, especially to provide potted and cut flowers in competitive flower worlds (Victoria et al., 2016). This helps to meet the supply-demand for potted and cut flowers in the world flower market.

In general, orchid flowers are distributed around the world except for the Antarctica region due to temperature differences (Pridgeon, 2001). Although orchids are a diverse and widespread flowering plants in nature, only a few genera are cultivated in mass propagation as a commercial ornamental crops like *Dendrobium, Oncidium, Cymbidium ,* and *Phalaenopsis* (Sarmah et al., 2017). Some orchids like *Dendrobium,Bletilla,* and *Gastrodia* contain medicinal values which are widely used in traditionalChinese medicine while *Vanilla* species are also taken as food (Cardoso et al., 2020*).* The beauty of the Orchidaceae family attracts the interest of commercial growers. Especially genera like *Phalaenopsis* Bellina and *Phalaenopsis violacea* due to the flower structure and colour with some stunning “blue” forms. Approximately 121

million pots of genus *Phalaenopsis* have been sold in the year 2016 which were US$ 5000 million (Chen., 2020).

The botanical classification and commercial classification of orchids have to be set separately because although genera have a superior genetic and morphological influence on commercial plants, most commercial orchid flowers were produced through the formation of hybrids from interspecific crossing (Cardoso., 2017). As an instance, the production of the hybrid genus of *Doritis* in crossed with *Phalaenopsis* became *Doritaenopsis* (Ho et al., 2016) but still commercially known as *Phalaenopsis* as the hybrid morphological resemblance and commercialization value remain the same.

In several orchid families, it is achievable to attain many viable and fertile progenies combinations from different morphological species and genera. So, this criterion allows breeders to combine many traits of interest into the formation of a new plant. This method allows the breeders to bring the innovative aspect of orchid production and advance in the breeding of new hybrids. This advanced hybridization capacity can be achieved by embryogenic initiation and later protocorm production that occur in the orchids cycle (Yeung., 2017).

The floriculture market of Malaysia consists of orchids (*Dendrobium, Aranda, Phalaenopsis, Oncidium*, and other types of orchids) and temperate flowers such as Chrysanthemum, Rosa, Lili, Gerbera. Malaysia has been placed top ten exporting countries in the floriculture market worth RM 11.9 million up to RM 13.2 million in 2017 (The Sun Daily, 2017). Among all the orchids, *Dendrobium* species occupied 85% of the total tropical international orchid trade market (Sheela, 2008).

2.2 *Phalaenopsis* orchid

2.2.1 History

Orchidaceae is well-known for its spectacular flower and diverse plant families. Most evolutionary biologists and botanists worked on orchids after the publication of *Fertilization of orchid* in 1862 by Darwin (Niu et al., 2021). The first discovery of *Phalaenopsis* orchid genus was in 1825 by a German botanist and entomologist called Dr. Karl Ludwig Blume (Niu et al., 2021). The name of orchid is initially derived from the Greek word "phalaina," which means "a moth," and "opsis" translates to "appearance."

Later, *Phalaenopsis* genus habitat is broadly distributed across Southeast Asia, which includes Sri Lanka, South India, and Southern China to Taiwan, Indonesia, Malaysia, Philippines, and Northern Australia (Tsai et al., 2011). *Phalaenopsis* is a type of tropical plant due to its wide-ranging distribution throughout Southeast Asia. Places like Peninsular Malaysia, Sumatra, and Borneo Island show three different forms of rainforest corresponding to the altitude (tropical rainforest 1000 meters, tropical mountain forest up to 2000 meters, and cloud or mist forest up to 3000 meters). In past studies (Tsai et al. 2010), the cultivation of *Phalaenopsis* has a high level of CO_2 absorption capacity under high humidity at night time and more photosynthetic rate occurs at 30°C during daytime. The adaption of temperature and climate of *Phalaenopsis* is similar in both wild species and hybrid plants, although the morphology of the native species differs from that of the hybrid (Zahara et al., 2019).

2.2.2 *Phalaenopsis* hybrid orchid

Interspecific hybridization between subgenera or sections has long been applied in breeding cultivars to obtain novel traits in the *Phalaenopsis* orchid. In the horticulture industry*, Phalaenopsis* is known as an economically significant pot and cut flower due to its maintenance mechanism and availability throughout the year, and the long-lasting shelf life of flowers up to 3-4 months (US Department of Agriculture (USDA, 2018). The *Phalaenopsis* hybrid orchid is formed as a result of hybridization between *Phalaenopsis* Purple Heart (Figure 2.1) and *Phalaenopsis* Fuller's Light (Figure 2.2). *Phalaenopsis* Purple Heart is white with a red lip in the middle. It will have one flower spike and bloom during the spring, fall, and winter seasons. *Phalaenopsis* Fullers Light flowers are purple and pink in colour with some patterns on the edges and centre. This flower prefers cool to intermediate temperatures. Generally, *Phalaenopsis* orchids can grow in indoor environments as orchids are exposed to an adequate amount of light and air circulation.

Figure 2. 1 : *Phalaenopsis* Fullers Light

Figure 2. 2 : *Phalaenopsis* Purple Heart

2.2.3 Physiology and classification

Phalaenopsis usually contains 3 to 5 broad dark pigmented green leaves with thick and leathery structures. This can grow up to 50 cm long and 10 cm wide under semi- light exposure. The thick flesh root emerges from the lower basal portion of the pendulous stem. The flowering stem usually grows up to 1 m long from the base of the leaves along with the flowers, which can last between 2 to 6 months. The flower comprises three petals and sepals, the flower petals are known as labellum along with three-lobed and are usually brighter in colour than the flower parts.

Classification

Kingdom : Plantae (Plant)
Subkingdom : Tracheobionta (Vascular plants)
Superdivision : Spermatophyta (Seed plants)
Division : Magnoliophyta (Flowering plants)
Class : Liliopsida (Monocotyledons)
Subclass : Liliidae
Order : Orchidales
Family : Orchidaceae (Orchid)
Genus : *Phalaenopsis* (Moth orchid)

2.3 Plant tissue culture

The culture of plant tissue, also known as micropropagation, includes the technique of propagating cells, tissues, or the entire mother plant under aseptic conditions (Hussain et al., 2021). The culture of plant tissue acts as an effective plant multiplication tool in which the true-to-type clones of the chosen plant can be propagated in large quantities within a short duration (Mohapatra et al., 2017).

Thisthe technique is mainly used in the fields of horticulture, agriculture, and forestry in particular. In addition, the tissue culture technique is also important in eliminating the growth of pathogens in the culture to produce secondary plant metabolites (Oseni et al., 2018).

The effectiveness of the plant culture is primarily due to the totipotency of explant cells under sufficient nutrient conditions by cell division autonomously and regeneration by organogenesis into a new plantlet (Feher, 2019). The genetic materials of the regenerated explants are identical to the mother explants with specialized roles to ensure exact plant cellular functions. However, due to their ability to undergo dedifferentiation, differentiated cells maintain their permeability, a stage where the cells transition from a differentiated phase to a more juvenile stage. At this stage, the specialised function of explants could be altered and adapted to a new environment (Sugiyama, 2015). Eventually, the explants will undergo the process of dedifferentiation followed by trans/redifferentiation of the cell cycle (Grafi, 2004). *In vitro* propagation requires several conditions for optimum plant growth like temperature, humidity, light intensity, and adequate nutrient composition (Bhoite et al., 2014).

The primary advantage of micropropagation is maintaining and restoring the genetic material of the endangered species and, most importantly, preserving the balance of the ecosystem and biodiversity (Usher, 2000). Plant growth regulators (PGRs) such as auxins, cytokinins are incorporated with micropropagation techniques to study the morphogenesis and organogenesis of explants under sterile conditions (Skoog et al., 1957; Gaba, 2005). This helps the plant biologist to develop modified protocols to study the structure of shoot, root, and apical regions effectively. Plant cell requires macronutrients, micronutrients, vitamins, and carbon sources to grow

better under optimum condition, and such requirement of nutrients varies from one plant to another (Saad et al., 2012). Therefore, each species needed to be identified and optimized to improve the success rate of micropropagation.

The development of transgenic plants with desirable characteristics can also be accomplished using the technique of plant tissue culture. This was proven by Yarra and colleagues (2019) using the plant genetic modification method, which could increase the resistance level towards diseases and abiotic stress in oil palms. Due to environmental factors, plants can undergo several stress levels, leading to variations ingene expression or regulation. Furthermore, studies like DNA microinjection in oil palm (Masani et al., 2014), microprojectile bombardment into *Lilium longiflorum* (Watad et al., 1998), *Agrobacterium*-mediated genetic transformation of asparagus (Chen et al., 2019) help scientists to explore in a more detailed and preciseway.

Plant tissue culture also serves as a suitable approach for the production of secondary metabolites and is useful to propagate natural resources (Gonçalves et al., 2018). This approach is beneficial for seasonal plants, which are mostly dependent on seasonal environmental changes. The micropropagation technique helps in overcoming such a situation, wherein the explant source can be accessible all year round (Isah et al., 2018). Generally, the plant tissue culture technique is contamination-free from fungal and bacterial infection. So, this will help the plants to grow in better conditions without pesticide application (García-Gonzáles et al., 2010).

2.4 Phytochemistry

Flavonoids, alkaloids, phenanthrenes, and bibenzyl derivatives are the most common phytochemicals found in the orchid family (Singh and Kumaria, 2021).

Various active compounds were found in *Dendrobium nobile*, mainly in the stems and leaves such as dendrobine, moscatilin, gigantol, denbinobine, nobiline, and dendrophenol (Miyazawa et al., 1997 ; Zhao et al., 2001). Meanwhile, in *Phalaenopsis equestris* two phenanthrenes derivatives were successfully isolated (Manako et al., 2001).

Compounds found in these orchids possess anti-carcinogenic properties against lung carcinoma, ovarian adenocarcinoma, and promyelocytic leukemia (Lee et al.,1995). Different extracting solvent methods like petroleum ether, ethyl acetate, methanol, and distilled water have been used to find the presence of compounds like phenols, alkaloids, flavonoids, tannins, tannins, phlobatannins, saponins, terpenoids, glycosides, and phytosterols in *Coelogyne stricta* leaf extracts (Lyudmyla et al., 2016).

2.5 Orchid Propagation

Orchid seeds are dust-like structure in nature with a size range of 0.1- 6mm and it lacks endosperm (Barthlott et al., 2014). Generally, endosperm acts as a nutrient source to the radicle and plumule at the early stage of seed germination before photosynthesis could take place. Seeds also can obtain nitrogen and phosphorus as a nutrient through the symbiotic relationship with mycorrhizal fungi. As a product of a symbiotic relationship, ammonium and sugar will be produced and this will be used for the germination process (Yeh et al., 2019).

Explants such as protocorm, protocorm-like bodies (PLBs), nodal segments, root tip, shoot tips have been developed in the tissue culture technique. The growth percentage of PLBs as a starting material is relatively high compared to other explants (Tantasawat et al., 2015). Therefore, PLBs were used to establish *in vitro* culture of

orchids and it consists of undifferentiated tissue parts which as identical to somatic embryos (Julkifle et al., 2012). The unorganized cell masses known as callus is difficult to induce cultures and easy to undergo necrosis (Roy et al., 2007). Therefore, callus is not suitable to serve as an explant for micropropagation until it reaches embryo state. The root tip of *crytopodium paranaense* was used as an explant to develop an efficient *in vitro* protocol and it was established from PLB culture at the initial stage (Guo et al., 2010). *Dendrobium* orchid can also be regenerated from leaf tip explant which is initially derived from PLB stock culture (Goswami et al., 2015). Cytokinin is a hormone that could induce shoot culture while auxin helps to induce the PLBs or somatic embryo cultures effectively. Regeneration of *Dendrobium densiflorum* Lindl was established via *in vitro* shoot tips of the seedling by incorporating hormones like 6-benzylaminopurine (BAP), 1-napthaleneacetic acid (NAA), and indole-3-butyric acid (IBA) to enhance the growth rate
(Pradhan et al., 2013).

2.6 Protocorm-like bodies (PLBs)

A somatic embryo is formed from vegetative cells which are further differentiated into the whole plantlet and PLB also carries the same characteristic as a somatic embryo which can be induced directly from shoot meristems, root tip, or apical bud (Tokuhara and Mii, 2003). Parenchyma cells usually consist of thin-walled cells with photosynthetic tissue. The same morphological structure was found in PLBs with actively dividing meristematic cells. The formation of secondary PLBs highly found in the tip of apical regions of PLBs. Due to this, the propagation of secondary PLBs is relatively large in number in a short duration (Sheelavanthmanth et al., 2005). As PLBs are bipolar in nature, shoot and root can be induced by the addition of hormones.

In 2003, Tokuhara and Mii stated that *Phalaenopsis* orchid PLBs originated from single cells by transferring the suspended cells from liquid to solid medium. The induction of PLB derived from shoot tip, root tips, and lateral buds on *Phalaenopsis* (Ichihashi, 1992) and leaf epidermal cells of *Doritaenopsis* (Park et al., 2002). PLBs are now being widely used for secondary metabolites production (Park et al., 2002).

Table 2. 1 : : *Phalaenopsis* explant on different medium composition

Species / hybrid	Mother explant	Medium composition	Photoperiod condition	Results	References
Phalaenopsis amabilis var. 'Manila	Leaf segments (1 cm × 0.5 cm) obtained from *in vitro* flower stalk nodes	MS added 15 mg/L BA and 3 mg/L NAA	Temp 25 ± 1 ◦C; 16-hr	50.65 number of PLBs per explant	Balilashaki and Ghehsareh 2016
Phalaenopsis amabilis cv. 'Surabaya'	Leaf segments from *in vitro* shoots obtained from inflorescence stalk segments	MS added 5 mg/L BA + 2 mg/L NAA and TDZ	Temp 25 ± 1 ◦C; 16-hr	22.45 number of PLBs	Balilashaki et al., 2015
Phalaenopsis 'R11 × R10'	Leaves, root tips and stem explants	MS ½ + 15% coconut water + 0.01% activated charcoal + 0.03% polyvinylpyrrolidone (PVP) + 88.8 µM BA + 5.37 µM NAA + 0.025%	Temp. 25 ◦C, 16-hr	71.2 PLBs was more effective	Antensari et al., 2014
P. aphrodite subsp. *Formosana*	Seedlings	Liquid MS ½ for 2 months and then transferred to solid MS (half strength) with 1 cm of medium.	Temp 25 ± 2 ◦C; 16hr	44 PLBs per seedling	Feng et al., 2014

NP (New *Phalaenopsis*)	Protocorm, root and leaves	MS medium added 3 mgL−1 TDZ	25 ± 1 ºC with 1000 lux intensity	23.3 PLBs/explant; Leaf: 100% explants with PLBs and 7.75 PLBs/explant; Root: 80% explants with PLBs and 8.25 PLBs/explant;	Mose et al., 2017
Phalaenopsis gigantea	Leaf tip segments	NDM medium added 0.1 mg L^{-1} TDZ, 10 mg L^{-1} chitosan	Temp 25 ± 2 ◦C, 16 hr	353 PLBs/explant 4.8 g PLBs FW	Samarfard et al., 2014
Phalaenopsis hybrids	Transversally cut protocorms 1.0–1.5 mm width	MS + 15% coconut water	Temp 25 ± 2 ◦C, 16-h	40 and 44% PLB formation and 11.7 and 13.3 PLBs per explant	Soe et al., 2014
Phalaenopsis Nebula	Calluses	0.1 mg L^{-1} thiamine + 2.0 mg L^{-1} glycine + 170 mg L^{-1} NaH_2PO_4 + 20 g L^{-1} sucrose	Temp 26 ± 2 ◦C, 16-hr	74 numbers of PLBs / callus	Chen et al., 2000

2.7 Thin cell layer (TCL) method

A thin cell layer is a method used to enhance the mass propagation of *in vitro* explants efficiently and was proposed by Van in 1973 (Tripathi et al., 2018). The regeneration of desired explants can be manipulated via the thin cell layer method (da Silva, 2003). The study conducted by da Silva and Dobránszki (2011) used two ways to measure the regeneration capacity of explants (i) growth correction factor (GCF) and geometric factor (GF). This can be done by measuring parameters like the size range of explant, number, and size of the explants. Furthermore, both measurements allow directcomparison among several explants of different studies (da Silva et al., 2015). Hence, the cost of labour and the time needed for effective micropropagation may also be minimised.

TCL method has been applied for *in vitro* culture of orchid species over the years such as *Rhynchostylis gigantean* (Van Le et al., 1999), *Doritaenopsis* (Park et al., 2002) and *Cymbidium aloifolium* (Nayak et al., 2002). Micropropagation of many varieties of plants including vegetables, leguminous plants, fruits, crops like rice, maize, and sorghum used TCL as an efficient method for better growth (Nhut et al., 2003).

The growth correction factor (GCF) is a proportional the number that expresses how many times the target organs can be regenerated from a source organ in a comparison of two explants. Theoretically, each plant or cultivar would have its own GCF for each explant type because the regeneration capacity (RC) is influenced by many different elements, including explant type, position, age, and size, as well as many others, including sucrose, plant growth regulators (PGRs), light, and temperature.

2.8 Plant growth regulators (PGRs)

Several factors such as culture medium, genotype, and plant growth regulators affect the micropropagation of explants (Muktadir et al., 2016). Plant hormones or phytohormones are chemical messengers that help to coordinate cellular activities by flowing throughout the plants and can penetrate cells via cell membranes easily (Vanneste and Friml., 2009). There are several types of plant growth regulators such as cytokinin, auxin, gibberellin, abscisic acid, ethylene, and salicylic acid. In most studies, cytokinin and auxin have been applied to evaluate the morphology structure of explants (Chin et al., 2019).

Indole 3-acetic acid (IAA) is an auxin phytohormone that is naturally found in plants, along with other endogenous auxins like indole-3-butyric acid (IBA), 4-chloroindole-3-acetic acid (4-Cl-IAA) and phenylacetic acid (PAA). Among these, due to the convertibility of IBA into IAA via β oxidation, IBA is known as the IAA storage medium (Woodward and Bartel., 2005). In addition, a synthetic auxin, for instance, 2,4- dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), 3,6 dichloro-2-methoxy benzoic acid (dicamba) are also largely used to propagate plant growth efficiently (Korasick et al., 2013). Usually, the production of endogenous auxin occurs at the site of the vascular pathway of plants. This movement of endogenous auxin is placed in a cell-to-cell manner through active transport with the help of carrier protein while passive diffusion can be actively done by transporting auxin to adjacent cells (Armengot et al., 2016).

Generally, the shoot induction can be achieved by using cytokinin, which can be further divided into two groups: adenine and phenyl urea-type (Mok et al., 2001). The configuration of the adenine group may differ depending on the location of the N6 side chain. Zeatin exists as stereoisomers in *trans*-form. However, *cis*- is another type that

presents in plants abundantly (Gajdošová et al., 2011). Besides this, N-phenylureas are compounds containing a moiety of N-pheylureas that are structurally distinguishable by a phenyl group connected to a single urea group of the nitrogen atom. Mostly, plant tissue culture studies involved synthetic phenylurea-type cytokinin such as thidiazuron (TDZ) in stimulating plant apical growth and development.

2.9 Addition of amino acids

Protein is known as macromolecules made up of amino acids. In general, the molecules are crucial for living organisms which carry more than half of the total dry weight of a single cell (Yildiz, 2010). Polypeptides are made up of 40-50 amino acid monomers to form a single chain by peptide bonds. The series of amino acid arrangement is coded by the gene as the building blocks of proteins varies between proteins (Voigt et al., 2002). Protein is a molecule that comes in various conformations, including primary, secondary, tertiary, and quaternary structures due to its pattern of folding and coiling of polypeptides chain.

Protein occurs in linear form in its primary structure shape, where the building blocks coordinate and link themselves in a linear sequence via covalent bonds (Eisenhaber et al., 1995). The secondary structure of a protein refers to the three-dimensional protein pattern due to the formation of hydrogen bonds between the amino acid carbonyl and a hydrogen atom. This also can be further divided into alpha (α) helix and beta (**β**) pleated sheet, following their dimensional form. Proteins with coiled polypeptide chains are rod-shaped alpha helix proteins (Wardah et al., 2019). It is formed as a result of the hydrogen bonding of amino acids between N-H and C-O of different amino acids in a similar polypeptide chain (Bai & Englander, 1994). Beta

pleated sheet proteins, on the other hand, are structured in such a way that polypeptide chain segments are placed back to back and linked by hydrogen bonds that form a sheet between the backbone groups. Interaction of hydrophobic between secondary protein causes the molecules to fold into the compact globular structure (Wardah et al., 2019). The tertiary interaction of amino acids can be strengthened by the formation of disulphide bond, hydrogen bond, and salt bridge (Kumar et al., 2021).

Protein consists of a different range of biomolecules with several biological activities associated with them. The unstable protein levels in plants are induced by due to different stressors such as temperature, light, water, salinity, and heavy metals. Protein profiling in a systematic way to study proteins in various aspects, including their post-translation changes, protein interaction, and their responses to a modifier, is also known as proteomics (Gulcicek et al., 2005). Proteomics is an analysis of whole protein complements within a biological entity that can be found. In controlling the state of homeostasis, different proteins are involved and endow plants with the adaptive capacity to survive in stressful conditions. For example, proteins abundant in-late embryogenesis (LEA), which are the hydrophilic proteins being produced during drought and have been postulated to take part in a protective role in maintaining enzymatic function (Amara et al., 2014). LEA proteins inhibit the aggregation of hydrophobic proteins that appear to be denatured because of heat-induced stress due to their hydrophilic characteristics (Tunnacliffe et al., 2007).

Moreover, hydrophilic proteins reduce the production of citrate synthase enzyme under extreme temperatures. In addition, proteins like thioredoxins help in monitoring the redox potential of different proteins via the reversible process of exchange of cysteine thiol-disulfide (Dos Santos et al., 2006). Furthermore, the kinetics of biomolecules have increased under heat stress as temperature increases and

contributes to misfolding and aggregation of proteins. The nature of protein molecules like chaperonins allows denatured enzymes to be folded or re-folded by supplying a cavity sequestered from the cytoplasm surroundings (Specht al, 2010).

2.9.1 The effect of amino acids on plant tissue culture

The essential amino acids for optimal growth are synthesized by most plants, however, the incorporation of certain types of amino acids is crucial for establishing the cella and protoplasts. Addition of amino acids provides plant cells with a source of nitrogen that is easily assimilated by tissues and cells quicker than inorganic nitrogen sources. Types of amino acids like casein hydrolysate, L-glutamine and adenine frequently used in culture medium for the shoot and root growth of plants. The frequent concentration of casein hydrolysate is usually used for 0.25–1 g/L. Glycine was used for the enhancement of cell growth in culture media and included glycine at 2 mg. l^{-1}, glutamine up to 8 mM, asparagine at 100 mg/L, L-arginine, and cysteine at 10 mg/Land L-tyrosine at 100mg/L (Kenneth,1989)

By altering plant physiological characteristics in response to stressful environmental conditions, the regulation of plant proteins affects the phenotype (Kosova et al., 2018). A protein molecule is responsible for several biological functions of plants. Furthermore, it is characterized into five classes: - glycine-rich proteins, proline-rich proteins, arabinogalactan solanaceous lectins, and proteins extensins (hydroxyproline-rich protein) (Showalter, 1993). Due to their association with receptors linked to the plasma membrane or by the liberation of signaling molecules, proteins embedded in cell walls are often engaged in plant signaling (Fry, 2004). Additionally, in modulating plant epigenome, transcriptome, and metabolome functions, proteins also act as regulators.