INFLUENCE OF LIGHT EMITTING DIODES ON *Dendrobium* HYBRID ORCHID PLANTLETS

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2021

INFLUENCE OF LIGHT EMITTING DIODES ON *Dendrobium* HYBRID ORCHID PLANTLETS

by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

January 2021

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to Prof Dr. Sreeramanan Subramaniam, who has been very supportive throughout my study. He has always been doing his utmost in offering helps that eased my research project. His effective superintendence and valuable advice have been guiding me on the right path in accomplishing all of the planned milestones on time. My sincere gratitude goes to Dr. Chew Bee Lynn, who has been giving me constructive advice with her expertise in this field.

I thank Collaborative Research in Engineering, Science and Technology (CREST) and P-Plus Sdn Bhd for offering me the scholarship which covered my tuition fees for 3 years. This helped a lot in alleviating my financial burden and allowed me to stay focused on my study. I sincerely appreciate the assistance offered by all of my lab mates and the staff from School of Biological Sciences.

I am grateful for the unconditional support given by my parents and family, without which the completion of this study would be impossible. The encouragement and trust from them served as a source of motivation for me to overcome the obstacles and difficulties I have encountered during my study.

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LIST OF ACRONYMS AND ABBREVIATIONS

PLBs	Protocorm-like bodies
MS	Murashige and Skoog
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
ISSR	Inter simple sequence repeat
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
DDMP	Flavonoid 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-

KESAN DIOD PEMANCAR CAHAYA TERHADAP PLANLET ORKID HIBRID *Dendrobium*

ABSTRAK

Tempoh peringkat muda yang panjang dan pertumbuhan orkid yang lambat menyebabkan propagasi vegetatif orkid mengambil masa yang panjang dan memerlukan tenaga kerja yang banyak. Dalam perkara tersebut, mikropropagasi berfungsi sebagai kaedah yang berkesan untuk penghasilan anak pokok orkid yang serupa dari segi genetik dalam masa yang singkat. Penggunaan diod pemancar cahaya (DPC) membolehkan penyelarasan profil cahaya dan membantu dalam meningkatkan pertumbuhan kultur *in vitro*, memandangkan penyinaran cahaya dengan spektrum yang spesifik membantu dalam mendorong fotomorphogenesis. Kajian ini bertujuan untuk meningkatkan pertumbuhan orkid kacukan *Dendrobium* (*Dendrobium* Enopi x *Dendrobium* Pink Lady) melalui kaedah mikropropagasi. Teknik sel lapisan nipis meningkatkan pembentukan jasad seperti protokorm (JSP) sekunder dalam kadar 1.56 lebih tinggi berbanding dengan eksplan JSP konvensional. Berbanding dengan pengawalanturan pertumbuhan tananam yang lain, chitosan (0.25 mg/L) membantu dalam meningkatkan kapasiti percambahan (15.69 cambah) dan pembentukan akar (5.15 akar). Pendedahan JSP pra-rawat dengan DPC putih bawah penyinaran DPC merah intensiti tinggi (29.30 µmol/s) merangsangkan kadar peningkatan proliferasi JSP tertinggi, dengan berat kering 0.152 g. Untuk JSP pra-rawat dengan lampu pendarfluor merah, penyinaran seterusnya dengan DPC merah dalam intensity tinggi (29.30 µmol/s) mendorong percambahan pucuk dalam purata tertinggi, dengan 13.65 percambahan. Kapasiti pembentukan akar dipertingkatkan oleh DPC biru-merah (1:1) dengan intensity 20.30 µmol/s, nombor akar tertinggi iaitu 4.580 dicatat pada hari ke-

180 selepas hari inokulasi. Informasi genetik anak pokok terjana telah dinilai dengan menggunakan penanda DAMD dan ISSR yang masing-masing menunjukkan lebih daripada 85 % dan 92 % persamaan informasi genetik. Gas kromatografi spektrometri jisim (GCMS) mendedahkan kewujudan sebatian bioaktif yang mempunyai fungsi antikanser. Antaranya adalah DDMP, phenol-2,4-bis(1,1-dimethylethyl) dan 1,2-benzenedicarboxylic acid, mono-(2-ethylhexyl) ester. Pembiasaan JSP terhadap sumber pencahayaan berkemungkinan berlaku pada kajian ini memandangkan pra-rawatan JSP di bawah sinaran lampu pendarfluor merah untuk satu atau dua kitaran subkultur menunjukkan pengurangan daripada segi pengumpulan metabolit sekunder, selepas disinari oleh pelbagai rawatan DPC. DPC hijau dengen intensiti tinggi (16.9 µmol/s) berpotensi untuk digunakan sebagai sumber pencahayaan dalam meningkatkan kandungan fenolik. Kajian ini mendapati bahawa asid amino seperti L-leucine, glycine and proline (25 - 100 mg/L) menunjukkan tiada kesan yang memberangsangkan dalam mendorong kandungan metabolit sekunder. Kajian ini menunjukkan fungsi DPC yang menonjolkan dalam menjana anak pokok orkid dengan kesan variasi somaklonal yang minima. Di samping itu, DPC turut berfungsi sebagai sumber pencahayaan yang baik dalam memberangsangkan pengumpulan metabolit sekunder yang tinggi dalam kalangan kultur JSP *in vitro*.

INFLUENCE OF LIGHT EMITTING DIODES ON *Dendrobium* HYBRID ORCHID PLANTLETS

ABSTRACT

The long juvenile stage and slow growth rate of orchids make their vegetative propagation to be time consuming and labour-intensive. In this context, micropropagation serves as an eminent approach for large scale production of genetically identical orchid plantlets within a short time. The engagement of light-emitting diode (LED) permits the adjustment of light profile and aids in fostering growth and development of *in vitro* cultures, as narrow light spectrum is anticipated to induce photomorphogenesis. The present study aimed to promote the growth of a *Dendrobium* orchid hybrid (*Dendrobium* Enopi x *Dendrobium* Pink Lady) through micropropagation means. Thin cell layers (TCLs) technique promoted the formation of 2˚ protocorm-like bodies (PLBs) by 1.56-fold as compared with the conventional PLB explant. As in comparison with the other phytohormones, chitosan (0.25 mg/L) enhanced both the shooting (15.69 shoots) and rooting (5.15 roots) capacity of the *in vitro* cultures. The exposure of white LED-treated PLBs under high intensity (29.30 µmol/s) red LED irradiance stimulated the highest proliferation rate, with 0.152 g of recorded dry weight. For PLB explants pre-treated with red fluorescent light (FL), their further exposure to high intensity red LED illumination induced the highest shoot induction rate, with 13.65 shoots. The rooting capacity was improved under blue-red LED (1:1) at the intensity of 20.30 µmol/s, where the highest root number of 4.580 was recorded at 180 days after inoculation. Regenerated plantlets were genetically assessed by using DAMD and ISSR DNA markers which correspondingly detected genetic similarity percentage of 85 % and 92 %. Gas chromatography-mass

spectrometry (GCMS) revealed the presence of several bioactive compounds with anticancer properties within the *in vitro* PLB cultures. These included DDMP, phenol-2,4-bis(1,1-dimethylethyl) and 1,2-benzenedicarboxylic acid, mono-(2-ethylhexyl) ester. PLB explants could have exhibited habituation towards illumination source in the present study as PLBs pre-cultured under red FL for one or two subculture cycles displayed a reduction in secondary metabolites accumulation, after being irradiated with different LED spectra. Green LED at high intensity (16.9 µmol/s) could be engaged as the illumination source to promote phenolics production. Study herein discovered that amino acid supplements such as L-leucine, glycine and proline (25 - 100 mg/L) exhibited no significant effects in promoting secondary metabolites production. The present study displayed the prominent role of LED in orchid plantlets regeneration with minimal effects on somaclonal variations. Moreover, LED also serves as an excellent illumination source in inducing high secondary metabolites accumulation among the *in vitro* PLB cultures.

CHAPTER 1

INTRODUCTION

Orchidaceae is among the most diversified families of angiosperms which comprises of more than 30 000 species, with hundreds of new species being introduced every year (Hinsley et al., 2018). Approximately 850 genera are grouped under Orchidaceae, encompassing 10 % of the angiosperms species (Lucksom, 2007). The vegetation of orchids occurs on almost all inhabitable continents as marked by Dressler (Dressler, 1981). However, the distribution of orchids is densely found within tropic regions. More than 720 orchid species are inhabiting on Mountain Kinabalu, Sabah in Malaysia. Being highly diversified, orchids exhibit various distinct variations in terms of their life forms, distributions of the population as well as trophic patterns (Gardes, 2002). They can exist as terrestrials, lithophytes or saprophytes, although about 70 % of the known species are epiphytes (Kartzinel et al., 2013).

Floriculture industry flourishes in Malaysia as time progresses. In 2000, a total of RM 33 million worth of orchids were exported from Malaysia to other countries. This value elevated to RM 40 million in 2005 (Malaysia, 2003). Every year, an estimated export value of RM 150 million is anticipated in Malaysia, wherein the majority of the orchids are *Dendrobium* (Ahmad et al., 2010).

Other than being prized for their exquisite and long-lasting flowers, orchids have been extensively used as traditional medicine. The medicinal properties of orchids are mainly attributed to numerous phytochemicals present within them, for instances, alkaloids, flavonoids, terpenoids, phenanthrenes and bibenzyl-derived compounds (Gutiérrez, 2010). These compounds confer orchids the capacity to display over a wide array of biological activities. For examples, anti-inflammatory, anticancer, antimicrobial, and anti-rheumatic. Owing to their phytochemical constituents which

retain bioactive effects, orchids have been engaged as natural remedies for various illnesses. The medicinal effects of *Dendrobium* species, commonly known as Shi-Hu in Chinese, have been well-documented in previous studies. Among them, *Dendrobium nobile* is found to be rich in bioactive compounds such as dendrobine, dendrine and nobilonine. It has been used in treating fever, gastritis, stomach and lung cancer (Lin et al., 2003) as well as diabetes by elevating insulin levels and insulin sensitivity (Shi et al., 2004). Besides that, other *Dendrobium* orchids which include *Dendrobium aurantiacum* and *Dendrobium candidum*, also possess antidiabetic effect (Gutiérrez, 2010). The presence of bioactive compound fimbriatone in *Dendrobium fimbriatum* has also been discovered to be effective in hindering the growth of human stomach cancer cell line (Bi et al., 2003).

In nature, propagation of orchids are often limited by various factors. The orchid seeds, although being produced in large quantity with up to 3 million per seed capsule, are miniscule and lack of endosperm. This greatly restricts the germination rate of the seeds. Furthermore, a symbiotic association has to be established in between orchid seeds and mycorrhizal fungi in order to heighten the germination rate. However, the presence of symbionts is scarce in nature. Orchids can be propagated through vegetative means such as cutting and splitting of clumps and shoots (Utami et al., 2017). These approaches retain several drawbacks as it requires a relatively long time to establish adequate tillers. The cutting also increases the chances of pathogenic infections as the wounded regions are exposed to the external environment. Moreover, over-harvesting of wild orchids threatens their population as the requirements for their growth is constrained in nature and thereby demand a long time to restore the population (Hinsley et al., 2018).

Nevertheless, the acquisition of the bioactive compounds with pharmaceutical effects is mainly from different organs or the intact adult orchid plant. This imposes difficulties on the extraction procedure as some of the exotic species are inhabiting in spatially limited regions which are difficult to get access. Moreover, the intact plants tend to be impaired during the extraction process, and over-harvesting of wild orchids could have disrupted the ecosystems by causing disturbance to the populations in nature. The restoration of the wild orchid population is time-consuming as orchids have a long juvenile stage and a relatively low photosynthetic capacity which makes them being classified as slow-growing angiosperms (Zhang et al., 2018). The vegetative growth period of orchids can last for 4 to 7 years or longer before the flowering stage (Wang et al., 2010). Thence, the limited natural sources incline to restrain the obtainment of phytochemicals in an adequate amount wherein their capacities in inducing biological effects tend to be limited.

1.1 Rationale of study

Micropropagation overcomes several natural barriers which limit orchid propagation in nature. It proffers an alternate approach which is particularly useful in preserving as well as restoring wild orchid populations. Furthermore, the cultivation of orchids rich in bioactive compounds via protocorm-like body (PLB) cultures through micropropagation means also serves as an excellent source for the attainment of valuable phytochemicals, since PLB retains a high proliferation capacity (Sheelavanthmath et al., 2005).

Despite orchid micropropagation has been well-established, the regeneration rate of *in vitro* cultures is often restrained by several factors such as habituation. As

time progresses, the plant tissue cultures might turn recalcitrant by losing dependency on exogenously applied growth factors, for instance, phytohormones (George, 1993).

The present study aims to establish an effective artificial illumination source in plant tissue culture through the engagement of light emitting diodes (LEDs), which is anticipated to overcome the shortcomings of conventional orchid micropropagation. On top of that, the role of chitosan as plant growth stimulator has also been studied in the study herein to improve the growth of a *Dendrobium* orchid hybrid (*Dendrobium* Enopi x *Dendrobium* Pink Lady), which is relatively recalcitrant towards the effects of exogenously applied plant growth regulators (PGRs).

As the most crucial abiotic factor, artificial illumination system provides *in vitro* cultures the energy source necessary to ensure healthy growth and development as well as determining photomorphogenesis and biochemical profile of the cultures. LED prevails over the other artificial lights such as fluorescent, metal halide, incandescent and high-pressure sodium lamps due to several advantages it possesses. Being a semiconductor diode, LED conveys electrical energy into light energy in the form of photons. LED exhibits a higher energy efficiency due to the linearity of electrical current input with photon output (Pal et al., 2013). Additionally, due to their small size, the diodes display high flexibility as they can be fitted into plant tissue culture chambers of various structures (Kozai et al., 1997). Besides, LED is also a superior choice as an artificial light source because of its longer lifespan, relatively lower heat radiation and most importantly, the adjustable light wavebands and intensity (Nhut et al., 2003b).

The prominent role of LED in enhancing the growth of *in vitro* cultures is highly anticipated, as it emits over a narrow light spectrum which stimulates both physiological and biochemical changes within the cultures. The accumulation of

various phytochemicals, such as secondary metabolites, within the *in vitro* cultures under different light spectra is studied in depth. The present study aims to demonstrate the correlation between fluctuation of phytochemical contents and physiological changes of the *in vitro* orchid cultures, as in response towards the irradiation of different narrow light spectra. Findings of the study herein proffers insight into orchid micropropagation through LED irradiance which could be extrapolated for further studies.

1.2 Research objectives

The present study was conducted to accomplish the objectives as stated below:

i. To ascertain the efficiency of thin cell layer (TCL) in enhancing protocorm-like bodies (PLBs) proliferation capacity,
ii. To assess the capacity of chitosan in promoting PLBs growth of various stages as a plant growth stimulator,
iii. To inspect the role of light spectra and intensity in dictating physiological, biochemical and molecular profiles of *in vitro* plant cultures,
iv. To reveal the presence of bioactive compounds within PLBs and elevation of secondary metabolites production through light-emitting diode (LED) irradiation.

CHAPTER 2

LITERATURE REVIEW

2.1 Orchidaceae

Orchidaceae, a phylum grouped under Angiospermophyta, is one of the largest families of flowering plants. Orchids are vastly diversified, either geographically and taxonomically. The first recorded colonization of orchids on earth can be dated back to 4th millennium B.C. Throughout histology, orchids have been evolved and thrived to become the second-largest flowering plant family after Asteraceae (Chase et al., 2015). Orchid itself comprises of 10 % of the angiosperms, with 29 199 recognized species (Govaerts et al., 2014) spanning 750 to 850 genera (Hossain, 2011).

Orchids are found colonizing all the continents, except for Antarctica and desert regions. The wide distribution of orchids around the world is attributed to their developed life histology strategies, in which they can exist in many forms via terrestrial, epiphytic, saprophytic or lithophytic. Tropical and sub-tropical regions are enriched with diverse orchid varieties as orchids are pantropic flowering plants, and more than 10,000 species can be discovered in these regions with most of them are epiphytes.

2.1.2 Economic importance of Orchidaceae

The eye-catching and long-lasting inflorescence of orchids makes them an eminent ornamental plant which accounts for a large share of global flower trade, either as potted or cut flowers. The international trade value of cut orchids recorded at US$ 464 million in the year 2007, the value fluctuated and reached US$ 504 million in 2012 (De et al., 2014). The Netherlands is the major orchid exporting country and contributed 39.67 % of global orchid exportation, followed by Thailand and Taiwan

with 28.41 % and 10 % respectively (De et al., 2014). Thailand, a country with a tropical climate and extensively rich in orchid species, is the origin for 1,300 species and more than 180 genera of orchids. As one of the largest orchid exporting countries, Thailand exported US$ 60 million worth of cut orchid flowers out of the country in the year 2014 (Thammasiri, 2016). Orchids have also gained popularity in Europe countries. In 2015 alone, an estimation of € 76 million worth of cut orchid flowers have been imported into Europe markets, with Italy occupied the largest market share of € 19 million, followed by United Kingdom, France and Germany with € 16 million, € 12 million and € 7.5 million respectively (CBI, 2016).

2.1.3 *Dendrobium* orchids

Dendrobium is the largest genus of Orchidaceae with about 1 200 species around the world (da Silva et al., 2014). *Dendrobium* orchids belong to the subfamily Epidendroideae, which is also the largest orchid subfamily that comprises of 21 160 orchid species or 76 % of the family (Govaerts et al., 2014). The distinct floral parts of *Dendrobium* orchids make it an excellent ornamental plant. Moreover, numerous *Dendrobium* orchids also possess medicinal values and widely used to cure diseases such as stomach ache, blood purification, abscesses, malignant, ulcers and breast cancer (Zhang et al., 2006).

Dendrobium is one of the genera of Orchidaceae which is native to China, Hong Kong, Taiwan, India, Thailand, Vietnam, and other temperate and tropical Asian regions (Sujjaritthurakarn et al., 2011). These orchids are diverse plants that have a vast geographical origin, ranging from various continents. *Dendrobium* is also known as the “tree of life” in Greek, where “Dendron” means “tree” and “bios” means “life” as *Dendrobiums* are epiphytic orchids which live on other host plants. Pseudobulbs, a

specialized part which primarily functions as a water storage organ, are present among *Dendrobium* orchids. The inflorescence of *Dendrobium* orchids grows laterally, where the petals start arising from the upper part of the shoot. In contrast, the lateral sepals tend to form a mentum with column foot (Xiang et al., 2013). The ornamental characteristics of *Dendrobium* orchids make them among the most highly valued cut flowers, especially in Southeast Asian countries (Akter et al., 2007). *Dendrobium* orchids have gained their popularity due to their diverse flower colours and shapes. Other than that, *Dendrobium* orchids can be kept for a long time due to their long lifespan, which makes them available all year round (Dehgahi et al. 2017).

However, overexploitation of orchids solely for their ornamental or medicinal values is threatening the population of wild orchids. Furthermore, any disruptions to the natural terrain of wild orchids because of human activities can also endanger their populations. Some of the orchids might be particularly vulnerable to overexploitation as their inhabitation is often limited or confined by the specificities they possess. For instances, the pollination mechanisms and the accessibility to mycorrhizal symbionts (McCormick et al., 2014).

2.2 Plant tissue culture

Plant tissue culture which also known as micropropagation, involves the technique of cultivating cells, tissues or the whole mother plant itself under aseptic conditions. It has been widely applied mainly in the fields of agriculture, horticulture and forestry. Plant tissue culture serves as a powerful tool for the propagation of the plant of interest, in which the true-to-type clones of the selected plant can be multiplied in large number within a short period of time. Other than plant multiplication, plant

tissue culture is also being employed as an approach to eliminate pathogens as well as the production of plant secondary metabolites (Oseni et al., 2018).

The success of plant tissue culture is greatly attributed to the totipotency ability of plant cells, which refers to the ability of a plant cell to autonomously undergo cell division and regeneration into a new plantlet through organogenesis (Fehér, 2019), provided with adequate nutrients and conditions. The cells will be differentiated into distinct cell types with specialized roles in order to ensure plant cellular functions. The regenerated plantlets through this mean are genetically identical with that of the mother plant. Differentiated cells, however, retain their plasticity owing to their capability to undergo dedifferentiation, a process where the cells regress from the differentiated stage to a more juvenile stage. The specialized functions or characteristics of the differentiated cells might be altered after going through dedifferentiation (Sugiyama, 2015). Cells undergone dedifferentiation will subsequently re-enter the cell cycle or undergo trans/redifferentiation or cell death (Grafi, 2004). The explants, being the starting materials, are only required in small amount and can regenerate into large number of plantlets. As opposed to the conventional propagation approaches, *in vitro* propagation offers a strict control over the growth determining factors, which include the light, humidity, temperature as well as nutrient compositions (Bhoite et al. 2014).

Micropropagation is fundamentally essential for the conservation of phytodiversity, especially the endangered species. It provides an alternative to restore or conserve the threatened species and helps in retaining the balance of the ecosystem by maintaining the genetic, species as well as the biodiversity (Usher, 2000). Micropropagation has developed rapidly followed by the discovery of plant growth regulators (PGRs) such as cytokinins and auxins, which are also the essential additives in stimulating plant morphogenesis and organogenesis (Skoog et al., 1957). The

cultivation of plant under controlled environmental factors is more conducive for plant growth. Furthermore, substantial nutrients, which are required to support plant growth and development, are easily accessible by the cultures through micropropagation approach. The media used in micropropagation are often fortified with essential nutrients such as macronutrients, micronutrients, and vitamins. However, optimum biotic, as well as abiotic factors, varied among different plant varieties and needed to be optimized in order to improve the success rate of micropropagation. Previous plant tissue culture studies have provided insights on the multiplication of plants of different varieties and categories through several morphogenic pathways (Anis et al., 2016).

The production of transgenic plants with desired traits can also be achieved by using plant tissue culture technique. Yarra and colleagues (2019) advocated that genetic modification of plants such as oil palm, aids in enhancing plant resistance towards diseases and abiotic stress, which in turn elevates the crop yield. Being sessile, plant counteracts environmental stress through genes regulations, which can be up- or down-regulated, depending on the degree of stress (Wang et al., 2003). The expression of these genes will then confers plant the resistance against the stressors. The incorporation of foreign genes into the plant genome can be done through various approaches. For examples, microprojectile bombardment into *Lilium longiflorum* (Watad et al., 1998), *Agrobacterium*-mediated genetic transformation of asparagus (Chen et al., 2019), and DNA microinjection in oil palm (Masani et al., 2014). These alternatives are more preferable as compared to the natural selection method, which takes a long time in developing plant genetic modification.

The massive collection of plants rich in bioactive compounds merely for the medical properties they possess exerts a negative impact on the survival of their wild populations. Furthermore, over-harvesting of certain medicinal plants which take years

to grow can endanger the whole population. Micropropagation provides a better alternative by propagating the desired plants in large scale within a short period of time without harming the plant population in the wild. Plant tissue culture approach is particularly useful for the production of plant secondary metabolites as it overcomes the limiting environmental factors such as limited natural resources (Gonçalves et al., 2018) and low natural propagation rate. As highlighted by Yue and associates (2016), the acquisition of plants which are grown only on specific regions restricts the commercial production of secondary metabolites. Moreover, seasonal plants which growth stages are highly dependent on seasonal changes might also limit their collection either for ornamental or medicinal purposes. However, these drawbacks can be overcome by using micropropagation means, wherein the plant source can be made available all year round through an established plant tissue culture system, without having to encounter the constraints as mentioned earlier (Isah et al., 2018).

Established plant cultures through micropropagation means are generally free of fungal and bacterial diseases as well as the other potential natural pests. Hence the application of pesticides can be avoided (García-Gonzáles et al., 2010). Additionally, plant tissue culture prevails over the conventional propagation methods as it retains the potential in altering the biosynthesis of plant bioactive compounds. This makes it a useful tool in the production of herbal medicines with improved quality (Debnath et al., 2006). Moreover, the phytochemical constituents of field-grown plants vary and fluctuate in accordance with environmental, geographical and seasonal factors (Murthy et al., 2014). On the contrary, plant tissue culture permits the production of bioactive compounds with uniform quality and yield in a continuous manner (García-Gonzáles et al., 2010).

2.3 Orchid micropropagation

Protocorms are greenish tuber-like bodies with spherical shape which emerge from germinating orchid seeds. The seeds of orchids are minute with only about 0.1 to 6 mm (Barthlott et al., 2014) and appear to be in dust-like form. Unlike other angiosperms, orchid seeds lack endosperm. The absence of endosperm makes it difficult for seed germination as nutrients and hormones required for embryonic growth are not present. In order to compensate with the inherent drawbacks, orchid seeds possess several characteristics to elevate their dispersal rate. They are wind-dispersed and present in huge quantity with 2 to 3 million seeds per capsule. However, the germination rate (0.3 %) is relatively low in nature (Arditti, 1992). Due to the limited storage reserves, orchid seeds often establish an association relationship with mycorrhizal fungi. This symbiotic relationship allows orchid seeds to obtain mineral nutrients such as nitrogen and phosphorus from the fungi to support germination. In return, orchid seeds provide fungi with ammonium and sugar (Yeh et al., 2019). The vegetative propagation of orchids involves the splitting of shoots and clumps or the keikis. Nevertheless, these conventional approaches are time-consuming and labour-intensive. Hence, an efficient propagation method is needed.

Micropropagation of orchids has been long established for large scale production of plantlets via plant tissue culture techniques. Various orchid micropropagation protocols using different parts such as protocorms, protocorm-like bodies (PLBs), nodal segments, shoot tips, seeds, and flower stalk nodes have been developed. Among the explants or starting materials, PLB cultures are the preferable approach for the establishment of *in vitro* orchid cultures as these bodies consist of undifferentiated tissues and resemble orchid somatic embryos morphologically (Julkifle et al., 2012).

Apart than PLBs, several other vegetative orchid plant parts also serve as the explants for plantlets regeneration purposes. Pradhan and co-workers (2013) established an efficient protocol to regenerate *Dendrobium densiflorum* Lindl. plantlets by using *in vitro* shoot tips of the seedlings. The incorporation of exogenous hormones such as 6-benzylaminopurine (BAP) and 1-napthaleneacetic acid (NAA) in combination into culture media gave rise to stimulating results for shoot formation rate whereas indole-3-butyric acid (IBA) enhanced the rooting capacity of this particular medicinal orchid plant. Leaf tip explants have also been employed as the starting materials to regenerate *Dendrobium* plantlets as demonstrated in the work of Goswami and colleagues (2015), where the shoots arose from leaf tip explants-induced PLBs. Additionally, *Cyrtopodium paranaense* plantlets can be regenerated from root tip explants (Guo et al., 2010) through PLBs formation. Callus formation arising from *Dendrobium crumenatum* axillary buds, and also appeared as an intermediate stage for orchid plantlets regeneration as presented by Meesawat and Kanchanapoom (2002) in their work. Calli are masses of undifferentiated cells formed as a result of wounding. These unorganized cell masses hold the capacity to undergo embryogenesis to form somatic embryos, which further develop and regenerate into whole plantlets through indirect somatic embryogenesis pathway. However, callus formation in orchids possesses several difficulties in terms of induction, cultures maintenance and necrosis (Roy et al., 2007).

2.3.1 Protocorm-like bodies (PLBs) culture

As indicated by Mishiba and colleagues (2005), the engagement of PLBs in orchid micropropagation shortens the intermediate period of time required for plantlets regeneration. PLB can be induced directly from several explants such as shoot meristems, leaves, flower stalks and root tip while indirect PLBs formation often

occurs through callus cultures (Tokuhara et al., 2003). Similar to protocorms, PLBs consist mainly of parenchyma cells. The external tissues layers of globular PLBs are composed of actively dividing meristematic cells which are responsible for the formation of secondary PLBs. The high proliferation capacity of PLBs in giving rise to the formation of secondary PLBs in large quantity in a short space of time (Sheelavanthmath et al., 2005), makes them a prevailing explant choice for orchid micropropagation. PLBs display bipolar structure and undergo differentiation with the formation of shoots and roots, in which the organogenesis eventually leads to plantlets regeneration.

2.3.2 Thin cell layer

Thin cell layer (TCL) concept was first proposed by Van (1973 b) and has been applied to many studies in plant tissue culture over the past few decades. TCL is a simple yet effective concept in enhancing the productivity of *in vitro* plants. It permits mass propagation of a species of interest. Besides, the regeneration of particular plant organs can be manipulated as well (da Silva et al., 2003). Hence, the labour cost and the time required for successful micropropagation can be reduced. Two new concepts proposed by da Silva and Dobránszki (2011) have been applied to evaluate the efficacy of TCLs on the micropropagation of *Dendrobium* species via protocorm-like bodies (PLBs). The growth correction factor (GCF) and geometric factor (GF) are used to express the actual potential regeneration capacity of the explants, by taking parameters such as explant size and shape into account. The application of GCF and GF allows direct comparison among various explants of different studies (da Silva et al., 2014).

TCL technique has been applied in the micropropagation of a wide range of plants, and these include leguminous plants, vegetables, fruits, cereals and grasses such

as rice, sorghum and corn (Nhut et al., 2003). Nevertheless, TCL method has also been practised for *in vitro* culturing of orchid plants over the past few years. For instances, *Aranda* Deborah (Lakshmanan et al., 1995), *Rhynchostylis gigantean* (van Le et al., 1999), *Cymbidium aloifolium* (Nayak et al., 2002) and *Doritaenopsis* (Park et al., 2002).

2.3.3 Plant growth regulators

In response to the ever-changing environmental conditions, plants produce mobile signalling chemicals such as phytohormones. These endogenous molecules are occurring in low concentrations and play an essential role in signalling and regulating plant growth and development. Phytohormones are chemical messengers that coordinate cellular activities by circulating throughout plants, and they can diffuse with ease into plant cells through the membranes (Vanneste et al., 2009). The five major common plant hormones are auxins, cytokinins, gibberellin, ethylene and abscisic acid. However, there are also other new classes of hormone that have been identified, and these include brassinosteroids, jasmonate, strigolactones and salicylic acid.

Cytokinins can be classified into two groups: the adenine-type and phenylurea-type. The endogenous adenine-type cytokinins are further divided into isoprenoid and aromatic cytokinins, depending on the side chain attached to the N^6-side chain of the adenine ring (Mok et al., 2001) which gives rise to their configuration. Naturally occurring adenine-type cytokinin such as zeatin exists as stereoisomers, with the *trans*-form being the active form. Nonetheless, *cis*- form can also be found abundantly in plants (Gajdošová et al., 2011). On the other hand, phenylurea-type cytokinins are characterized by derivatives attached to the nitrogen atom of a phenylurea group.

Unlike the adenine-type, phenylurea-type cytokinins are not naturally occurring cytokinins in plants. Synthetic phenylurea-type cytokinin such as thidiazuron (TDZ) is applied exogenously to stimulate plant growth and development.

Auxins are low molecular weight mobile compounds bearing a carboxyl group and an aromatic ring. The carboxyl group and the aromatic ring should be at a distance of 0.55 Å, in order for auxin to be in active form (George et al., 2008). Indole 3-acetic acid (IAA) is the predominant naturally occurring active auxin that produced in plants, together with the presence of other endogenous auxins such as indole-3-butyric acid (IBA), 4-chloroindole-3-acetic acid (4-Cl-IAA) and phenylacetic acid (PAA). Among those, IBA is known as the storage form of IAA due to the convertibility of IBA into IAA through β oxidation (Woodward et al., 2005). Other than that, synthetic analogues of auxins such as 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 4-amino-3,5,6-trichloropicolinic acid (picloram) and 3,6-dichloro-2-methoxybenzoic acid (dicamba), are also being introduced and widely applied exogenously to promote plant growth and development, as these analogues are capable of inducing auxin responses in plants (Korasick et al., 2013). IAA is vulnerable to stimulants such as light and oxidants, hence it has been suggested that IAA is less effective and stable as compared to those synthetic analogues (George et al., 2008). The endogenous auxins produced at the sites of production travel over a long distance to the other plant parts through vasculature means. The delocalization of auxins from one site to another occurs in a cell-to-cell manner via two distinct pathways: passive diffusion and active transport in which auxins are actively transported to neighbouring cells with the aid of influx and efflux protein carriers localized in the plasma membrane (Armengot et al., 2016).

2.3.4 Chitosan

Chitosan is a biopolymer composed of glucosamine and N-glucosamine units. It is a cheap and easily accessible biomaterial (Szymańska et al., 2015) resulted from chitin deacetylation, a substance found abundantly in the exoskeleton of crustaceans, insects, or fungi (Ifuku, 2014). Chitosan has been reported to stimulate a range of metabolic changes that enhance plant immunity against various infections (Al-Hetar et al., 2011). The nature of chitosan can be degraded through several approaches, primarily via oxidation, chemical or through enzymatic reactions (Ma et al., 2014). There are various determining factors which will affect the stability of chitosan, some examples are the moisture content, humidity and temperature. The preferable storage temperature for chitosan is 2 - 8°C in closed condition as it is sensitive to environmental conditions. Storage of chitosan solution in ambient and high temperature (40°C) tends to accelerate the degradation rate of chitosan chains (Nguyen et al., 2008). However, several studies exhibited that there is no significant alternation on the chemical structure of chitosan after the exposal to autoclaving. This indicates that autoclaving is an applicable sterilization method for media incorporated with chitosan (Yang et al., 2007).

Chitosan is a biomaterial with great uses. It can function as water purifier, antibacterial as well as food preservatives (Restanto et al., 2016). The role of chitosan in promoting inflorescence and orchids plant defence mechanisms against microbial has been reported in previous study (Chandrkrachang, 2002). The function of chitosan as antimicrobial is greatly attributed to its capacity in inducing cell walls liginification which further inhibit fungi and/or bacteria penetration (Reddy et al., 2005). Besides from cell wall thickening, chitosan also exhibits its antimicrobial property through several mechanisms. These include cytoplasmic aggregation, hyphae disintegration

and increased vacuolation, all of which are detrimental to microbial (Laflamme et al., 2000).

2.4 Plant metabolites

Plants produce various phytochemicals throughout their lives. These biologically active compounds are vital in supporting the growth and development of plants as well as to sustain their basic life functions. Plant metabolism encompasses all the biochemical reactions that occurred within an organism. Metabolites formed as a result of metabolism, either intermediate or end products. (Thirumurugan et al., 2018). Generally, there are two main categories which phytochemicals can be grouped into primary and secondary metabolites. Among the two, primary metabolites are primarily involved in the maintenance of cellular functions and possess a direct impact on plant growth and development. Some of the primary metabolites include carbohydrates, proteins, and lipids. All of which play a role in determining the primary metabolic activities of plants such as cellular division, storage, respiration and reproduction (Hussein et al., 2018). Primary metabolites are present in all living cells as indicated by Seigler (1998).

2.4.1 Secondary metabolites

Secondary metabolites such as phenolic acids, flavonoids, alkaloids, steroids, are phytochemicals produced by the derivative pathways of primary metabolic pathways. In other words, they are derived from plant primary metabolism (Thirumurugan et al., 2018). Unlike primary metabolites, the constituent of plant secondary metabolites fluctuates and occasionally occurs (Isah, 2019). The absenteeism of secondary metabolite does not lead to the immediate death of plants.

However, it greatly impairs the survival ability of plants (Thirumurugan et al., 2018). Secondary metabolites play an essential role in aiding plants to counteract with the ever-changing stressful environmental conditions. They confer the plants with defence mechanisms against pathogens, herbivores, ultraviolet rays and other hassles. Secondary metabolites can be found abundantly in some specialized tissues or structures of plants. Their production, however, may vary depending on the genotype, plant physiological status and the environmental factors (Gonçalves et al., 2018). The biosynthesis of secondary metabolites fluctuates at different plant developmental stages (Shitan, 2016).

Plant secondary metabolites are capable of inducing numerous biological effects. The pharmacological effects they displayed makes them a prominent source of modern medicines. Plants with therapeutic values have been used for medicinal purposes since 5 000 years ago (Mahesh et al., 2008). The medicinal properties of these compounds are mainly attributed to the antioxidant potential they possess. This antioxidant property aids in reducing the risk of developing certain diseases. Plants are rich sources of antioxidant compounds with beneficial health effects and are exceptionally useful in pathogenesis. They help in combating several chronic diseases such as cardiovascular diseases, cancers, arteriosclerosis, neurodegenerative diseases, diabetes as well as osteoporosis (Pandey et al., 2009). Despite being such a valued source for phytochemicals, plant secondary metabolites are difficult to be synthesized artificially due to their structural and chemical complexity (Bhatia, 2015). The production of plant secondary metabolites through tissue culture approaches are well-documented and have been further applied into a variety of fields including pharmaceuticals, agrochemicals, flavours, insecticides, fragrances, and cosmetics (Whitmer et al., 2002).

2.4.2 Phenolics

Phenolic compounds occur naturally in plants and appear to be the most pronounced plant secondary metabolites. Phenolics composed of the largest phytochemical group which are accountable for most of the plant antioxidant activities (Okpuzor et al., 2009). They comprise of over 8 000 forms which chemically differ from each other (Dai et al., 2010). Phenolics are molecules bearing one or more phenol moieties and can be further categorized into different groups depending on their chemical structures. Phenolics with one aromatic ring such as benzoic acids and cinnamic acids are known as simple phenol. In contrast, compounds such as flavonoids and tannins, which bear 2 or more aromatic rings with higher molecular weight, are known as complex phenol (Anantharaju et al., 2016). Phenolics encompass a variety of diverse compounds, for instances, flavonoids, phenolic acids, tannins, lignans, lignin, stilbenes, coumarins and curcuminoids (Gan et al., 2019). Among those, flavonoids form the largest group and have been studied extensively. These compounds are involved in a wide array of plant activities in which they play significant roles as antioxidants, plant structural strengthening agents, colouring agents as well as signalling compounds in plant defence mechanisms.

2.5 Oxidative stress

The intake of oxygen by plants is necessary for the usage of mitochondria for energy production. Furthermore, the accumulation of oxygen occurs within plants as a result of photosynthesis. The occurrence of oxygen metabolism in plants leads to the formation of reactive oxygen species (ROS) in a continuous manner (Vainonen et al., 2015). The by-products of oxygen metabolism such as superoxides, peroxides, hydroxyl radicals and singlet oxygen, are especially rich in concentrations within plant

organelles which are actively involved in aerobic metabolism. For examples, chloroplast, mitochondria and peroxisomes (Maurino et al., 2008). ROS are highly reactive, thereby overproduction of ROS tends to induce oxidative stress and disrupt plant cellular functions through various means, such as oxidising lipids, proteins and nucleic acids which often leads to cell death. Although their presence in high concentrations is detrimental for plant cells, ROS have been proposed to play a crucial role in cell signalling (Das et al., 2015). Intracellular redox state serves as an indicator in triggering various cellular signalling pathways, which in turn activates cellular functions such as plant defence mechanism (Chiu et al., 2012).

The upsurge of ROS is detrimental to cells. However, plants have developed several mechanisms to act upon the over-accumulation of ROS. Various enzymatic or non-enzymatic antioxidants present within plants are being employed in detoxifying or scavenging the free radicals (Das et al., 2015) in order to ensure redox balance. The exposure of plants towards stressful environmental conditions often leads to oxidative stress. Redox homeostasis is initiated through the accumulation of non-enzymatic or enzymatic antioxidants which vary in terms of biochemical properties and localization. This provides more flexibility in regulating ROS levels, both temporally and spatially (Das et al., 2015). Extensive studies have proven that oxidative stress is linked to the development of several diseases, for instances, cancer, atherosclerosis and various cardiovascular diseases (Taniyama et al., 2003).

2.6 Plant defence mechanism

Plants often interact with external environmental conditions and are therefore being regularly exposed to various stressors of different extents. In order to ensure their survivability, plants have developed mechanical as well as biochemical measures to overcome the hassle conditions they encounter. As the first line of defence

mechanism, waxy cuticle on the leaf surface, thorns and trichomes are often present and serve as the physical barrier to protect plants against herbivores. Besides, plants display a defence mechanism through biochemical means. This approach offers a broader array of activities which is more dynamic. The involved active compounds can be produced constitutively within the makeup of plant cells, or as a result of plant damage (War et al., 2012).

Exposure of plants towards abiotic stresses for a prolonged period is hostile to plants. As in response to the stressors, phytochemicals are manoeuvred within the plant for homeostasis purposes, thereby heightening their resistance towards stressors such as salinity, water stress, extreme temperatures and ultraviolet radiation.

2.6.1 Chlorophyll in plant defence mechanism

Chlorophyll is an essential green pigment which can be found abundantly among autotrophs. Other than harvesting light energy to drive the chemical reaction necessary for the synthesis of glucose as the energy source, chlorophyll plays a significant role in conferring plants the protection against oxidative stress (Yuan, 2007). Chlorophyll comes in different forms, namely chlorophyll a, chlorophyll b, chlorophyll c and chlorophyll d. Among those, chlorophyll a is the core photosynthetic pigment while chlorophyll b and chlorophyll c are the accessory pigments which help in transferring light energy to chlorophyll a (İnanç, 2011). Chlorophyll a and b are commonly found in higher plants such as orchids and green algae whereas chlorophyll c and d are more common in brown and red algae. Chemical structure of chlorophyll a and b composed of a “head”, a porphyrin ring with magnesium atom bound at the centre of it, and a “tail” which comprises of a phytol with 20-carbon chain.

Owing to the antioxidant and chemopreventive features, chlorophyll aids in diminishing the detrimental effects of excessive ultraviolet radiation exposal in algae,

hence reducing oxidative stress (Yuan, 2007). Chlorophyll quenches and neutralizes the deleterious hydrophobic compounds which are carcinogenic. For examples, were polycyclic aromatic hydrocarbons, heterocyclic amines, and aflatoxins (Koutsaviti et al., 2018), and thus it helps to prevent the development of various diseases. The antioxidant properties of chlorophyll can be attributed to the porphyrin ring with the intact metal atom, whereby the antioxidant potential is more superior as in comparison with metal-free chlorophyll derivatives (Hoshina et al., 1998). Furthermore, chlorophyll functions in inhibiting lipid autoxidation, a process in which lipids are broken down as a result of a chemical reaction between oxygen and unsaturated lipids (Ahmed et al., 2016).

Apart from the natural occurring chlorophylls, a wide range of synthetic compounds deriving from chlorophylls have been introduced for their antioxidant potential. Some of the metallo-chlorophyll derivatives, with the magnesium atom within the chlorins being substituted with other metals such as copper, retain a higher antioxidant capacity and is more stable than the natural ones (Lanfer-Marquez et al., 2005). Chlorophyllin, an analogue of chlorophyll, exhibits anti-mutagenic and anti-carcinogenic activities against mutagens, probably due to its ability to bind with the mutagenic substances or the metabolite(s), which subsequently reduces their availability in cells (Dashwood et al., 1998).

2.6.2 Carotenoid in plant defence mechanism

Carotenoid, which also known as tetraterpenoids, is a group of polyene type natural occurring pigments (Landrum, 2009) that endows plants with red, yellow and orange colours. Aside from being an essential accessory photosynthetic pigment, carotenoids possess antioxidant potential which is beneficial in plant defence mechanism. Carotenoid consists of fat-soluble compounds of diverse structures and

functions. There are more than 700 compounds, with most of them bearing two terminal rings on a 40-carbon chain (Bell et al., 2000) which is made up of 8 isoprene units (C_5H_8). The presence of conjugated double bonds in the carbon chain confers carotenoids the ability to serve as antioxidants. Delocalization of electrons throughout the whole system promotes the chemical reactivity of carotenoids, thereby enhancing the isomerization and oxidation capacity of carotenoids (Oliver et al., 2000).

Natural occurring carotenoids exist in two forms, namely carotenes and xanthophylls, which differ in terms of their chemical structures. Carotenes comprise of compounds with hydrocarbon chains without oxygen atom while xanthophylls are oxygen-containing carotene derivatives (Botella-Pavía et al., 2006) such as lutein, violaxanthin, antheraxanthin and zeaxanthin. Carotenoids possess photo-protective ability and aid in protecting plants from photo-oxidative damage induced by light-mediated stress. Carotenoids help plants in countering with ROD-induced oxidative stress by quenching free radicals such as singlet oxygen ($^1O_2$), which are deemed to be deleterious for plant growth and development (Fiedor et al., 2014). Furthermore, carotenoids are effective scavengers of triplet state chlorophylls, an excited chlorophyll state that reacts readily with oxygen molecules to yield oxygen free radicals in the chloroplast (Young, 1991). In addition, Demmig-Adams (1990) provoked that carotenoids help in dissipating excess energy which excites and promotes triplet chlorophyll states, through the inter-conversion of violaxanthin, antheraxanthin and zeaxanthin.

2.6.3 Proline in plant defence mechanism

Proline is an amino acid which involved in protein biosynthesis. Nevertheless, it is better known as imino acid due to its distinct chemical structure with other amino