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Development of *In-Vitro* Culture of *Phalaenopsis* Blackjack Orchid.

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A report submitted in fulfillment of the requirements for the degree of Bachelor of Applied Science (Agrotechnology) with Honours.

Faculty of Agro Based Industry

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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I certify that the report of this final year project entitled "**Development of *In-Vitro* Culture of *Phalaenopsis* Blackjack Orchid**." by **Nur Fazilah Bt Musa**, matric number **F15A0144** has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Agriculture Technology) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

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Development of *In-Vitro* Culture of *Phalaenopsis* Blackjack Orchid

ABSTRACT

Phalaenopsis Blackjack orchid, originates from Taiwan has high demand due to its popularity as cut flowers and potted plants. However due to its low seed formation and low conventional breeding, the demand for *Phalaenopsis* Blackjack could not be fulfilled, hence this study was done. Aseptic technique to develop *in-vitro* culture of *Phalaenopsis* Blackjack was carried out by using leave as explant. There were three surface sterilization used to investigate the effect of different surface sterilization technique towards *in-vitro* culture of *Phalaenopsis* Blackjack orchid which was method A (sonication), method B (shaker) and method C (shaker and activated charcoal in media). Apart from that, to investigate the effect of hormone combinations toward *Phalaenopsis* Blackjack orchid multiplication, different hormone combinations with different concentrations Naphthaleneacetic acid (NAA; 0, 0.1, 1 mg/l) and Thidiazuron (TDZ; 0, 1, 3 mg/l) were used. In this experiment, survival rate, contamination rate and appearance were analysed and observed. Sonication method was found to be the most effective surface sterilization method compared by using shaker while ½ MS media supplemented with 0.1 mg/l NAA and 1 mg/l TDZ was the best treatment in multiplication of *Phalaenopsis* Blackjack orchid.

Keywords: *Phalaenopsis* Blackjack, Sonication, Shaker, NAA, TDZ

Pembangunan Anak Pokok *Phalaenopsis* Blackjack Orkid Secara Tabung Uji.

ABSTRAK

Orkid *Phalaenopsis* Blackjack yang berasal dari Taiwan mempunyai permintaan yang tinggi disebabkan oleh kepopularitiannya sebagai bunga potong dan tanaman pasu. Oleh kerana pembentukan benih dan pembiakan konvensional yang rendah, permintaan untuk *Phalaenopsis* Blackjack tidak dapat dipenuhi, maka kajian ini telah dilakukan. Teknik aseptik untuk membangunkan anak pokok tabung uji *Phalaenopsis* Blackjack telah dilakukan dengan menggunakan daun sebagai eksplan. Terdapat tiga pensterilan permukaan yang digunakan untuk mengkaji kesan teknik pensterilan permukaan yang berbeza ke arah anak pokok tabung uji *Phalaenopsis* Blackjack iaitu kaedah A (sonikasi), kaedah B (*shaker*) dan kaedah C (*shaker* + arang aktif dalam media). Selain itu, untuk mengkaji kesan gabungan hormon ke arah multiplikasi *Phalaenopsis* Blackjack, kombinasi hormon yang berlainan yang berlainan kepekatan, asid Naphthaleneacetic (NAA; 0, 0.1, 1 mg/l) dan Thidiazuron (TDZ; 0, 1, 3 mg/l) telah digunakan. Dalam eksperimen ini, kadar hidup, kadar pencemaran dan rupa daun telah dianalisis dan diperhatikan. Kaedah sonikasi didapati sebagai kaedah pensterilan permukaan paling berkesan berbanding penggunaan *shaker* manakala media ½ MS ditambah dengan 0.1 mg/l NAA dan 1 mg/l TDZ adalah rawatan terbaik dalam multiplikasi orkid *Phalaenopsis* Blackjack.

Kata Kunci: *Phalaenopsis* Blackjack, Sonikasi, *Shaker*, NAA, TDZ

TABLE OF CONTENTS

CHAPTER 4 RESULTS AND DISCUSSION

CHAPTER 5 CONCLUSION AND RECOMMENDATION

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

SYMBOL	DESCRIPTIONS
G	Gram
Mg	Miligram
L	Litre
^{O}C	Celcius
%	Percentage
Mg/l	Milligram per litre
Ac	Activated charcoal
PLBs	Protocorm-like-bodies
NAA	Napthaleneacetic acid
TDZ	Thidiazuron
MS	Murashige and skoog
BA	6-benzyladenine (

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Phalaenopsis or phals for short is the genus under the family of orchid (*Orchidaceae*). With 27 000 confirmed species around the world, *Orchidaceae* is the largest family of flowering plants in the plant kingdom beating the sunflower family, *Asteraceae* (Arditti, 2008). Balilashaki et al. (2014) mentioned that orchid has both interest in term of economic and ecological. Based on Chin (2016), until today, there are 43 000 of orchids hybrids, alone, are existed and listed. Ten percent (10%) of flower in the world are filled with orchids and thirty percent (30 %) of orchids are monocotyledons. These Orchidaceae are well-known flowers mainly as cut flowers and potted plants. Out of many genera in *Orchidaceae*, only a few are fit to be used as cut flowers which are grown commercially to fulfil the market request (Arditti, 2008). *Cattleya*, *Cymbidium*, *Phalaenopsis*, *Dendrobium* and *Vanda* are examples of the most common orchids (Chin, 2016). The orchid production in the world is highest in few regions in USA, followed by the UK and the Holland (Schiff, 2018). Asia and Africa also have big production of these exotic flowers (Khoddamzadeh et al., 2011) and the productions of orchid are highly profitable in several Asian country such as Australia, Thailand, Singapore and Malaysia.

Orchids are native throughout southeast of tropical Asian countries such as Philippines, Borneo, Java and elsewhere. They are available at the other regions too but scientist believed the plants are greater in number at tropical areas (Schiff, 2018).

Orchidaceae originates from Greek word, “orkhis” which means testicle, that are shown by the roots when are exposed to reveal a pair of tubers, hence explained the name (Schiff, 2018). Schiff (2018) added again, different varieties with different types of shapes, sizes and colours of flowers are what make them unique from other flowers. Its colourful and big flowers attached on the long stalk make it important in forest ecosystem (Balilashaki et al., 2014). These doyens in ornamentals hold the mystique and perfection vibes that emitted from the flowers of each species, make orchid unique and different from the other flowers. Along with its elegance looks, orchids also have a compelling history that make it as a favourite plants among the horticulturist which makes its economic value in the flower market skyrocket (Chin, 2016). According to Shah, Shrestha, Pandey and Joshi (2017), among the Chinese and Indian community especially at least developed area, the orchid are often used as remedy for various types of disease such as bronchitis, nervous disorders, cancer, microbial infection, rheumatism, piles and inflammation where roots, leaves and pseudobulbs are the most common and often used parts from the orchid plants. Some species also have medicinal value and certain essence that can be used in flavouring. Apart from that, some of the species also fragrance and often extracted to become perfume. Orchids are second after rose in popularity and it is proved by the availability of orchids, where it can be seen and found planted anywhere in the world except in deserts and Antarctica (Chin, 2016).

In orchid conventional breeding, small quantities can be produced at one time is already a major problem but if added with other problems such as rare development of

orchid seed pods, exudation of phenolics and somaclonal variation, it make the propagation by natural seems impossible based on Balilashaki et al. (2014). Shah et al. (2017) added, in nature, orchid propagate through seed germination and vegetative propagation only make the population of orchid always in danger of extinction. Furthermore, the rate of natural propagation is very low. Even if natural propagation successfully propagate, both of these process take long time, years, for seedling to become adult plant. During the growth, the plants could be eliminated when any intrusion on habitat or physical environment occur thus resulting in failure to meet their high demand in national and international market. To solve this problem, tissue culture seems like the best way to overcome the problems. Although in tissue culture techniques has its own obstacle, but compared to the natural propagation, tissue culture has more advantage such as genetically identical, short period, plantlets with high photosynthetic or photoautotrophic ability and higher survival rate at ex-vitro environment. Furthermore, through tissue culture, it is possible to alter the DNA either by improve, modify or optimize the culture (Balilashaki et al., 2014).

The numbers of species in genus *Phalaenopsis* are always grow mainly due to the effort shown by the orchid breeders in crossing the flowers of desired species that have distinctive traits. Without some help, some species or due to some circumstances, the plants cannot cross by itself. Although this genus is old and the species under it are various and extensive, the lack of attention given and the inability to grow rapidly will be the reasons of their extinction. Orchids are special. Although their flowering time are different, but they still share the same similarities. They tend to last longer than any other flowers so all orchid flowers are long-lasting and available in every color except for blue. Some of the blooming phals flower has sweet fragrance coming along but some are not

but nevertheless, they are still sought by orchid lovers. The diverse shapes of the flowers are also one of the contributors to its popularity, because it's pleasing to the eye.

1.2 Problem Statement

Orchids are slow-growing plants and listed as one of top ten in international market, making it popular thus increase the demands. In natural propagation, productions of orchid are limited due to the infrequent seed formation (Mose, Indrianto, Purwantoro & Semiarti, 2017). The situation that only depend on natural propagation resulting in insufficient clones especially in short period of time. So, in order to solve the problem, the popular method known nowadays which is *in-vitro* propagations are used where it is believed to be the best option in increase the multiplication of orchids in short period of time. In addition, it is more efficient than natural propagation.

The development of *in-vitro* culture of *Phalaenopsis* Blackjack orchid by using leaves were done to increase the production. In this study, three types of surface sterilization method were tested to find out which method is the most suitable to develop *in-vitro* culture. Apart from that, two types of hormone combinations, Thidiazuron (TDZ) and Naphthaleneacetic acid (NAA) with different concentration were used to observe the effects of hormone combinations on the leaves.

1.3 Hypothesis

H0: Different surface sterilization technique has same effect towards *in-vitro* culture of *Phalaenopsis* Blackjack orchid.

Ha: Different surface sterilization technique has different effect towards *in-vitro* culture of *Phalaenopsis* Blackjack orchid.

H0: Different hormone combinations has same effect on multiplication of *Phalaenopsis* Blackjack orchid.

Ha: Different hormone combinations has different effect on multiplication of *Phalaenopsis* Blackjack orchid.

1.4 Significance of Study

The aesthetic value in *Phalaenopsis* are irreplaceable, so in order to continue the legacy of *Phalaenopsis*, along with to supply and fulfil the demand both locally and internationally, *in-vitro* tissue culture were used as the growth rate for conventional vegetative propagation is too slow.

By doing this study, further information regarding *Phalaenopsis* was able to be provided related to method of surface sterilization and best combination of hormones to develop cultures. A research was conducted which was to develop *in-vitro* culture of

Phalaenopsis Blackjack orchid by using different surface sterilization method and combination of two different types of hormones, NAA and BAP, each with different concentrations. The study also highlight the general protocol that has been established by previous research.

1.5 Objectives

The objectives of this study are:

a) To study the effect of different surface sterilization technique towards *in-vitro* culture of *Phalaenopsis* Blackjack orchid.

b) To investigate the effect of hormone combinations toward *Phalaenopsis* Blackjack orchid multiplication.

CHAPTER 2

LITERATURE REVIEW

2.1 Orchidaceae

According to Chin (2016), sympodial and monopodial are two types of growth habits that can be found in orchids (Figure 2.1). In sympodial, horizontal stem (rhizome) and swollen shoots (pseudobulbs) can be observed while these features are not available for monopodial. However, single main stem can be observed in monopodial and sometimes even branched stem. Rhizomes that bend upward produce small plants with leaves and flower scapes can be found at the base of plants. Water and nutrient are stored in Pseudobulbs. Examples of sympodial orchids are *Cattleya*, *Cymbidium*, *Oncidium* and *Dendrobium* while monopodial orchid is *Phalaenopsis*, *Vanda* and *Vanilla* (Chin, 2016). Apart from growth habit, orchids can be grouped into 4 more types which are epiphytes, terrestrial, subterranean and lithophytes. Most of the orchids are epiphytic followed by terrestrial by 25%. Orchids that are grown at tropical area said tend to be either epiphytic or lithophytes while orchids that are grown at temperate area mostly are terrestrial.

Figure 2.1: Difference between sympodial and monopodial

Source: http://www.orchidcarelady.com/orchid-care-basics-what-kind-of-orchid-is-it/

Orchid plants have root systems that grown aboveground. The plants which has cylindrical and unbranched root with apex is long and pigmented to protect it from the strong radiation is called as aerial root while when the roots are flattened and attached to something for support, it is called as epiphytic root system (Lafarge, 2015). Orchids are the perennial herbs with upright stems. The leaves can be either alternate, opposite or whorled on the stem or also may grow only at the base of the plants. The flowers arrangements are various, depend on species and are highly specialized. Capsule, represent as the fruits, responsible in releasing many tiny seeds. Series of leaves are formed at the apical bud of single stem. Roots and stem of flowers are arise from the nodes of each leaves. The branching stem in orchid is rare but possible (Chin, 2016).

According to Shah et al. (2017), orchid, a beautiful flowering plants that is famous for its multi usage either as ornamental, medicinal or food value is well known for its rare seed development making the propagation through nature is miraculous yet remarkable. Moreover, orchid is mycohetetrophic plants meaning that their lack of endosperm seeds greatly rely on endophytes to uptake carbon to be used in germination, growing and

adaptation to nature. Seed formed symbiotic interaction with endophytes. Endophytes not only functioned in the growth and development of plants by initiate the germination process but also supply nutrient and immune defence since the orchid seed has no reserved food for growth. What is more, endophytes not only helpful in germinate the seed in nature but they also capable in overpower high mortality rates along with poor growth and immunity during the acclimatization (Shah et al., 2017).

2.2 *Phalaenopsis*

Phalaenopsis, a genus under *Orchidaceae,* hold many importance in horticulture area. *Phalaenopsis* also known as moth orchid because of the flower structure that possessed by species under this genus which resemble the moth in flight (Figure 2.2) which make it as its distinctive features (Nash, 2003). Other than that, phals are hardier compared to other species of orchids which indirectly increase its value thus make them particularly popular among first-time orchid growers. Compared the other types of orchids, phals are among popular potted plants. Also, to add more its value, phals is the first tropical orchids in Victorian collections (Nash, 2003). *Phalaenopsis* is one of the main flower in floriculture product (Niknejad et al., 2011) hence are included in top export genera of orchids (Schiff, 2018). *Phalaenopsis* has high value in the floriculture market, therefore, to maintain and increase its value, the hybrids such as *Phal. I-Hsin* Blackjack which originates from Taiwan were used in this study (Figure 2.3).

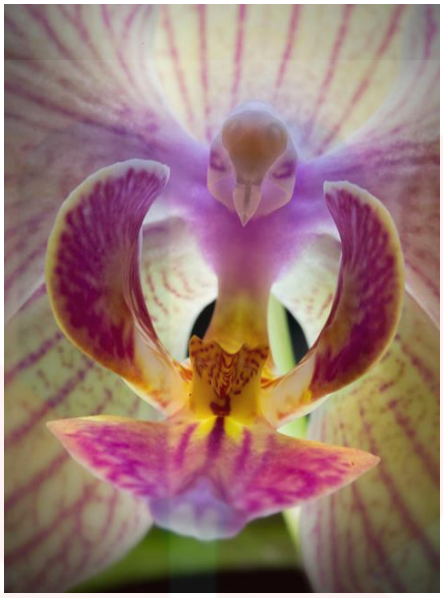

Figure 2.2: *Phalaenopsis* orchid that said resemble the moth in flight.

Source: https://imgur.com/91s3aRT

Figure 2.3: *Phalaenopsis* Blackjack orchid.

Leaves of moth orchids are differs in shape and size, depends on the species. However, they still share the same morphology with other species under different genus. For example, to cover their lack in pseudobulbs (storage mainly for water), the leaves is succulent and fleshy. This defence mechanism is their adaptations in order to retain the

water especially in arid climates. The leaves are olive dull green to purple or brownish. Other than that, long and few flowers or branch and full flowers are the two types of flower stems. However, there are stem that does not produce any flowers and it is called cylindrical stems (Lafarge, 2015).

Khoddamzadeh et al. (2011) stated, many researchers are using *P. amabilis* in their experiments. Pollen of *Phalaenopsis* has the ability to travel long distances (Li, Liao, Huang & Chan, 2015), however it took hours to days for pollen to reach an embryo sac (Chen & Fang, 2016). The continuous efforts of orchid breeder producing or aiding of hybrids are an act to create or improve the existing orchids especially in *Phalaenopsis*. Genetically, large genome are possessed by *Phalaenopsis* making the process to improve the traits using conventional breeding become difficult because of the 38 chromosomes and different length for each species possess resulting in the sexual incompatibility (Niknejad et al., 2011).

Phalaenopsis are available throughout the world including Taiwan (Khoddamzadeh et al., 2011). According to Lafarge (2015), Taiwan not only listed in one of the top country that export the orchids but also known as leaders in *Phalaenopsis* markets and that mainly due to the high quality production and continuous research. According to Lafarge (2015), the demand for *Phalaenopsis* at present are booming, the peak of being the most popular potted plants. *Phalaenopsis* market are expand each day and today, the production are dominated by Taiwan. The enormous greenhouse and latest technology in fertilizing, lighting and growing possessed by Taiwan make all the large production are possible. The value of hybrids are not less compared to the main species. The hybrids are done all the time by both professional or non-professionals. The impact to too many hybrids resulting in the lack of well documented hybrids (Lafarge, 2015).

2.3 *In-Vitro* Culture

Plants from *in-vitro* culture is the plants that grow from tissue culture laboratory by culturing the meristem or other parts of mother plants (Niknejad et al., 2011). According to Babaei, Nur Ashikin, Saleh and Abdullah (2013), tissue culture technique is known as the 'aseptic' method growing plants either by using plant cells, tissues or organs taken from the mother plant and then are cultured in artificially nutrient medium. In-vitro orchids are grown on various modified media with varied addition of chemical compounds, gelling agents, salts and cofactors (Shah et al., 2017). In-vitro propagation is known as the method to regenerate plants even for highly differentiates and functionally specialized cells. This method not only can use leaves, roots, stem, floral parts and endosperm as explant but also isolated gametic cells (Bhojwani & Dantu, 2013). Khoddamzadeh et al. (2011) stated that different techniques have been developed by using various parts. In orchid, the protocols have been established for few genus namely *Cymbidium, Vanda, Phaphiopedilum* and *Phalaenopsis* (Niknejad et al., 2011). For *Phalaenopsis*, culturing by using leaves, flower stalk buds, whole shoots, shoot tip, stems, nodes and roots have been studied (Niknejad et al., 2011). In a research by Babaei et al. (2013), cultures by using shoot tip has high rate of contamination mainly due to its location which is near with the ground.

Niknejad et al. (2011) stated that in-vitro culture are developed based on the concept of totipotency of the cell thus making the techniques widely used in conserving the plants. Organogenesis (shoot differentiation) or embryogenesis (adventive embryony) are the form that expressed by the cellular totipotency (Bhojwani & Dantu, 2013). Many improvement can be done by using tissue culture techniques. Based on Niknejad et al.

(2011), plants from in-vitro have the ability to resist the virus. Probability for plantlets to get somaclonal variation are low when using in-vitro propagation since the techniques able to produce true-to-type plants (Khoddamzadeh et al., 2011). Tissue culture is an essential approach used in plant reproduction, germplasm conservation, research and commercial production (Wang, Wang, Li, Song & Chen, 2016).

It is an advantage to the industry as mass multiplications are possible (Chin, 2016) and are available throughout the years (Chugh, Guha & Rao, 2009). However, small scale industries cannot afford since the cost to build the tissue culture buildings and hire expert are high (Chin, 2016). Due to the high cost needed to do *in-vitro* culture, it is only used for commercial purpose only (Gnasekaran et al., 2010). In order to lessen the time for plant to complete its life cycle, *in-vitro* propagation are used since the growth is slow in conventional propagation where it takes up two to three years (Niknejad et al., 2011). In addition, due to the restrictions and limitations possessed by the natural propagation, for the sake of conservation and commercial production, tissue culture technique has been opted as an alternative method to breed the orchid in large quantity (Shah et al., 2017). Moreover, conventional breeding sometimes can cause unorganized and irregular plant forms. Interference on the level of hormones by infection, insects, microorganisms or genetic tumours might be the cause (Bhojwani & Dantu, 2013). Callus can be induced *in-vitro* by adding the hormones to medium to any normal tissues taken from different part of plants for it to undergo irregular cell division (Bhojwani & Dantu, 2013). Chin (2016) also stated that the successfulness *in-vitro* culture depends on species as each species possess different genotype dependent nature.

Based on Chugh et al. (2009), propagation through tissue culture is greatly studied since 45 years ago for commercialization and *ex-situ* conservation of orchids. Based on

the authors, propagation through tissue culture has long been used on orchid and was the first species of flowering plants to use this technique (Shah et al., 2017).

Despite all the efforts, according to the authors, no matter how high the percentage of plantlets are formed *in-vitro*, plant tissue culture can only be declared successful only if the transfer of plants from *in-vitro* to *ex-vitro* are successful. Most plants from tissue culture are having hard times in adapting to the new environment resulting the plants to die. The acclimatization to greenhouse or field change the environment completely in term of humidity, intensity of light and septic environment (Chugh et al., 2009).

2.4 Murashige and Skoog (MS) Medium

Murashige and Skoog (MS) medium is the medium used *in-vitro* for plant growth. MS media is the most used media due to its components which are compatible with most of the plants. Khoddamzadeh et al. (2011) stated, the composition of medium can change the rate of protocorm-like bodies (PLBs) proliferation and explants survival. In the big scale, low price yet high quality of medium are what industry needed to maintain the protocols established (Gnasakaran, Rathinam, Sinniah & Subramaniam, 2010).

Also, PLBs proliferation are better in MS media. In addition, better development are observed in solid media compared in liquid media where it is believed to work best in proliferation (Khoddamzadeh et al., 2011). In addition, the proliferation of PLBs are effective in half strength MS compared to in Vacin and Went (VW) media (Khoddamzadeh et al., 2011).

Gnasakaran et al. (2010) stated, rapid growth are undergo by PLBs in tissue culture, however, the mechanical support by medium such as nutrients, vitamin and plant growth regulators (PGRs) should be monitored from time to time to avoid the plantlets growth stunted as the elements and quantity in media are important for explants to produce plantlets.

2.5 Hormones

Plant growth regulators (PGRs) or plant hormones are the chemical passengers used to stimulate somatic embryogenesis on the explants (Thomas & Jimenez, 2005). Types of plant parts used, concentration and combination of hormones plays remarkable role in tissue culture (Khoddamzadeh et al, 2011). Each species are differs in amount of hormones needed. Hormone combination of auxin and cytokinin at correct concentration and composition of media is the key to micropropagation (Kosir, Skof & Luthar, 2004).

2.5.1 Napthaleneacetic Acid

Napthaleneacetic acid (NAA) is a synthetic hormone from the auxin family. NAA commonly used to induce roots in plant. The process such as organogenesis, cell expansion, cell division, cell differentiation and gene regulation need auxin to work (Shah et al., 2017).

NAA at high concentrations usually are lethal to the plants. This statement is supported in study done by Khoddamzadeh et al. (2011) by using *Phalaenopsis bellina*

(Rchb.f.) Christenson. The finding shows to stimulate PLBs growth, auxin in low concentration are helpful. However, the data in research by Khoddamzadeh et al. (2011) shows no PLBs are observed in 0.1 mg/L NAA and 1 mg/L TDZ. Other than that, without any TDZ treatment even after the culture was left up to 16 weeks, it shows no proliferation, however browning on the leaf margins and necrosis in explants are observed instead.

Based on the result analysed by Talukder, Nasiruddin, Yesmin, Hassan and Begum (2003), shoot proliferate was better when combination of hormones BAP and NAA are used instead of single use on *Dendrobium* orchid.

2.5.2 Thidiazuron

Thidiazuron (TDZ) is cytokinin, used to induce shoot regeneration when the combination ratio of auxin is lower than cytokinin. A report by Chen, Chang and Chang (2000) stated that among other types of cytokinin, TDZ is the most effective hormone to be used to increase the production of orchids and induce direct somatic embryogenesis by using leaves as explants. The statement also proved by the experiment done by Niknejad et al. (2011) by using *Phalaenopsis Gigantea* where it shows that TDZ is more effective compared to BAP (6-Benzylaminopurine) and KIN (Kinetin) when using leaves as explants to induce callus or PLBs. Some response were shown in BAP but no respond are detected in media combined with KIN (Niknejad et al., 2011). The same study also proved that the combinations of TDZ and NAA at concentration 1 mg/L and 0.1 mg/L respectively produce the highest mean number however, when 0 mg/L or low concentration of TDZ are used, the data shows that no PLBs or callus are induced. In

Khoddamzadeh et al. (2011) study, they found that the best combination between TDZ and NAA are depends on the species used.

According to Khoddamzadeh et al. (2011), PLBs are able to form on the cut surfaces directly even with the presence of TDZ alone when *Phalaenopsis bellina* (Rchb.f.) Christenson are used. Chen and Chang (2006) reported for *Phalaenopsis amabilis*, TDZ are efficient to be used for direct somatic embryogenesis and secondary somatic embryogenesis since both are from epidermal cells and leaf respectively. The study by Niknejad et al. (2011) used BAP and Kin to accelerate the germination of seed and the result shows that combination of 1mg/L BAP and 2mg/L KIN supplemented along with coconut water (cw) works the best when cultured on Vacin and Went (VW) medium.

Chen and Chang (2006) found the induction of PLBs and callus are second most productive when combination of hormones between TDZ and NAA at 0.03 mg/L and 0.01 mg/L respectively are used. On the contrary, Niknejad et al. (2011) in his study revealed the most productive are when 0.03 mg/L TDZ combined with 0 mg/L NAA.

In Khoddamzadeh et al. (2011) and Balilashaki et al. (2014) research, *Phalaenopsis bellina* (Rchb.f.) Christenson and *Phalaenopsis amabilis cv. Cool 'Breeze'* were used respectively. The first emergence of PLBs reported ranged between 2 – 3 mm in diameter can be detected on the medium that use 3 mg/L TDZ as the treatment despite the difference in time of emergence, on 10^{th} week and 6^{th} week respectively. Combination of TDZ and NAA able to induce PLBs but the rate was way higher when using TDZ alone (Khoddamzadeh et al., 2011). After 7 weeks from culture, most media supplemented with TDZ shows growth while there are no protocorms growth detected on media supplemented with either BAP or NAA. There also no growth found at some of the media

with TDZ hormones and is believed due to the size of leaves and it proved that the production of protocorms increase with the augmented concentration of TDZ although the active sample become diminish (Balilashaki et al., 2014).

2.6 Sterile Technique

Contaminations in tissue culture are major setback that hinder the production of *in-vitro* culture. Due to that, steps to prevent or lessen the contamination are needed. Babaei et al. (2013) stated, contamination caused by microorganisms is one of the biggest factor that trammel the process in growing *in-vitro* plants. Contamination is lethal to cultures and it usually either starts from media, tools, working space or explants itself (Paek, Hahn & Park, 2011). Contaminations can appear anytime, not only during the early stage of culture but also during subcultures (Babaei et al., 2013).

As the technology develops, various ways are invented to reduce infections such as by using autoclaves, filtering, microwave ovens, open flame, ethanol or hypochlorite solution (Paek et al., 2011). Babaei et al. (2013) mentioned, to remove contaminations, usually chemicals are used such as sodium hypochlorite, mercuric chloride, antibiotics and fungicides. Paek et al. (2011) again stated, for media and tools, autoclave usually is used to maintain the sterility and prevent infection. Various model, shapes and size of autoclave are available at the market. Autoclave has the ability to sterile things by killing microorganisms and spores due to the combination of high temperature, pressure and time that are used together. Temperature at 121 ^{o}C are used at pressure 15 psi. When the temperature reached 121 ^{o}C, it must stay like that for 30 minutes to increase the effectiveness in killing microbes. The time taken might differs according to the weight or

volume of load. Before autoclave the media, it is advisable to check the types of media first since some media are not resistance to heat (Peak et al., 2011).

Apart from autoclave, open flame such as from Bunsen burner are often used in sterilizing the tools or any surface (Paek et al. 2011) of jars or petri dish because it does not produce any smoke and soot make it clean to be used. Other than that, high temperature flame produced from bunsen burner helps in killing the microorganisms. According to Paek et al. (2011), chemicals such as germicidal reagents also can be used as it is safe for both plants and human. The chemical usage in sterilization not only effective but also affordable for most peoples, make it popular.

2.7 Leave Explant

According to Smith (2013), explant is a small piece of tissue from plants that will be used in tissue culture. The tissues are consist of meristematic cells. Meristematic cells contain undifferentiated cells have the ability to give rise to new plants usually by formation of callus. There are few criteria that need to be considered when choosing the explants, namely the explant age, season, size, plant quality, goal of culture and genotype of plant.

Based on Balilashaki et al. (2014), flower stalk, flower stalk buds and leaves has been used in previous research of *Phalaenopsis* in inducing callus and culture. Various part of plants can be used but using leaves are the most beneficial because it is not only easy to get but also taking it not killing the plant at all (Chugh et al., 2009). In addition, compared to other parts of explants, foliar explants does not need the sacrificial of whole plants and available anytime unlike some parts which depends on the season like

inflorescence parts (Balilashaki et al., 2014) making the donor plant still can grow like normal.

The first experiment using leaves to produce PLBs from *Cymbidium* was first started on 1965 by Wimber (Balilashaki et al., 2014). Niknejad et al. (2011) stated that only few studies have been made using leaf as explant to induce PLBs. They added, when using mature leaves, the induction of PLBs are low even when taken from upper parts. Based on Paek et al. (2011), ethylene released by thin leaf sections are more efficient in forming PLBs and callus compared to when using thick leaf segments (over 5mm) in culture.

Based on Chugh et al, (2009), few researchers in the past tried to culture the mature leaf of *Laelio cattleya cv. Portia 'Mayflower'* and *Epidendrum cv. O'Brienianum*.by using different parts such as tips, petiole, and different sections of blade and base. The result shows that callus and PLBs formation only formed on the leaf tips. However, when the same experiment was done using Vanda hybrid, contrast to the previous result, the present result shows that callus and PLBs formed best on leaf base with 80%.

Explants at the end part of leaves near the stem are said to produce most protocorms and supported in research done by Pengow et al. (2010). Young leaves are better than old leaves for proliferation. Chugh et al, (2009) also stated, age of leaves used as explants plays the vital role in the inducing PLBs. This statement was proved by Balilashaki et al. (2014) in his experiment as it clearly shows age of plants play foremost effect in disinfection. It either can promote or impede the sterilization process. For instance, when old plants is used as the mother plant, the possibility for infection with any virus or bacteria is higher. Other factors are the elements in medium, the

concentration of hormones, source of donor plants, position of explants on medium and which part of leaves are used.

2.8 Phenolic Compound

According to Xu et al. (2015), browning in plant tissue culture is occur frequently and considered as common issue. Also, browning not only occur in tissue culture but in our daily life also as it can easily be seen on cut apples or potatoes. Plants have their own defence mechanisms mainly to protect them from insect oviposition by exudate phenolics, their secondary metabolites (Golan et al., 2017). In tissue culture, those unpleasing changes can results in death therefore the regeneration of explants are failed (Xu et al., 2015). As revealed by Wang et al. (2016), the quinones from the oxidation of phenolic compounds are lethal to the plant tissues, hence explaining why the browning is followed by subsequent death of explants.

Browning issue was also mentioned by Babaei et al. (2013) that another factor that limit *in-vitro* production apart from contamination is browning as it could occur on both explants and media. Browning is a recalcitrant problem that often encountered (Wang et al., 2016). Commonly, the poor growth of explants and even the failure in tissue culture are often linked with the browning (Wang et al., 2016). For the species that easily browning, such as plants with higher secondary metabolites content, although the addition of media supplements such as activated charcoal or ascorbic acid are said could reduce the browning, however the results are not fully satisfactory (Wang et al., 2016) contrasted with statement from Thomas (2008) that phenolic exudation can be reduced with the addition of AC and regular sub culturing. A research on how to reduce the exudation of

phenolics on shoot tip of *Curculigo latifolia* was successful by using ascorbic acid, citric acid, polyvinylpyrrolidone (PVP), bavistin and chloramphenicol for 9 hours straight however, different species and different explants might have different response when the same treatment given (Babaei et al., 2013).

Earlier study by Raghuvanshi and Srivastava (1995) showed the ability for explants to regenerate are tremendously hampered by the exudation of phenolics compound. Chugh et al. (2009) agrees too, although tissue culture are widely used nowadays, there are few limitations when culturing the orchids. Production of phenolic compounds is such a biggest obstacle in tissue culture and due to this, subculture in every 14 days is required (Balilashaki et al., 2014) correspond with what Chugh et al. (2009) revealed in his study that it is advisable to quickly transfer the explants to medium to avoid or lessen the excretion of phenolics which can damage the cells when the explants are oxidized.

In other words, browning is catastrophic because due to its deleterious effects, regenerative ability decrease and poor growth of explants or even death to the explants making the production of culture using *in-vitro* technique restricted (Wang et al., 2016). However there are no further research has been made on the initiating factor and on the basal molecular mechanisms of the *Phalaenopsis* browning (Xu et al., 2015).

2.9 Surface Sterilization

Apart from the tools and media, explants also need to be sterilized before placed in the media. This act is called as surface sterilization. Surface sterilization usually use

dilute chlorine bleach to disinfestation the explants, however the concentration and time to wash are varies depend on explants (Smith, 2013).

According to Paek et al. (2011), germicide is known for having best contact with plant material, however it is advisable to add few drops of wetting agent to the solution first. Even though Clorox, Javex and Purex are the household bleaches, they also said as the best solutions to sterile work area, tools or explants due to its chemical ingredients. If those bleaches are to be used on explants, the solutions must be dilute first to avoid from damaging the explants.

2.10 Activated Charcoal

In tissue culture, the media to the process such as micropropagation, germination of orchid seed, anther culture, elongation of stem, formation of bulb, rooting, protoplast culture, somatic embryogenesis and production of synthetic seed are often added with activated charcoal (AC) as it plays vital role (Pan & Staden, 1999) which is enhance the growth and development of explants (Thomas, 2008).

Activated charcoal is one of the ingredients frequently used in tissue culture as it is not only darken the medium as an act in aiding to validate the polarity but also adsorb any PGR, organic compounds or substance inhibit by the media or explant. AC also could be the cause to the release of natural substance or substance adsorbed by the AC (Pan & Staden, 1999). This statement was supported by Thomas (2008). He stated, AC is such a great absorber and able to adsorb various kind of substance is due to its abundant of very tiny pores and big inner surface area. In addition, AC also reported to be related with stimulatory and inhibitory of the substances that promote three things, first is adsorption of vitamins, second is metal ions and third is PGRs, growth and alteration and darkening

of medium which the substances are naturally released only with the presence of AC (Thomas, 2008).

Based on Thomas (2008), porous and tasteless are what the AC is. All the non-carbon impurities has been removed and has undergone surface carbon oxidation. With the addition of some types of charcoal, the media could turn from acid-catalysed sucrose to fructose and glucose. Fructose and glucose can be categorised as carbon source for *in-vitro* culture too apart from sucrose however the suitability also depends immensely on the type and age of the mother plants (Pan & Staden, 1999). AC also reported by Thomas (2008) to increase the rooting due the partial darkness created which is indistinguishable as the underground environment. Addition of AC to the media are said can reduce or prevent browning is because oxidation of phenols increase with the increase of brightness thus cause the browning (Thomas, 2008). From the literature, it is confirmed that AC indeed plays role in *in-vitro* morphogenic response but the concentrations to be used greatly influenced by things such as purpose, medium, species and explants. The concentrations of AC range between 0.002 g/l to 150 g/l (Thomas, 2008).

Although the addition of AC to the media could give positive effect, nevertheless, it still have negative effect. The study by Pan and Staden (1999) shows that the effect of AC are highly depends on media and tissues used. The statement also agreed by Thomas (2008). He claimed that there also negative or unwanted outcome result from the usage of AC. It may adsorb unwanted substances but it also may adsorb certain PGRs or hormones and making the quantity of PGRs added in the medium unknown. This situation can create problems to the researchers when certain amount are needed to be used since there are no method to know the exact amount of PGRs being adsorbed by AC. In other words, AC is a complex substance and careful study of its effect need to be reviewed before use it.

2.11 Shaker

Rotary shaker is known as micro-stirring technique which affect protein crystallization in cells and also increase flow in the droplet due to the vibration produced (Murai et al., 2008). In development of biotechnology, the most popular option is stirred bioreactors and shake flask however their functions are not the same (Azizan & Buchs, 2017).

Tennenhouse (2018) said constant agitation ensures that the cells are aerated and nutrients are available to help the cells grow uniformly along increase the surface area of the top of the liquid to maximize gas exchange between the liquid and the air due to the circular motion. Flow produced by rotary shaker are contradictory with natural convections where it circulate horizontally (Murai et al., 2008). Variable speeds are needed to accommodate different culture vessel sizes, volumes, and numbers and types of cells. All these factors explain why determining the optimal speed for a particular application is such a common challenge encountered in suspension cell culture (Tennenhouse, 2018)

According to Li and Kurata (2005), the agitation by shaker produce cell growth with better uniformity, better culture control and improved scale up making agitated suspension culture is better than static solid cultures, however, it could lead to hydrodynamic stress and stunting of plant cell growth, metabolites production and organogenesis. Other than that, the finding in a book published by Arditti and Yam (2017) stated that contaminated seed that already germinate could be cleaned by putting them in on shaker in solution and no rinse needed to transfer to media.

2.12 Sonication (Ultrasonic Cleaner)

Ultrasonic cleaner is one form of ultrasound that use inaudible sound frequencies that generally ranging from 20 to 100 kHz where manipulation of cells without destroy it by physical touching is possible due to the existence of ultrasound technology (Gaba et al., 2006). Sonication can manipulate cells and organs also supported by Shin, Bague, Elghanedi, Lee, and Paek (2011). In plant tissue culture, water bath sonicators are used to generate ultrasound as it not only simple and cheap but they also multipurpose (Gaba et al., 2006).

Gaba et al. (2006) mentioned that in the past, to disrupt biological material, many used ultrasound at high frequency. Due to that, during the year between 1950 and 1970s, various large scale research on the effect of low frequency radiation toward biological material were made. Hydrolysis and elimination of bacterial cells are stimulated by sonication making it effective technology to be used in inactivation of microbial (Venkatanagaraju, Kamal, Joy, Induresmi & Xavier, 2018). Gaba et al. (2006) once stated external bacteria were successfully eliminated when mild ultrasound were used along with detergent and it became an option when antibiotics or cleaning products failed to do so. This is because the thinning or cavitation in membrane cells, the release of hydrolytic isoenzymes and free radicals productions due to sonication are what make it able to eliminate microbes (Venkatanagaraju et al., 2018). In addition, to encourage growth on recalcitrant explants or to sort somatic embryos, ultrasound are often used and although ultrasound could put stress or agitate cell membrane, however no effect were observed on fungal growth (Gaba et al., 2006). Other than that, in Shin et al. (2011) published

findings, the usage of ultrasonic cleaner as pre-treatment increase seed germination and protocorm productions.

Based on Gaba et al. (2006), the duration in sonication influence the outcome and it varied from 0.2 seconds to 30 minutes although according to Shin et al. (2011), treatment with more than 8 minutes intensify damaged embryos. For the brief treatment duration, 30 to 120 seconds, shoot production and multiplication are possible both on recalcitrant and non-recalcitrant cells while in five to tenminutes treatment, regeneration and growth are promoted although through excessive water however no hyperhydration are reported. Waxy surface from cell surface and epidermal cell were removed on ten minutes sonication but no injuries are reported. For the maximum exposure which is 30 minutes, the process cause peeled off on external cell wall, explode cells isolation and rupture on explant surface. Apart from that, species and cultivars play big role in the outcome of treatment (Shin et al., 2011).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Material

3.1.1 Plant material

The materials used in this culture was the leaves of *Phalaenopsis* Blackjack. The mother plants were bought at Kota Baharu nursery and grown in nursery near the tissue culture laboratory.

3.1.2 Chemicals

Chemicals used in surface sterilization were tween-20, 70% ethanol, 3% hydrogen peroxide, 20% Clorox solutions and sterile distilled water whilst chemicals that were used in preparing half strength MS (1/2 MS) media was stated in Table 3.1.

3.1.3 Apparatus

The apparatus used were schott bottles, glass jars, beakers, measuring cylinders, conical flask, pipette, pipette pump, funnel, micropipette and tips, analytical and bench top balancer, petri dish, spatula, aluminium foil, hot plate stirrer, magnetic stirrer, 1L jug, scales, pH meter, sterile tape, parafilm, ultrasonic cleaner and rotary shaker.

Table 3.1: Components in Murashige and Skoog (MS) Media

A. Macronutrient	**1X (g/L)**
Ammonium nitrate (NH4NO3)	1.65
Potassium nitrate (KNO_3)	1.90
Calcium chloride (CaCl2.2H2O	0.44
Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$)	0.37
Monopotassium phosphate. (KH_2PO_4)	0.17
B. Micronutrient	**1X (g/L)**
Manganese(II) sulphate ($MnSO_4.4H_2O$)	0.02230
Zinc sulphate ($ZnSO_4.7H_2O$)	0.00860
Boric acic (H_3Bo_3)	0.00620
Potassium Iodide (KI)	0.00083
Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.00025
Cupric sulphate ($CuSO_4.5H_2O$)	0.000025
Cobalt (II) chloride ($CoCl_2.6H_2O$)	0.000025
C. Vitamin	**1X (g/L)**
Myo – inositol	0.1
Glycine ($C_2H_5NO_2$)	0.002
Thiamine HCL (Vitamin B1)	0.001
Nicotinic acid (Vitamin B3)	0.0005
Pyridoxine (Vitamin B6)	0.0005
D. Ferum Source	**1X (g/L)**
$FeSO_4.7H_2O$	0.0278
$Na_2EDTA.2H_2O$	0.0373
E. Others	**1X (g/L)**
Sucrose	30
Agar	8

3.2 Methodology

3.2.1 Stock Solution Preparation

Four set of stock solutions based on nutrient groups were prepared one by one. Medium concentration were set along with the amount, then the desired amount of ingredients were calculated and weighed. First preparation was macronutrients. Two-third distilled water of desired final amount was put in a jug and the ingredients were added, one by one, in sequence, based on the amount weighed. During the process of adding up the ingredients, the solution was constantly but slowly stirred by using hot plate stirrer. Next ingredients only were added when the previous ingredients were completely dissolved. Once all the ingredients were added and soluble, distilled water was added again until reach the final volume. Once the preparations are done, the solutions was poured in schott bottle. The bottle then will be labelled with information such as type of stock, media type, instructions, production date and it belongs to who. Once labelled, the stock solution was stored at 4 ℃ in refrigerator. The same procedures were repeated to prepare stock solutions for micronutrients, vitamin and ferum.

3.2.2 Hormones Preparation

For the hormones, 1 mg/ml stock solution were prepared. First, 100 mg of NAA hormone was weighed and added into a 100 ml volumetric flask. Then, 2-5 ml of solvent such as NaOH (depends on the hormone used) was added to dissolve the NAA. The

mixture was stirred until all the hormones was dissolved. Next, without stopping the stirring, distilled water was added little by little, until the solution reach 100 ml. The stocks then was kept in schott bottles then stored at 4° C in refrigerator. The same process was repeated again to prepare stock solution for TDZ hormones.

3.2.3 Half (½) Strength MS Media Preparation

Five hundred (500) ml of half (1/2) strength MS media to culture the leaves was prepared. First, 400 ml of distilled water was added into 500 ml conical flask. A magnetic stirrer was put in conical flask to aid in constant stirring. 15 g of sugar/sucrose was added in conical flask and was waited until it dissolved. Next, macronutrient, micronutrient, vitamin and ferum source will be added into the solutions one by one according to the calculation based on desired amount of final volume. Hormones then were added according to the treatment as stated in Table 3.3. Once all were added and dissolved, distilled water was added again until it reach 450 ml. The solution was continuously stirred. The next step was measure the pH solution by using pH meter. The pH were adjusted until it become pH 5.6 – 5.8 by either add 1 M NAOH or 1M HCl. One drop by one drop was added while stirring until desired pH is reached. Once the desired pH was reached, distilled water was added again to make up the final volume. Only after that, 4g of agar was added into the solution and was kept stirred until all elements are dissolve except agar. For the media that use activated charcoal (AC), 1 g/L AC was weighed, added and stirred into the solution. The solutions then was microwaved to melt the agar. Once the agar melt, the solutions will be let to cool down for a bit. Lastly, the medium was dispensed 30 – 40 ml each into 100 ml jars. Before autoclave the medium, the cap of

glass jars were closed loosely. Media in the jars was sterilized by autoclaving it at 121 °C for 15 minutes. The autoclaved media were stored in the dark room at room temperature maximum for 2 weeks.

Table 3.2: The combinations of hormones for each treatments

TDZ (mg/L) / **NAA (mg/L)**	**0**	**1.0**	**3.0**
0	T1	T2	T3
0.1	T4	T5	T6
1.0	T7	T8	T9

Notes: 3 replications were done for each treatments

T1: Control

T2: 0 mg/L NAA + 1 mg/L TDZ

T3: 0 mg/L NAA + 3 mg/L TDZ

T4: 0.1 mg/L NAA + 0 mg/L TDZ

T5: 0.1 mg/L NAA + 1 mg/L TDZ

T6: 0 mg/L NAA + 3 mg/L TDZ

T7: 1 mg/L NAA + 0 mg/L TDZ

T8: 1 mg/L NAA + 1 mg/L TDZ

T9: 1 mg/L NAA + 3 mg/L TDZ

3.2.4 Explant Preparation

Leaves was used as explants in this study. The explants was taken from the purchased plant that was grown in nursery. *Phalaenopsis* Blackjack originates from Taiwan was used as mother/donor plant. Young and healthy leaves of the *Phalaenopsis*

Blackjack from nursery was taken to be used as explant source. The leaves were excised from the stem by using scissor and was surface sterilized first before culture.

3.2.5 Surface Sterilization

Surface sterilization is needed to be done in order to provide sterile explants. There are 3 different method were used to surface sterilize the explants. Plant part which is leaves to be used as explant were harvested from the mother plant grown in nursery. Young and healthy leaves that excised from mother plants were washed three (3) minutes under running tap water. After three minutes, the washed leaves were put in schott bottles and moved under laminar flow to provide sterile conditions for the next step. The leaves were submerged five (5) minutes in few drops of Tween-20 solution followed by 30 seconds in 70% ethanol and five (5) minutes in 3% hydrogen peroxide. The next step of sterilization depends on the method used. For method (A), the leaves were submerged ten (10) minutes in 20% Clorox solutions while for method (B) and (C), the leaves were submerged 15 minutes in 20% Clorox solutions. During the submersion, for all the method A, B and C, rigorous shaking were done under laminar flow hood. After that, for method (A), the leaves were immediately sonicated ten (10) minutes at full power by using ultrasonic cleaner while for method (B) and (C), the leaves were shake 20 minutes by using rotary shaker (Table 3.3). Next, in method A, B and C, the leaves then were rinsed three (3) times with sterile distilled water, each after pouring off sterilant. Lastly, the leaves were transferred to petri dish for 5 minutes for brief drying. The explants then were ready to be inoculated on prepared media. All the dilution of solution in these steps were done under laminar flow by using sterile distilled water.

Table 3.3: Surface sterilization methods

METHOD	DESCRIPTION
A	10 minutes sonication + 10 minutes clorox
B	Shaker 15 minutes
C	Shaker 15 minutes + Activated charcoal in media

3.2.6 Culture the Explants

Once the surface sterilization process was finished, the surface sterilized leaves were cut into small square pieces, 1cm x 1 cm accordingly. The cut explants were quickly inoculated, horizontally, on ½ strength MS basal medium supplemented with combinations of TDZ and NAA at different concentrations. All the explants from surface sterilization method A and B were inoculated on ½ strength MS media added with combinations of NAA and TDZ at various concentration (Table 3.3) while the explants from method C were inoculated on ½ strength MS media added with combinations of NAA and TDZ at various concentration and 1 g/L activated charcoal (Table 3.4). Each glass jars contained three (3) explants. During the culture, sterile techniques were practiced. Three (3) replications were done for each treatment. The culture was incubated in growth medium at 25 ± 1 ^{o}C under 16 hours photoperiod using fluorescent tubes.

Young and healthy leaves were excised and washed 3 minutes under running tap water.

The process of surface sterilization then moved in the laminar flow.

The leaves were submerged 5 minutes in 1% tween-20, followed by 30 seconds in 70% ethanol and 5 minutes in 3% hydrogen peroxide. The dilution were made by using sterile distilled water.

For method A, the leaves were submerged 10 minutes in 20% clorox solutions.

For method B & C, the leaves were submerged 15 minutes in 20% clorox solutions.

During the submersion, rigorous shaking were done under laminar flow hood

After that,
for method A, the leaves immediately sonicated 10 minutes at full power by using ultrasonic cleaner.

For method B & C, the leaves were put in the shaker for 20 minutes

(refer Table 3.4)

Next, the leaves were rinsed 3 times with sterile distilled water, each after pouring off sterilant.

The leaves then were transferred to sterile petri dish for 5 minutes for brief drying.

Figure 3.1: Flowchart of surface sterilization method

3.2.7 Statistical Analysis

The development of *in-vitro* culture were monitored every week. There was three replication were made on each jar. Survival rate, contamination rate and appearance of explants were observed and analysed by using one way analysis of variance (ANOVA) through SPSS software application. The best method for surface sterilization of explants and best combination of hormones were discovered.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Result

To develop *in-vitro* culture for *Phalaenopsis* Blackjack orchid, a study was done to find out the best surface sterilization method along with the best NAA and TDZ combinations at different concentration. Leaves of *Phalaenopsis* Blackjack were dissected from the mother plant to be used as the explant. Each jar contained 3 explants each with 1 cm x 1 cm sized explants. Each treatment were replicated 3 times so the total explants in jars for each method would be 81.

There were nine treatments (Table 3.2) each with different concentration of hormones combination were used. Other than that, three methods (Table 3.3) of leave surface sterilization also were used to provide sterile explants for the culture which were (A) 10 minutes sonication with 10 minute Clorox method, (B) 20 minutes shaker and (C) 20 minutes shaker with AC in media. There were three main parameters that are discussed in this chapter. First is the survival rate of control explants between method A, B and C (Figure 4.1). Second is contamination rate between treatments and methods (Table 4.1 and Figure 4.2) and lastly, the appearance of explants. Based on these three parameters,

the best surface sterilization method and the best hormone combinations to develop *in-vitro* culture of *Phalaenopsis* Blackjack were determined.

4.1.1 Survival Rate

In the first parameter which is survival rate, only T1 (0 mg/L NAA + 0 mg/L TDZ) which act as control (Table 3.3) were used to compare survival rate between three surface sterilization methods in week 1. Survival were determined by the color of explants on first week after culture then were analysed by using One way ANOVA test. Explants that are still green, healthy and not undergo either chlorosis or necrosis were considered to survive.

Figure 4.1 shows survival rate of control explants in each surface sterilization method. The graph bar shows method A (10 minutes sonication) has the highest survival rate which is $56.79^{a} \pm 4.29$ followed by method C (20 minutes shaker with AC in media), $50.61^{a} \pm 4.12$. The lowest survival rate on week 1 was $45.67^{a} \pm 4.41$ which belong to method B (20 minutes shaker). From the Pos Hoc test, method A, B and C were not significantly different towards each other however since method A has the highest mean value, it is considered the most effective method in surface sterilize leave of *Phalaenopsis* Blackjack orchid.

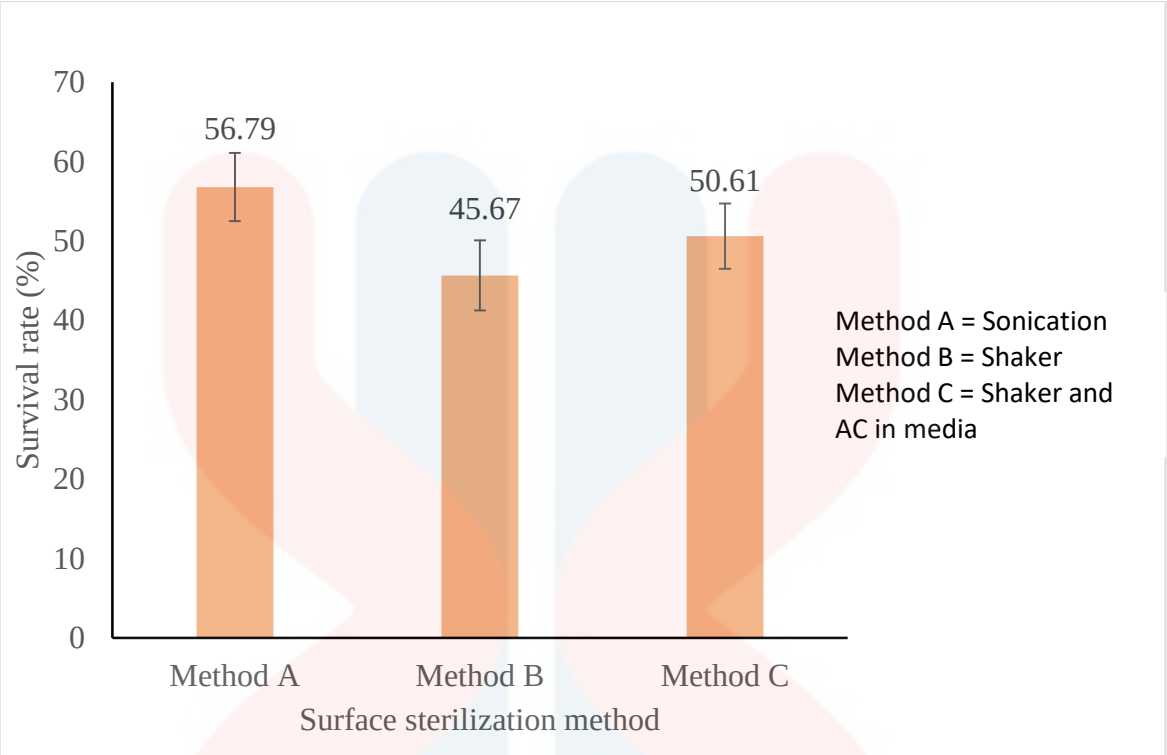

Figure 4.1: Survival rate of control explants in three surface sterilization methods.

4.1.2 Contamination Rate

Table 4.1 and Figure 4.2 shows the survival rate and contamination rate after 28th day of culture. T5 (0.1 mg/L NAA and 1 mg/L TDZ) has the highest survival rate, 55.55 ± 12.42 followed by T7 (1 mg/L NAA and 0 mg/L TDZ) and T4 (0.1 mg/L NAA and 0 mg/L TDZ), 37.03 ± 6.67 and 37.03 ± 12.96 respectively. T2 (0 mg/L NAA and 1 mg/L TDZ) has the lowest survival rate only with 7.40 ± 7.40. For the contamination rate, the highest contamination is T2 (0 mg/L NAA and 1 mg/L TDZ) with 92.59 ± 7.40. Second highest contaminations is T9 (1 mg/L NAA and 3 mg/L TDZ) with 81.48 ± 8.07 followed by T6 (0 mg/L NAA and 3 mg/L TDZ) with 77.77 ± 11.11. The lowest contaminations is T5 (0.1 mg/L NAA and 1 mg/L TDZ) with 44.44 ± 12.642.

Based on the Pos Hoc test of survival rate, there were only two treatments that consist of one subset. T5 (0.1 mg/L NAA and 1 mg/L TDZ) and T2 (0 mg/L NAA + 1 mg/L TDZ) shows the mean number of contamination rate and survival rate are significantly different compared to the mean number of other treatments. The mean number for T1 (0 mg/L NAA and 0 mg/L TDZ), T3 (0 mg/L NAA and 3 mg/L TDZ), T4 (0.1 mg/L NAA and 0 mg/L TDZ), T6 (0 mg/L NAA and 3 mg/L TDZ), T7 (1 mg/L NAA and 0 mg/L TDZ), T8 (1 mg/L NAA and 1 mg/L TDZ) and T9 (1 mg/L NAA and 3 mg/L TDZ) shows no statistically significance between each other since the mean number are exist on two same subset. Since T5 has the highest value compared to T2, it proved that T5 is better than T2.

Table 4.1: Survival rate and contamination rate between treatments.

Treatments	**Survival rate (%)**	**Contamination rate (%)**
T1	25.92 ± 10.79	74.07 ± 10.79
T2	7.40 ± 7.40	92.59 ± 7.40
T3	25.92 ± 9.25	74.07 ± 9.25
T4	37.03 ± 12.96	62.96 ± 12.96
T5	55.55 ± 12.42	44.44 ± 12.42
T6	22.22 ± 11.11	77.77 ± 11.11
T7	37.03 ± 6.67	62.96 ± 6.67
T8	29.62 ± 11.71	70.37 ± 11.71
T9	18.51 ± 8.07	81.48 ± 8.07

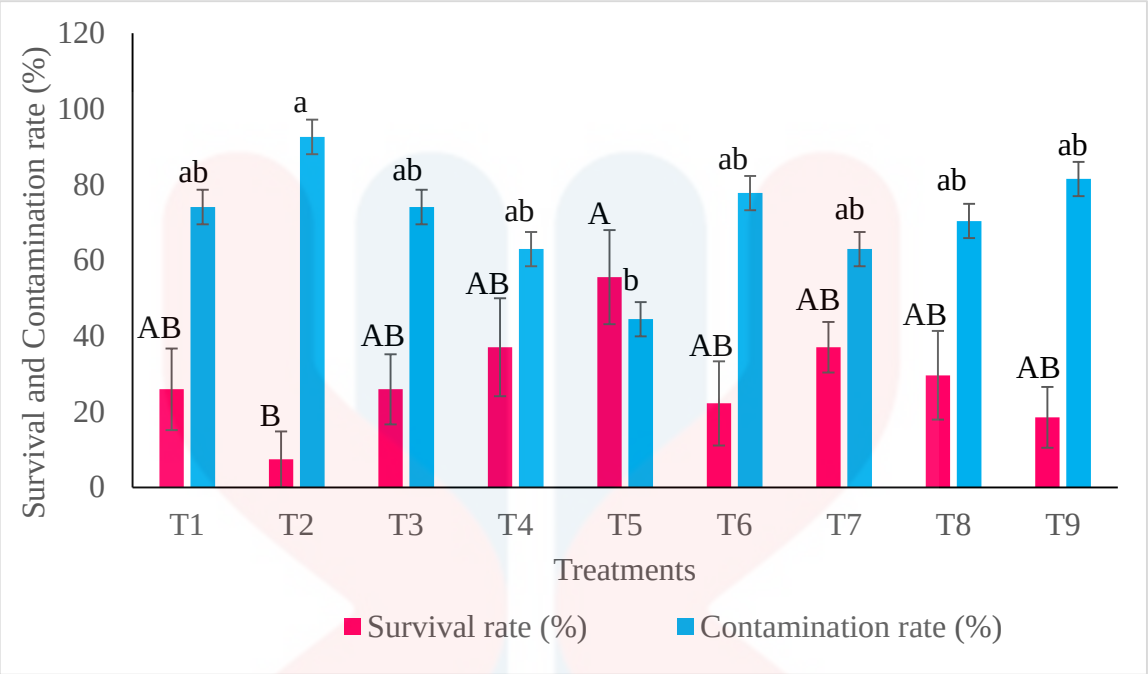

Figure 4.2: Survival rate and contamination rate.

4.1.3 Appearance

Other than survival rate and contamination rate, appearance of explants also were observed. For T1 (control), all explants from three methods has same appearance, browning, chlorosis, dark media browning and heavy contamination. For T2 (0 mg/L NAA and 1 mg/L TDZ), all the explants undergoes necrosis and heavy contamination however only method A, the media changed color due to the browning. In T3 (0 mg/L NAA and 3 mg/L TDZ), the explant stay greenish only in method A, other than the appearance of explants were browning, chlorosis, light media browning, heavy contamination. Both method B and method C in T4 (0.1 mg/L NAA and 0 mg/L TDZ), the media changed colour to cloudy and believed were the result from heavy contamination. However, the explants of method A were still greenish although undergoes browning.

In T5 (0.1 mg/L NAA and 1 mg/L TDZ), there were two methods that have greenish explants, in method B and method C although the media has turned to cloudy although both methods has light contamination only. All the explants in T6 (0 mg/L NAA and 3 mg/L TDZ) undergoes necrosis and heavy contamination. Media in method B and method C also changed color to cloudy. In T7 (1 mg/L NAA and 0 mg/L TDZ), all the explants from three different methods undergoes necrosis, the death of tissue. Heavy contamination were also detected. In method B and method C at T8 (1 mg/L NAA and 1 mg/L TDZ), heavy contamination were observed although green, healthy explants are still present. The chlorosis explants in method A has light contamination only. Lastly was T9 (1 mg/L NAA and 3 mg/L TDZ). All explants from three method of surface sterilization undergoes browning, chlorosis and heavy contamination. The media also changed in colour to browning.

Based on the observations on appearance (Table 4.2 and Figure 4.3), most explants undergoes the same changes. Generally, after 28th day after culture, most of the explants undergo either chlorosis or necrosis. Chlorosis is when the explants is yellow in colour due to the loss of green coloration while necrosis is when the explants turn colourless, the death of tissues. Other than that, most of the explants were heavily contaminated either by bacterial or fungal. Some of the media also changed in colour either it become cloudy or yellow.

Apart from that, browning also a major problem in this study. The browning eventually result in death of tissue making the development of *in-vitro* culture are failed (Xu et al., 2015). This also supported by Wang et al. (2016) that the failure in tissue culture is often related with the release of phenolic compounds.

As for the contamination, the only factor that affect the survival rate of explants is surface sterilization methods. Surface sterilization is very important step when *ex-vitro* plants were used as mother plant since it grows in uncontrolled environment and easily will get early contamination if the environment are favourable to the surrounding microbes such as from air or soil. *In-vitro* is a technique that greatly depends on aseptic conditions for the establishment of plants hence, due to that, when plants grown on field are used directly as source of plants, it considered as suicide. What is more is when the explants used are so close with the ground (Babaei et al., 2013).

Table 4.2: Appearance of explants in week four

TREATMENT	METHOD OF SURFACE STERILIZATION	APPEARANCE
1	A	Browning, chlorosis, dark media browning, light contamination
	B	Browning, chlorosis, dark media browning, heavy contamination
	C	Browning, chlorosis, dark media browning, heavy contamination
2	A	Necrosis, dark media browning, heavy contamination
	B	Browning, necrosis, heavy contamination
	C	Browning, necrosis, heavy contamination
3	A	Greenish, browning, chlorosis, light media browning, heavy contamination
	B	Browning, chlorosis, light media browning, heavy contamination
	C	Browning, chlorosis, light media browning, heavy contamination
4	A	Greenish, browning, light media browning
	B	Necrosis, medium turns cloudy, heavy contamination
	C	Necrosis, medium turns cloudy, heavy contamination
5	A	Browning, chlorosis, light media browning
	B	Greenish, browning, media turns cloudy, light contamination
	C	Greenish, browning, media turns cloudy, light contamination
6	A	Necrosis,, heavy contamination
	B	Necrosis, medium turns cloudy, heavy contamination
	C	Necrosis, medium turns cloudy, heavy contamination
7	A	Necrosis, dark media browning, heavy contamination
	B	Necrosis, chlorosis, media turns yellowish, heavy contamination
	C	Necrosis, chlorosis, media turns yellowish, heavy contamination
8	A	Browning, chlorosis, dark media browning, light contamination
	B	Greenish, browning, media turns yellowish, heavy contamination
	C	Greenish, browning, media turns yellowish, heavy contamination
9	A	Browning, chlorosis, light media browning, heavy contamination
	B	Browning, chlorosis, media turns cloudy, heavy contamination
	C	Browning, chlorosis, media turns cloudy, heavy contamination

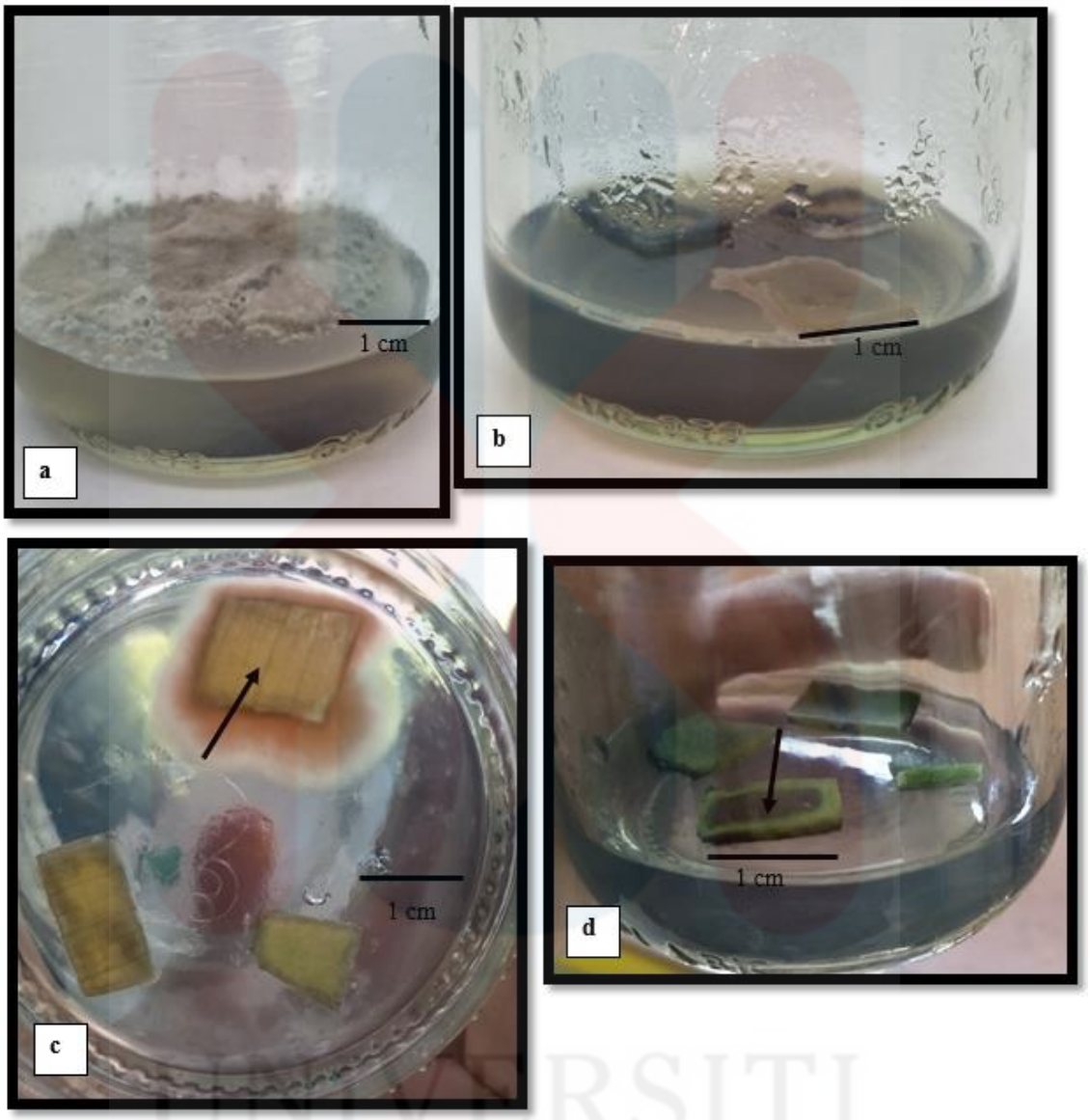

Figure 4.3: The appearance of *Phalaenopsis* Blackjack explants. a) The fuzzy growth of fungal on method B, T2. b) Necrosis of explant cultured on ½ strength MS media supplemented with AC. c) Necrosis explant and d) Browning of explants and change in the colour of media.

4.2 Discussion

4.2.1 Surface Sterilization

To develop successful culture and prevent attack of microbes on culture, surface sterilization of explants are essential. There are various methods and equipments used in other studies however the effectiveness and successfulness of the method used highly depends on species and explants used (Smith, 2013).

The first objective in this study was to study the effect of different surface sterilization technique toward *Phalaenopsis* Blackjack orchid. Based on the result obtained from ANOVA, the p-value is greater than 0.05 which mean there was no statistically significance between surface sterilization methods hence, null hypothesis, different surface sterilization technique has same effect towards *in-vitro* culture of *Phalaenopsis* Blackjack orchid is rejected. It then can be said that different surface sterilization technique has different effect towards *in-vitro* culture of *Phalaenopsis* Blackjack orchid.

Based on Post Hoc test, it shows there was no significance differences between surface sterilization method since only one subsets are present in all surface sterilization methods however method A (sonication) shows the highest value which means method A (sonication) is better than method B (shaker) and method C (shaker and AC in media) in leave surface sterilization of *Phalaenopsis* Blackjack.

There were two equipments used to surface sterilize the explants for the development of in-vitro culture of *Phalaenopsis* Blackjack orchid, ultrasonic cleaner and

shaker. The general idea for both equipments was to disrupt the cell of plants. The micro-vibration created are able to dissociate the contaminants on the leaves, removing any debris on it.

Explants that undergo sonication method has the highest survival rate compared to explants that used shaker because although shaker did agitated the solution which the which increase sterilized surface area however ultrasonic cleaner manipulate the cells by using ultrasound make it effectual in inactivate the microbial (Vengkatanagaraju et al., 2018). The effectiveness of using ultrasonic cleaner in surface sterilization process also supported by Gaba et al. (2006). In the published report, it stated the usage of mild ultrasound with detergent were successfully remove external bacteria compared to antibiotics or cleaning products when used alone. In the current study, ten minutes of sonication were used to reduce contamination of microbes thus increase development of *in-vitro* culture and it was correspond with Gaba et al. (2006) investigation, that the duration in sonication influence the outcome and in 5 to 10 minutes treatment, regeneration and growth were promoted.

4.2.2 Hormone Combinations

In the present study, apart from different surface sterilization method, different treatments were also used. Combination of hormones (NAA and TDZ) with different concentrations were used to find out which combinations were the best to develop *in-vitro* culture of *Phalaenopsis* Blackjack orchid. Kosir et al. (2004) revealed that the amount of hormones needed to micropropagate the explants are varies according to

species. Due to that, it is important to study the effect of the amount and concentration toward *Phalaenopsis* Blackjack.

There were statement by Khoddamzadeh et al. (2011) stated that auxin at low concentration are beneficial as high concentration of NAA are harmful to the plants. Contrasted with NAA, based on Balilashaki et al. (2014), the PLBs induction increase with the augmented of TDZ. Apart from that, there was also a report that suggest the use of BAP was more effectual compared by using TDZ in micropropagation and the statement was based on result when leaves of *Phalaenopsis amabilis* var. 'Manila' was used (Balilashaki & Ghehsareh, 2016). Contrasted with those findings, many findings in previous studies shows that under the same conditions, TDZ was far better than BA in induction of adventitious shoot (Fan, Zhu, Zhu, Xu & Li, 2010). This also supported in a research done by Tao, Yu, Kong and Zhao (2011) which stated the TDZ was better than BA on *Cymbidium faberi Rolfe*. They added, the combinations of TDZ and NAA on PLBs of *Cymbidium faberi Rolfe* could increase the rate of shoot germination. In Lee and Chen (2014) findings, they found that single use of TDZ at range 0.1 mg/L to 1 mg/L does not give any positive result in callus and protocorm-like-bodies (PLBs) of *Dendrobium huoshanense*, instead it needs to be combined with 1mg/L of hormone 2.4-D.

From the analysed results, T5, ½ strength MS media supplemented with 0.1 mg/l NAA and 1 mg/l TDZ was the best hormone combinations to be used to develop *in-vitro* culture of *Phalaenopsis* Blackjack orchid. This finding was in line with the findings by Niknejad et al. (2011) which in their study, combinations of 0.1 mg/L NAA and 1 mg/L TDZ was the best in production of callus and protocorms of *P. Gigantea*. In Chen and Chang (2006) findings, combination auxin and cytokinin at 0.03 mg/l TDZ and 0.01 mg/l NAA was effective in induce PLBs when explant from *Vanda* spp. leaf were used. Different result were found in Khoddamzadeh et al. (2011) when leaf of *Phalaenopsis*

bellina (Rchb.f.) Christenson were used. Combination NAA: TDZ at 1.0 mg/l: 3.0 mg/l were the best in induce PLBs.

In Chen and Chang (2004) published findings, they found 3 mg/L of TDZ was the most effective in embryogenesis when seed of *Phalenopsis amibilis* 'Formosa' were used contrasted with the result in the present study, T6, which also used 0 mg/L NAA and 3 mg/L TDZ where it does not show any positive result. On the fourth week, there were no development of culture detected on free-hormone media (T1). This result was in agreement with the findings from Balilashaki and Ghehsareh (2016), that there were no protocorms produced on control explants instead only chlorosis and necrosis were detected on *P. amibilis* var. 'Manila'. The same was also reported on older report, Vendrame and Maguire (2007) when using *Doritaenopsis* Purple Gem 'Ching Hua'.

Almost all the explants in the current study undergoes browning which was one of the major cause of failure in development of culture apart from chlorosis, necrosis and contamination. The browning which caused by the release of phenolic compounds was also reported on *Phalaenopsis amabilis* var. Formasa shimadzu. The phenolics had caused the oxidation to the medium which phytotoxic to the explants. One of the way to overcome is by constant subculture (Chen & Chang, 2004). As for the chlorosis or necrosis of explants, based on Balilashaki and Ghehsareh (2016), although increase in concentration of hormones were said to increase protocorms production, it also caused the chlorosis and necrosis of plants which at the end will cause failure to develop as culture.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Based on the recorded and analysed data, different surface sterilization methods and different treatments do have different effect on development of *in-vitro* culture of *Phalaenopsis* Blackjack orchid. Method A which was sonication method was the best surface sterilization technique towards *in-vitro* culture of *Phalaenopsis* Blackjack orchid while T5, 0.1 mg/L NAA and 1 mg/L TDZ was the best hormone combinations toward *Phalaenopsis* Blackjack orchid multiplication.

5.2 Recommendation

In the current study, there are few limiting factors that retard the development of *in-vitro* culture of *Phalaenopsis* Blackjack orchid. In tissue culture, aseptic techniques and environment are needed to develop and maintain *in-vitro* plants making asepsis condition is crucial. As everyone aware, contaminations is one of the major problems in tissue culture and it also major problem in this current study. For the further studies, here

are some recommendations to be practiced and few precautions should be taken for the better result.

First is when taking care of mother plant in the greenhouse. As we all well aware, there are many microbes in the air and in the soil. Few days prior taking the explants from mother plant, avoid from excess watering and soil splashing during watering as these two can increase the initial contamination. If initial contamination increase, the probability for it to contaminate when cultured also increase.

Second is the usage of sodium hypochlorite. In the current study, Clorox were used. The concentration and time used in surface sterilization in this study might not fit for this species and explants thus leading to either inadequate sterilization or phytotoxicity which at the end caused contaminations and chlorosis to explants making explants unable to develop.

Other than that, the size of excised explants should be cut slightly larger than intended size so that after the surface sterilization process and during subculture, the damaged cut or browning cut could be removed for the better development of culture. By doing this, damaged cut at the sides could be removed for better explant development.

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APPENDICES

Dilution Equation

By using this equation, the volume or concentration of solutions used were to find out.

$$M_1V_1 = M_2V_2$$

Where;

M_1 = Stock concentration

V_1 = Stock volume

M_2 = Media concentration

V_2 = Media volume

Table A 1: Survival rate of control explants

Surface sterilization method	Survival rate
Method A	56.79 ± 4.29
Method B	45.67 ± 4.41
Method C	50.61 ± 4.12

Table A 2: Mean and standard error

Descriptives

Survival_Rate

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Method A	27	56.7904	22.29556	4.29078	47.9705	65.6102	33.33	100.00
Method B	27	45.6785	22.92555	4.41202	36.6095	54.7476	.00	100.00
Method C	27	50.6167	21.42694	4.12362	42.1405	59.0929	33.33	100.00
Total	81	51.0285	22.41657	2.49073	46.0718	55.9852	.00	100.00

Descriptives

Contamination_Rate

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
T1	9	74.0756	32.39380	10.79793	49.1755	98.9756	.00	100.00
T2	9	92.5922	22.22333	7.40778	75.5099	109.6746	33.33	100.00
T3	9	74.0744	27.77867	9.25956	52.7219	95.4270	33.33	100.00
T4	9	62.9644	38.88905	12.96302	33.0717	92.8572	.00	100.00
T5	9	44.4456	37.26892	12.42297	15.7981	73.0930	.00	100.00
T6	9	77.7789	33.33292	11.11097	52.1569	103.4008	.00	100.00
T7	9	62.9644	20.03254	6.67751	47.5661	78.3628	33.33	100.00
T8	9	70.3711	35.13673	11.71224	43.3626	97.3796	.00	100.00
T9	9	81.4822	24.21617	8.07206	62.8680	100.0964	33.33	100.00
Total	81	71.1943	31.95204	3.55023	64.1291	78.2595	.00	100.00

Table A 3: Post Hoc test

Survival_Rate

Tukey HSD[a]

Surface_Sterilization_Method	N	Subset for alpha = 0.05
		1
Method B	27	45.6785
Method C	27	50.6167
Method A	27	56.7904
Sig.		.164

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 27.000.

Contamination_Rate_Week_4

Tukey HSD[a]

Treatments	N	Subset for alpha = 0.05	
		1	2
T5	9	44.4456	
T4	9	62.9644	62.9644
T7	9	62.9644	62.9644
T8	9	70.3711	70.3711
T3	9	74.0744	74.0744
T1	9	74.0756	74.0756
T6	9	77.7789	77.7789
T9	9	81.4822	81.4822
T2	9		92.5922
Sig.		.227	.522

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

Survival_Rate_Week_4

Tukey HSD[a]

Treatments	N	Subset for alpha = 0.05	
		1	2
T2	9	7.4078	
T9	9	18.5178	18.5178
T6	9	22.2211	22.2211
T1	9	25.9244	25.9244
T3	9	25.9256	25.9256
T8	9	29.6289	29.6289
T4	9	37.0356	37.0356
T7	9	37.0356	37.0356
T5	9		55.5544
Sig.		.522	.227

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

Table A 4: ANOVA test

ANOVA

Survival_Rate

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1673.759	2	836.879	1.694	.190
Within Groups	38526.445	78	493.929		
Total	40200.204	80			

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Contamination_Rate_Week_4	Between Groups	13277.701	8	1659.713	1.747	.102
	Within Groups	68396.939	72	949.957		
	Total	81674.640	80			
Survival_Rate_Week_4	Between Groups	13277.701	8	1659.713	1.747	.102
	Within Groups	68396.939	72	949.957		
	Total	81674.640	80			