



Faculty of Resource Science and Technology

**EFFECTS OF BENZYLAMINOPURINE (BAP) CONCENTRATIONS ON
PROLIFERATION OF *Dendrobium* HYBRID ORCHID AND *Arundina graminifolia***

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**This project is submitted in partial fulfilment of the requirements for the degree of
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DECLARATION

I declare that this work entitled “Effects of benzylaminopurine (BAP) concentrations on proliferation of *Dendrobium* hybrid orchid and *Arundina graminifolia*” is the result of my own work except as cited in the references. I have properly cited all the sources of information that have been used in this thesis.

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TABLE OF CONTENTS

ACKNOWLEDGEMENT		II
DECLARATION		III
TABLE OF CONTENTS		IV
LIST OF ABBREVIATIONS		VI
LIST OF TABLES		VII
LIST OF FIGURES		VIII
ABSTRACT/ABSTRAK		IX
CHAPTER I	INTRODUCTION	1
CHAPTER II	LITERATURE REVIEW	
2.1	Family Orchidaceae	3
	2.1.1 <i>Dendrobium</i> Hybrid Orchid	4
	2.2.2 <i>Arundina graminifolia</i>	5
2.2	Propagation of Orchids	7
	2.2.1 Conventional Propagation of Orchids	7
	2.2.2 <i>In vitro</i> Propagation of Orchids	8
2.3	Micropropagation of Orchids	9
	2.3.1 Selection of Explant	9
	2.3.2 Media Composition	10
2.4	Protocorm Proliferation	11
2.5	Benzylaminopurine (BAP)	12

CHAPTER III	MATERIALS AND METHODS	
3.1	Plant Materials	13
	3.1.1 <i>Dendrobium</i> Hybrid Orchid	13
	3.1.2 <i>Arundina graminifolia</i>	14
3.2	Apparatus and Materials	15
3.3	Preparation of Media	15
3.4	Surface Sterilization of Explants	16
	3.4.1 Leaf Explant	16
	3.4.2 Protocorm Explant	16
3.5	Inoculation of Explants	16
	3.5.1 Leaf Explant	16
	3.5.2 Protocorm Explant	17
3.6	Data Collection	17
	3.6.1 Leaf Explant	17
	3.6.2 Protocorm Explant	17
3.7	Statistical Analysis	18
CHAPTER IV	RESULTS AND DISCUSSION	
4.1	Effect of BAP on Proliferation of <i>Dendrobium</i> Hybrid Orchid	20
4.2	Effect of BAP on Proliferation of <i>A. graminifolia</i>	27
CHAPTER V	CONCLUSIONS AND RECOMMENDATIONS	34
REFERENCES		35
APPENDICES		39

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BAP	6-benzylaminopurine
DAC	Days after culture
DMRT	Duncan's multiple range test
HCl	Hydrochloric acid
MS	Murashige and Skoog (1962)
NAA	1-naphthaleneacetic acid
NaOH	Sodium hydroxide
PLB	Protocorm-like body
PGR	Plant growth regulator
SPSS	Statistical Package for the Social Sciences
TDZ	Thidiazuron

LIST OF TABLES

Table		Page
4.1	Effect of BAP on the protocorm proliferation of <i>A. graminifolia</i> at 60 DAC	27

LIST OF FIGURES

Figure		Page
2.1	<i>Dendrobium</i> hybrid orchids. (A) <i>Dendrobium</i> Sonia and (B) <i>Dendrobium bigibbum</i> hybrid	5
2.2	<i>Arundina graminifolia</i> . (A) Flower of <i>A. graminifolia</i> and (B) <i>A. graminifolia</i> plantation	6
3.1	<i>Dendrobium</i> hybrid mother plant	13
3.2	Leaf explants. (A) Young leaves of <i>Dendrobium</i> and (B) Leaf explants cut in size of 0.2-0.4 cm	13
3.3	<i>Arundina graminifolia</i>	14
3.4	Formation of green protocorms (arrow) at 65 days after culture (DAC)	14
4.1	Browning of leaf explants of <i>Dendrobium</i> at their cutting edge after 30 days of culture	20
4.2	Effect of combination of BAP and NAA on leaf explants of <i>Dendrobium</i>	21
4.3	Swelling of the leaf explant of <i>Dendrobium</i> on ½ MS supplemented with 1.0 mg/L BAP and 1.0 mg/L NAA. (A) Swelling of leaf explant after 60 days of culture and (B) Swelling of leaf explant under microscope	22
4.4	Proliferation of protocorms on MS medium supplemented with 1.0 mg/L BAP. (A) Formation of new protocorm (arrow); (B)-(C) Proliferation of protocorm, at 30 and 40 DAC respectively; (D) Multiple shoot formed on protocorm at 50 DAC; and (E) Multiple leaves primordial emerged from the protocorm	28
4.5	Percentage of protocorm proliferation of <i>A. graminifolia</i> at different concentration of BAP	29
4.6	Average number of new protocorms produced per protocorm of <i>A. graminifolia</i> at different concentration of BAP	29

Effects of Benzylaminopurine (BAP) Concentrations on Proliferation of *Dendrobium* Hybrid Orchid and *Arundina graminifolia*

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ABSTRACT

Dendrobium hybrid orchid and *Arundina graminifolia* are valuable flowering plants that have been used for cut flower industry due to their attractive and long lasting flower. However, their propagation rate under conventional methods is slow and unable to meet the increased demand of plantlets. This study was conducted to determine the best concentration of BAP for developing an efficient protocol for mass production of planting materials. Young leaves excised from the mother plant of *Dendrobium* and protocorms of *A. graminifolia* produced from *in vitro* germinated seeds were selected as the explants and inoculated on MS medium supplemented with BAP at different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L). The results obtained showed that the protocorms of *A. graminifolia* were proliferated on all tested media where the highest percentage of proliferation was found on MS medium containing 1.5 mg/L BAP (33.33±16.67%) with an average of 7.00 ± 2.00 new protocorms formed per protocorm at 60 DAC. For *Dendrobium*, the leaf explants only changed their colour from green to yellowish or brownish instead of proliferating or PLBs formation at 60 DAC on all tested media.

Key words: *Arundina graminifolia*, BAP, *Dendrobium* hybrid orchid, proliferation.

ABSTRAK

Dendrobium hibrid orkid dan *Arundina graminifolia* merupakan tumbuhan berbunga yang berpontensi bagi industri bunga keratan kerana mempunyai bunga yang menarik dan mekar lebih lama. Tetapi, pembiakan orkid melalui kaedah konvensional sangat perlahan dan tidak mampu memenuhi permintaan anak benih yang semakin meningkat. Kajian ini dijalankan untuk mengkaji media pengkulturan terbaik untuk meningkatkan hasil anak benih orkid dalam masa yang singkat. Daun muda dari tumbuhan matang *Dendrobium* dan protokom daripada percambahan biji benih secara *in vitro* telah digunakan sebagai eksplan dan dikulturkan pada media MS yang ditambah dengan BAP pada kepekatan berbeza (0.5, 1.0, 1.5, 2.0 dan 2.5 mg/L). Hasil kajian menunjukkan bahawa protokom *A. graminifolia* berproliferasi pada semua media. Rawatan 1.5 mg/L BAP mencatatkan peratus protokom berproliferasi yang tertinggi (33.33±16.67%) dengan purata 7.00 ± 2.00 biji protokom baru terbentuk per eksplan pada hari ke-60 pengkulturan. Bagi *Dendrobium*, hanya perubahan daun warna yang dapat diperhatikan iaitu dari hijau muda kepada kuning dan mengalami nekrosis pada semua media yang dikaji selepas 60 hari pengkulturan.

Kata kunci: *Arundina graminifolia*, BAP, *Dendrobium* hybrid orchid, proliferasi.

CHAPTER I

INTRODUCTION

Orchidaceae is the largest flowering plant family that consists of more than 880 genera and 25000 species (Moudi *et al.*, 2013). They are distributed abundantly in the tropical regions. Over the years, orchid cultivation has been developed from a hobbyist market into a highly commercial market and today orchids are located at the top position among flowering plants valued for cut flowers and potted plants that sold in the world. Besides that, most of them have a number of great medicinal characteristics that have been used in traditional Chinese medicine and health care food production (Moudi *et al.*, 2013).

Among orchid genera, *Dendrobium* hybrid orchids and *Arundina graminifolia* that distributed in Malaysia are having a high commercial value. *Dendrobiums* are the second largest group of orchids that popular in cultivation for both orchid enthusiasts and cut flower industry due to their uniqueness and long lasting beautiful flowers (Banks, 2003). *A. graminifolia* is a terrestrial orchid that native to Asia with attractive flower that have been used as cut flowers and potted flowers (Alsarhan *et al.*, 2014). Both of them are considered as valuable plant in the cut flower and medicinal industry. Since the orchid propagation is one of the main supports for the mass orchid production, the consistent supplies of plant materials will be a major concern in the orchid industry. According to Hew and Yong (2004), the total demand for orchid plant materials has accounted nearly to 1600 million units of plant stock in the year 2000.

However, the propagation rate of orchids through vegetative methods is very slow and difficult to obtain the desired number of orchid clones for commercial purpose within a short

period of time. Besides, a pollinator is needed to enhance the capsule to form and mycorrhizal relationships are needed for the seed to germinate (Pradhan *et al.*, 2013; Sugiura, 2014). Furthermore, about 12 years may take by the seedlings to grow and become an adult plant (Parmar, 2014).

Thus, micropropagation is extensively used for mass producing of plantlets within a short period of time for commercial and conservation purposes. The shoot tips and axillary bud explants are mostly used as starting materials to initiate the shoot formation and multiplication. The regeneration of orchids into a complete plantlet has also been achieved by using PLBs culture, callus culture and somatic embryogenesis (Košir *et al.*, 2004; Niknejad *et al.*, 2011). However, there are some limitations of the existing protocols to produce a large number of plantlets at a time due to the low rate of protocorm-like bodies (PLBs) formation (Talukder *et al.*, 2003).

Therefore, a great effort in the manipulation of culture medium, especially the potential use of plant growth regulators (PGRs) has been carried out in order to develop a best culture medium for efficient micropropagation of orchids through shoot regeneration and proliferation. The plant growth regulator, benzylaminopurine (BAP) is widely used and showed markedly enhanced in cell division and shoot regeneration (Chawla, 2009).

The objectives of this study were to determine the effects of different concentrations of BAP on proliferation of *Dendrobium* hybrid orchid and *A. graminifolia* in order to identify the optimum concentration of BAP for developing an effective protocol for the proliferation and mass production of planting materials of *Dendrobium* hybrid orchid and *A. graminifolia*.

CHAPTER II

LITERATURE REVIEW

2.1 Family Orchidaceae

Orchidaceae is widely distributed throughout the tropical countries with 880 genera and more than 25000 species (Moudi *et al.*, 2013; Teoh, 2005). A majority of them are epiphytic plants that grow on the branches of trees and associated with symbiotic relationships in their life cycle (Chan *et al.*, 1994). They can be classified into two types of growth patterns which are important to be identified in order to grow and propagate them successfully. Orchids with monopodial growth are mostly grown upward which can up to several meters in length as in *Arundina*, *Phalaenopsis* and *Vanda* (McDonald, 1999). They grow continuously throughout the year and produce their new leaves at their tip. On the other hand, orchids with sympodial growth are growing laterally with formation of new pseudobulbs from the previous pseudobulb as in *Cymbidium* and *Dendrobium* (McDonald, 1999).

In Malaysia, the plantation and production of orchids and their hybrids is increasing and contributed to the economy. Fadelah (2007) stated that the export of orchids has increased from RM 12.8 million to RM 14.3 million from year 2003 to 2005. The orchids are popular as ornamental plants especially for cutting flowers and also play an important role in commercial and medical use. However, the population of some important species is decreasing and being extinct due to overexploitation or habitat destruction.

2.1.1 *Dendrobium* Hybrid Orchid

Dendrobium orchid is a sympodial epiphytic orchid that spread throughout Southeast Asia. In general, it has elongated roots that formed from the nodes, flattened smooth leaves and triangular lateral sepals (Teoh, 2005). The stems are flourishing with two to three clustered flowers at each node (Rajkarnikar, 2014). Most of them are produced annually and flowering during May to July (Banks, 2003).

Dendrobiums have accounted for 85% of the total trade in tropical orchid cut flower market (Sheela, 2008). The hybrids such as in soft cane, nobile and phalaenopsis type as shown in Figure 2.1 are typically used for sale. They are produced through the developed breeding methods such as protoplast isolation and chromosome doubling (Arditti, 2008). Among them, *Dendrobium* Sonia is one of the most popular and grown for many years as a cut flower (Arditti, 2008). Besides that, many of *Dendrobium* species such as *Dendrobium fimbriatum*, *Dendrobium nobile* Lindl. and *Dendrobium amoenum* Wall. Ex Lindl have been used as traditional medicines to relieve or treat burnt skin, fever and throat problems (Pant, 2013).

Dendrobium phalaenopsis hybrid that typically used for sale was used in this study. It has elongated roots, flattened smooth leaves and flower with colour combination of white, pink and purple that can be lasted for four to eight weeks (Arditti, 2008). They are usually reproduced by offshoots division. However, the multiplication rate is low. Furthermore, the production of complete plants through *in vivo* seed germination is difficult since symbiotic fungi are needed for the seed to germinate.

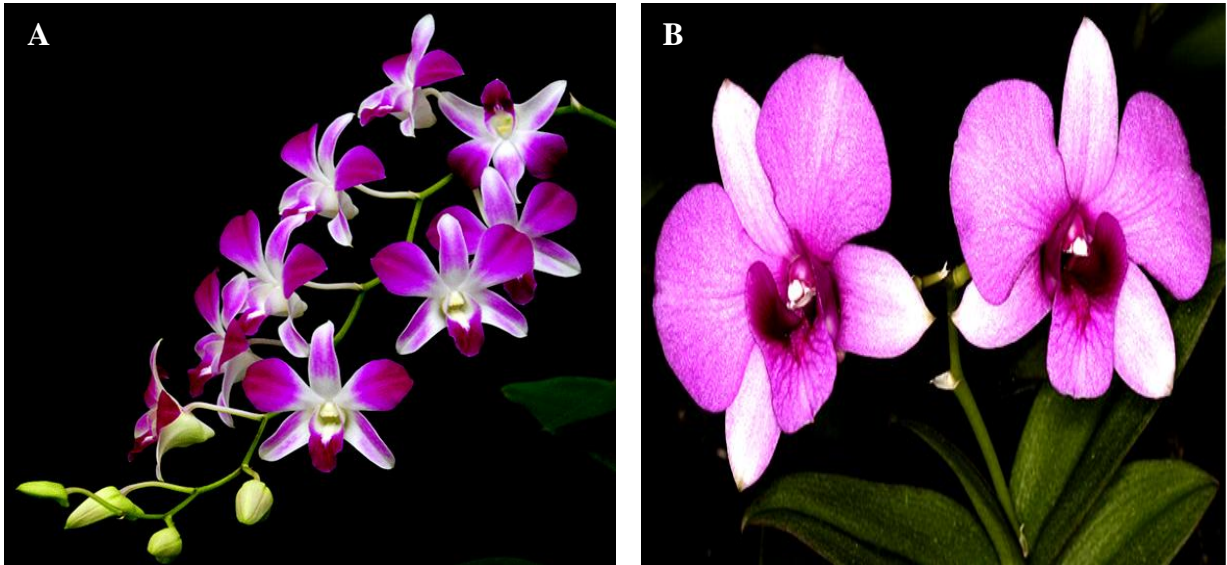


Figure 2.1 *Dendrobium* hybrid orchids. (A) *Dendrobium* Sonia and (B) *Dendrobium bigibbum* hybrid (Source: Bhide, 2010; Smith, 2010).

2.2.2 *Arundina graminifolia*

Arundina graminifolia or commonly known as bamboo orchid is a terrestrial orchid (Das *et al.*, 2013). It can be found along roadside, rocky sites or open grassy areas (Sugiura, 2014). It is distributed in China, India, Malaysia, Myanmar, the Philippines and Sri Lanka (Arditti, 2008). However, its population is decreasing due to intensive harvesting and habitat destruction (Sugiura, 2014).

It has long and narrow leaves and largely clumped erect stem which able to grow to a height up to 2m (Das *et al.*, 2013). The flowers have an outer whorl with 3 sepals and an inner whorl with 3 petals. Both sepals and petals are pinkish-white to pale pink. It blooms in summer and autumn with many flowers bloom at a time (Das *et al.*, 2013). Sugiura (2014) stated that the flowers bloom year-round in Malaysia.

The flowers of *A. graminifolia* are beautiful and able to persist for about one week and therefore can be used as cut flowers for decorative purposes. It is also popular as potted plant and cultivation in rural area as shown in Figure 2.2.

Besides that, it is also widely used for medical purposes for clearing heat, detoxicating, and dissipating blood stasis because it is able to possess activities of detoxification, antiarthritis and abirritation (Das *et al.*, 2013; Li *et al.*, 2013). The stilbenoids compounds such as diphenylethylene contain phenolic compounds and also exhibit the potential cytotoxicity. It has been extracted from *A. graminifolia* to study the cytotoxicity against the human tumor cell lines (Li *et al.*, 2013).



Figure 2.2 *Arundina graminifolia*. (A) Flower of *A. graminifolia* and (B) *A. graminifolia* plantation (Source: Miranda, 2013).

2.2 Propagation of Orchids

The methods of orchid's propagation can be grouped into two types: conventional and *in vitro* methods.

2.2.1 Conventional Propagation of Orchids

In conventional methods, orchids are propagated by vegetative methods and seed germination. Vegetative propagation of orchids through clumps division, bulbs or cutting has advantages of producing genetically identical plants and blossom quickly than plants from seed, but it is difficult to obtain the desired number of orchids for commercial purposes (Parmar, 2014; Venturieri & Pickscius, 2013). Some of the species are disappearing from their natural habitat due to these difficulties in vegetative propagation.

Generally, orchids are able to produce a large number of tiny seeds which are extremely light and able to move to a long distances by wind at maturity stage. However, only very few or less than 5% of them germinate under natural condition. The seeds contain only little or no food reserves. The mycorrhizal relationship is needed for the small and non-endospermic seeds to germinate (Parmar, 2014; Rajkarnikar, 2014). This symbiotic relationship provides nutrition for orchid growth while fungi get the shelter from the orchid at the same time. According to Fadelah (2006), more than 5 years is needed for the conventional breeding cycle of *Dendrobium* as it has a long juvenility period. Furthermore, the time required for the seedpod to reach maturity is still unknown as there are many factors which can affect it such as environmental and genetic factors. Thus, seed germination of orchid under *in vivo* conditions is difficult and time consuming.

Besides that, pollinating agents such as bees and wasps are required for pollination and capsule formation because some of the species are unable to self-pollinate spontaneously, although they are self-compatible (Sugiura, 2014). Furthermore, most of the flowers of orchids are only open for a few days. Thus, it is difficult for seedpod to form in nature.

2.2.2 *In vitro* Propagation of Orchids

The development of tissue culture techniques is essential to provide an excellent alternative for large scale propagation of genetically identical clones of desired orchid species and their hybrids. The method for non-symbiotic orchid seed germination on simple nutrient medium containing minerals and sugars was discovered by Knudson in 1922 and became a standard procedure for orchid seed germination (Rajkarnikar, 2014). Mass cultivation of orchids within a short time becomes possible with micropropagation and *in vitro* seed germination.

The mycorrhizal relationship that needed for seed germination is not common in nature. Therefore, a high proportion of the seeds fail to survive. In order to produce the seedlings of the desired orchid in mass scale, asymbiotically seed germination is used to overcome this association. The seeds are cultured on a suitable culture medium under controlled conditions in the laboratories. The medium is provided with all the necessary nutrients, vitamins and plant growth regulators to support the seed germination and seedling growth as well. The asymbiotic seed germination has been used for species rescue and conservation. However, the long juvenile period is required and heterozygous plants may produce in the seed culture. Furthermore, the rate of *in vitro* seed germination might also affected by the time in harvesting the capsules (Rajkarnikar, 2014).

2.3 Micropropagation of Orchids

Micropropagation is widely used for mass propagation of desired orchids for commercial purpose. The regeneration of orchids from various parts of mother plants is taken in order to produce similar clones from the mother plants. An orchid meristem culture that developed by Morel in 1960 is one of the techniques that completely changed the conventional concept of orchid culture for a number of genera and hybrids (Rajkarnikar, 2014). The growth and morphogenesis of tissue cultures is mainly influenced by the genotype of the explant, substrate and environment factors (George & Sherrington, 1984).

2.3.1 Selection of Explant

The selection of explant is depending on the final goal of the tissue culture. For clonal propagation, direct shoot regeneration from leaf explants is mostly used as it can also prevent somaclonal variation. In leaf culture, shoot buds are formed as small green protuberances on the bases of the explants before developing into shoots (Nayak *et al.*, 1997). According to Arditti (2008), a green mass may form within 45 days and 3 months are required for the protocorm-like bodies to form when the leaves taken from *in vitro* grown seedlings of *Dendrobium* were used as the explants.

Different explants such as leaf axillary, shoots (Venturieri & Pickscius, 2013), flower stalk node (Martin *et al.*, 2005), axillary bud (Dohling *et al.*, 2012), protocorm like bodies (Dutta *et al.*, 2011), nodal segment (Pradhan *et al.*, 2013), axenic pseudobulb segments (Sunitibala & Kishor, 2009) and nodes (Rajkarnikar, 2014) have been selected in micropropagation of orchids. They are being selected to induce the shoot and root formation

for large scale propagation and *ex situ* conservation of orchids. The shoot and root were initiated and multiplied from the explants when cultured on the medium supplemented with plant growth regulators in an appropriate concentration.

2.3.2 Media Composition

The medium composition is important to provide sufficient nutrient for the proliferation of the explants (Arditti, 2008). MS medium that rich in microelements and vitamins has been shown to yield better results than the other media for tropical orchids by giving the highest shoot length mean and number of multiplied shoots (Sheela, 2008). Asghar *et al.* (2011) found that more than 12% of PLBs are growing better in half strength MS media in a semi-solid culture compared to full strength MS media.

However, different types of culture may require different nutritional components. Therefore, different types of basal media have been studied and modified by adding of plant growth regulators (PGRs) and additives. PGRs are applied at a low quantity to control the growth and development of the cells and tissues. Auxin and cytokinin such as benzylaminopurine, 1-naphthaleneacetic acid (NAA) and thidiazuron (TDZ) are the mostly used PGRs in multiplication system of commercial orchids such as *Dendrobium*, *Phalaenopsis* and *Cymbidium* (Arditti, 2008; Sheela, 2008).

2.4 Protocorm Proliferation

Protocorms that formed from germinated seeds and protocorm-like bodies (PLBs) that induced from initial explants are typically used in mass production of orchids. The regeneration of shoot from protocorm or PLB able to give raises a complete plant which is essential for the stock plant production in the cut flower industry. The regeneration of plantlets for commercial purpose in *Cymbidium*, *Dendrobium*, and *Phalaenopsis* orchids have been successfully established by the use of protocorm and PLBs as starting material (Arditti, 2008).

Protocorm that formed from seed germination has been used as explant sources for many species such as *Dendrobium* and *Vanda* (David *et al.*, 2010) to develop efficient protocols for multiplication of orchid. New protocorms are formed and proliferated from the cultured protocorm and eventually developed into shoots and roots to give complete plantlets when suitable media are used.

Protocorm-Like Bodies (PLBs) are also widely used in the orchid industry as it can be multiplied indefinitely and also grows into a complete plantlet. PLB is defined as a spherical tissue mass that resemble the early stage of orchid embryo development which is induced from initial explants (Arditti, 2008). A large number of PLB can be obtained within a short period of time when appropriate medium was used. Each formed PLBs can be further cut into pieces and subcultured to give rise to several protocorm for further multiplication. They can proliferate and regenerate rapidly then grow into complete plantlets when cultured on appropriate medium and placed at proper cultural conditions.

2.5 Benzylaminopurine (BAP)

Benzylaminopurine (BAP) is a common plant growth regulator that has been proven to be efficient in the enhancing and promoting shoot regeneration, somatic induction, cell division and growth of axillary buds (Chawla, 2009). It is a first-generation synthetic cytokinin that commonly applied to apical dormancy for stimulating shoot proliferation and controlling the cell division when combined with auxin (Habiba *et al.*, 2014).

Arditti (2008) stated that the success of early attempts to culture cells and tissue explants could be depend on the addition of plant growth regulators into the culture media. The potential effects of BAP on *Dendrobium* have been studied on shoot regeneration (Talukder *et al.*, 2003), flower induction (Nambiar *et al.*, 2012), protocorm like bodies (PLBs) regeneration and plantlet formation (Khatun *et al.*, 2010). These studies indicated that medium supplemented with appropriate concentration of BAP has increased the rate of shoot proliferation, earlier flowering induction, and plantlet formation.

The combinations of BAP with other PGRs also have been conducted and developed to establish the optimal concentration of each growth regulator in culture medium for shoot proliferation. For direct shoot regeneration, induction medium that contain BAP or TDZ and NAA are mostly used. MS medium supplemented with a combination of 2.5 mg/L BAP and 0.5 mg/L NAA was found to be the most efficient media that give the highest percentage of shoot proliferation for *Dendrobium* orchid (Talukder *et al.*, 2003).

CHAPTER III

MATERIALS AND METHODS

3.1 Plant Materials

3.1.1 *Dendrobium* Hybrid Orchid

A healthy and free from visual disease symptoms *Dendrobium* hybrid mother plant was collected from the Charlie Orchid Garden, Sarawak (Figure 3.1). The young leaves were excised from the newly generated shoot of the mother plant and used as the explant (Figure 3.2) in this study.



Figure 3.1 *Dendrobium* hybrid mother plant.

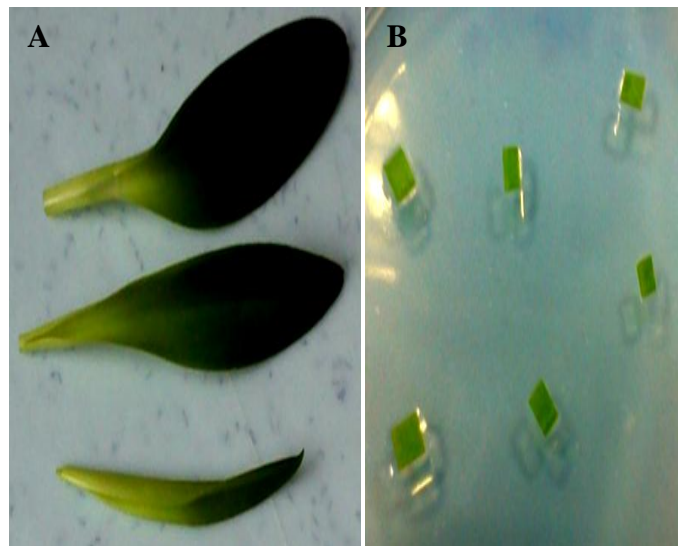


Figure 3.2 Leaf explant. (A) Young leaves of *Dendrobium* and (B) Leaf explant cut in size of 0.2-0.4 cm.

3.1.2 *Arundina graminifolia*

A green capsule of *A. graminifolia* (Figure 3.3) was collected from a village located at Serian, Sarawak. The capsule was surface sterilized by soaking in 20% bleach solution with 2 drops Tween-20 for 15 min followed by a dip in 70% ethanol for 30 seconds. After three times rinsed with sterile distilled water, the capsule was dried and dissected longitudinally with the help of a sterilized forceps and scalpel.

The seeds were scooped out from sterilized capsules and inoculated on MS medium supplemented with 20% coconut water by spreading over the surface of the culture medium. The seeds were germinated after 42 days of culture and become green protocorm after 65 days of culture as shown in Figure 3.4. These green protocorms were chosen as the starting material.



Figure 3.3 *Arundina graminifolia*.

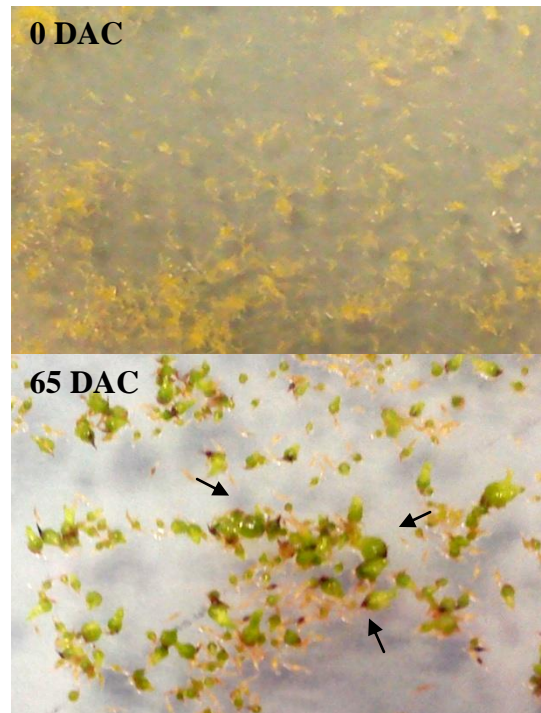


Figure 3.4 Formation of green protocorms (arrow) at 65 days after culture (DAC).