Effect of BAP and NAA Concentration on Seed Germination and Growth of *Cymbidium finlaysonianum* orchid

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DECLARATION

I declare that this thesis entitled "Effect of BAP and NAA Concentration on Seed Germination and Growth of *Cymbidium finlaysonianum* orchid" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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LIST OF ABBREGIATIONS AND SYMBOLS

PGR	Plant Growth Regulator
BAP	6-benzylaminopurine
NAA	1-Naphthaleneacetic acid (NAA)
DSG	Dendrobium Seed Germination
MS	Murashige and Skoog Medium (MS)

Kesan Kepekatan BAP dan NAA pada Percambahan Benih dan Pertumbuhan Orkid

Cymbidium finlaysonianum

ABSTRAK

Cymbidium finlaysonianum adalah orkid epifit yang berasal dari Malaysia. Bagaimanapun, disebabkan oleh penebangan hutan dan eksploitasi manusia, pertumbuhan spesies ini di hutan Malaysia telah berkurang. Percambahan orkid seperti ini sukar kerana ia bergantung kepada simbion kulat mikoriza tertentu yang membekalkan karbohidrat untuk bercambah. Ia adalah kerana orkid tidak mempunyai endosperma. Disebabkan kesukaran ini, percambahan benih *in vitro* diperlukan untuk mendapatkan propagul. Oleh itu dalam kajian ini, komposisi media pertumbuhan yang berbeza telah digunakan untuk meningkatkan percambahan benih orkid *Cymbidium finlaysonianum*. Benih orkid yang steril dibiakkan pada medium percambahan benih yang ditambah 1 mg/L, 2mg/L dan 3mg/L BAP masing-masing. Percambahan benih maksimum dengan permulaan kalus dicatat dalam medium Dendrobium Seed Germination (DSG) dengan 3 mg/L BAP. "Protocorm" telah diperhatikan secara berterusan menggunakan media Murashige and Skoog Medium (MS) yang ditambah dengan 0 mg/L, 1 mg/L, 2 mg/L dan 3mg/L BAP masing-masing dan ditambah dengan 0 mg/L dan 1mg/L NAA . Bilangan akar, panjang akar, bilangan daun, panjang daun dan ketinggian data frekuensi tumbuhan dikumpulkan selepas 6 minggu untuk menentukan media terbaik untuk percambahan benih. Kesan kepekatan BAP yang berbeza telah dinilai pada PLB menunjukkan bahawa 2 mg/L BAP telah terbukti sebagai kepekatan terbaik dalam pertumbuhan pucuk manakala MS basal (0 mg/L BAP) menunjukkan kepekatan terbaik utuk pertumbuhan akar. Selepas 6 minggu pemerhatian, tanaman orkid di subkultur menggunakan media MS ditambah dengan 2 kepekatan yang berlainan NAA (0 mg/L dan 1 mg/L NAA). Ia adalah untuk menentukan kepekatan terbaik untuk pemanjangan akar.

Kata kunci: Pertumbuhan benih; *Cymbidium finlaysonianum*; BAP, *in vitro*, Media.

Effect of BAP and NAA Concentration on Seed Germination and Growth of

***Cymbidium finlaysonianum* orchid**

ABSTRACT

Cymbidium finlaysonianum is an epiphytic orchid native in Malaysia. However due to deforestation and human exploitation, the distribution of this species in Malaysian forest has been decrease. The germination of this orchid in nature is difficult because it depends on specific mycorrhizal fungal symbionts that supply the carbohydrates in order to germinate. It is because orchids lack of endosperms. So, due to these difficulties an *in vitro* seed germination is required in order to get the propagules. Therefore in this study, different growth media composition was used to enhance the germination *Cymbidium finlaysonianum* orchid seeds. Sterile orchid seed was cultured on seed germination medium supplemented with 1 mg/l, 2mg/l and 3mg/l of BAP respectively. The maximum seed germination with callus initiation was recorded in Dendrobium Seed Germination medium(DSG) with 3 mg/L of BAP . The protocorm was continuously observed using the Murashige and Skoog Medium (MS) media medium supplemented with 0 mg/L, 1 mg/l, 2mg/l and 3mg/l of BAP respectively and 0 mg/L and 1 mg/L of NAA respectively. Number of root, length of root, number of leaf, length of leaf, and height of plant were collected after 6 weeks to determine the best media for seed germination. The effect of different concentration of BAP was evaluated on PLB shows that 2 mg/L of BAP has been proved as the best concentration on shoot development while MS basal (0 mg/L of BAP) for root development. After 6 weeks of observation,the plantlet of orchids was subcultured using MS media supplemented with 2 different concentration of NAA (0 mg/L and 1 mg/L of NAA). It is for determined the best concentration of NAA for root elongation.

Key words: Seed culture; *Cymbidium finlaysonianum*; BAP, *in vitro*, Media**.**

CHAPTER 1

INTRODUCTION

1.1 Research Background

Orchids are well known flower among the world with their beautiful long lasting flowers and varieties. They are among the most diverse family in the flowering plants in the world. Orchid also known as Orchidaceae. These orchids are native flower in tropic regions of Asia and Australia such as Himalayas and the Philipines. “Butterfly orchid” is the term that used by Chinese in Asia. It shows the aesthetics of this particular flower (Hala, 2009). Orchid is one of important group in ornamental plants consist several thousand species and hybrids. These flower are attractive to everyone especially to breeder, amateurs and flower lovers due to their unique and beautiful flower with a wide range of vibrant color (Zahara, Datta, Boonkorkaew, & Mishra, 2017). These beautiful flowers have the largest family in flowering plant about 30,000 different species and 200,000 hybrids. Most of these places are not protected and unmanaged makes most orchids endangered or threatened.

Orchids need a combination of multiply factor to continue reproduction in nature. The seeds are one of special types and poorly develop. For germination in nature, seed of orchid need more endosperm and require a suitable fungal stimulant. The fungus is accepted to increase the carbohydrate, auxin, and vitamin transport in the orchid (Arditti & Krikorian, 1996).

Research by Shahinul, Islam, Bhattacharjee, Mondal, and Subramaniam (2015) stated that only 0.2 to 0.3% of seeds germinate in nature. Seed also takes a long time to germinate and any disturbance in habitat or physical environment will destroy whole population of orchid. The seedling process of orchids also takes a long period of up to 12 years to become an adult. They are exceedingly heterozygous and their vegetative propagation through division is slow.

Former researchers (Autónoma De Yucatán et al., 2006) described "This difficulty in natural population drives some of the indigenous species to extinction" (as cited in Shahinul et al., 2015). *Cymbidiums* are one of the most important and well known orchid in cultivation and they are versatile plants, buttonholes, marketed as cut-flowers and as pot plants, it also producing many large and long-lasting flowers (Du, Cribb, & Tibbs, 2007). They also stated that the genus of *Cymbidium* currently consists of some 52 species dispersed all over South and Eastern Asia, the Malay Archipelago and north and east Australia. *Cymbidium finlaysonianum Lindl.* are listed as vulnerable to extinction species because it is the one that rapidly disappearing. This species also commercial important orchid. Conventional propagation is slow and might difficult. To overcome the problem, *in vitro* culture techniques are adopted for quick propagation of orchid species. For make a micropropagation process, seed of orchid needed. Mass propagation give the alternative to fulfill the demand. The important to have disinfection procedure to obtain the composition

of seed. The developing of mass propagation of *Cymbidium finlaysonianum* using *in vitro* seed culture were established (Shahinul et al., 2015).

1.2 Problem Statement

Normally, orchids hook up themselves to the bark of trees, or on surface of plant but most of them not be able to survive due to the unfavorable environment. In nature, orchids usually grow through seed. Unfortunately, the seed may not germinate on appropriate hosts in adequate percentages. Due to these difficulty, the tissue culture technique was introduced. The essential components in tissue culture is *in vitro* regeneration (Aktar, Nasiruddin, & Hossain, 2008). The difficulty of orchid seed to germinate has been recognized for a long time and the success rate is found to be low in nature. The difficulties for orchid seeds to germinate is due to environmental factor but sometimes the genetics also may lead the problem (Nambiar, Siang, & Mahmood, 2012). Therefore, the *in vitro* propagation in culture medium shall be an alternative method to speed up multiplication. *In vitro* cultures of plant embryos are used as in research tool for several purposes, such as rescue of rare hybrids, genetic manipulation, propagation of germplasm, and physiological, morphological and anatomical studies.

One of the most important factors for the growth and morphogenesis of plant tissues *in vitro* is the composition of the culture medium. The basic nutrient requirements of

cultured plant cells are very similar to those of whole plants. Therefore, the optimum condition should be established in order to enhance the seed germination.

1.3 Hypothesis

H_0 : There is no effect of different media composition on the seed germination and growth of *Cymbidium finlaysonianum* orchid.

H_1 : There is an effect of different media composition on the seed germination and growth of *Cymbidium finlaysonianum* orchid.

1.4 Objective

The objective of this study is to identify the effect of different 6-benzylaminopurine and 1-Naphthaleneacetic acid (NAA) concentration on germination and growth of *Cymbidium finlaysonianum* orchid.

1.5 Scope Of Study

This study was mainly focus on germination of seed of wild orchid, *Cymbidium finlaysonianum* with different seed germination media composition. Seed germination media was added with different BAP and NAA concentration to grow the seed.

1.6 Significance Of Study

Cymbidium finlaysonianum orchid owing to its high commercial value in the floricultural industry, natural populations are under threat from over-exploitation. Furthermore, seed of orchid difficult to find in forest. Moreover, orchid seeds usually a slower grower in nature. Orchid seed also may not germinate properly in vivo. The tissue culture technique was introduced to germinate the seed of orchid and *in vitro* technique introduced to the seed germinate rapidly and produce more explant. Therefore, the *in vitro* propagation through seed can produce a lot of orchid propagules.

CHAPTER 2

LITERATURE REVIEW

2.1 *Cymbidium Finlaysonianum* Orchid

Cymbidium finlaysonianum orchid comes from Orchidaceae family that priceless green and aesthetic in Malaysia. Orchids is one of the largest family in the flowering plant kingdom. There are about 20 000 to 35 000 species in five subfamilies in this family and about 1000 species are found in Peninsular of Malaysia. The largest genera are *Bulbophyllum* (2,000 species), *Epidendrum* (1,500 species), *Dendrobium* (1,400 species) and *Pleurothallis* (1,000 species). Members of this family are found on every continent except Antarctica, with the highest diversity in tropical regions of Southeast Asia, South America, and Central America. Seventy percent of all orchid species are epiphytes, but terrestrial, aquatic, and lithophytic species can also be found (Kauth et al., 2008).

Orchids can be easily to differentiate among other plants. It is a bilateral symmetry, resupinate flower and have very extreme small seeds. Orchid is epiphytic which grow on support and have modified aerial roots. Orchids also have simple leaves and parallel veins.

The structure of the leaves will depend on the specific habitat of the plant. The Orchidaceae are famous with the variety of flowers. Some of orchid may have single flower but most have inflorescence and large number of flowers. Orchid contain very numerous number per capsule. When it's ripening, they will blow off like dust particles and spores. The seed of orchid are lack of endosperm and must have symbiotic relationship with the fungi that will help to give them nutrient to germinate. Orchids are perennial herbs. People have high demand on wild species of orchid so the flower can lead to be an extinction species (Go & Akmal, 2017).

Cymbidium finlaysonianum is one of the orchid species that grow in island of Borneo. They believed that this species able to ward off evil spirits (Michael, 2006). The orchid seed comes in small size with no endosperm that give the lack to germinate in nature. Horticultural techniques have been created to immerse the orchard seeds in artificial nutrient mediums, eliminating fungal requirements for germination and greatly assisting the propagation of ornamental orchids. *In vitro* technique helps the seed from get limited food reserves. Knudson (1946) introduce this technique of an asymbiotic seed germination in increasing the viability of orchid in nature. This technique is commercially used by grower. Seed of orchid need a proper sterilization process to avoid contamination. The specific nutritional and suitable environment condition are need for *in vitro* germination of orchid seed (Tawaro, Suraninpong, & Chanprame, 2004).

The taxonomy classifications of *Cymbidium finlaysonianum* are as follows:

Kingdom: Plantae

Phylum: Tracheophyta

Class: Liliopsida

Order: Asparagales

Family: Orchidaceae

Genus: Cymbidium

Species: *finlaysonianum*

2.2 Plant Tissue Culture

Haberlandt (1902) implies the concept of plant tissue culture and provides the basis for the cultivation of plant cells, tissues and organs in culture. Initially, plant tissue culture appeared as a research tool and focused on experiments to culture and study the development of small and isolated tissue cells. Plant tissue culture application is for horticulture and shows the achievement and limitations of tissue culture and some views on current and future developments. With the rapid population growth, the number of fruits,

vegetables and various ornamental plants cannot meet the needs of people in developing countries. In a relatively short period of time, many commercial laboratories have been established around the world to exploit the potential for micro-dispersion of mass production of clonal plants for the horticultural industry (Haberlandt, 1902).

Today, plant tissue culture applications are more than clonal propagation. Various routine technologies have been developed to include somatic embryogenesis, somatic hybridization, virus removal and the use of bioreactors for mass dissemination (Idowu, Ibitoye, & Ademoyegun, 2002). Seed of orchid are used to culture *in vitro.* Germination of seed asymbiotically will be the best way to study the growth and development of orchid seeds and seedling.

2.3 Micropropagation

The mass propagation provides an alternative way of meeting demand. Unfortunately, the conventional spread is slow and tough, indicating *in vitro* methods for mass multiplication may be more appropriate. The development of *in vitro* seeds is much influenced by several factors such as the age of the seeds, nutrient media and organic carbon sources, and others. The development of protocorms from germination seeds and induction of secondary protocorm or subsequent PLB, from different tissues as a discovery has been a reliable method for breeding orchids. The spread by the formation of PLB is the preferred option because a large amount of PLB can be obtained within a short period of

time. Protocorm-like bodies can grow and quickly regenerate into plantlet. The regeneration and multiplication of orchids through natural seedlings is limited due to endosperm suppressed and the needs of fungal partners. Germination of orchids in *in vitro* is often used to produce commercially important orchids and is shown as an efficient tool for producing orchids for conservation and recovery (Mohanty, Paul, Das, Kumaria, & Tandon, 2012).

2.4 Seed Germination Media

Plant tissue culture media should usually contain some or all of the following components: macronutrients, micronutrients, vitamins, amino acids or nitrogen supplements, carbon sources, undetermined organic supplements, growth regulators and reinforcing agents. According to the (Physiology, 1976),elements in concentrations greater than 0.5 mM/L are defined as macroelements and are required in concentrations less than 0.5 mM/L as microelement. It is important to consider that the optimum concentration of each nutrient to achieve maximum growth rate varies between species (Saad & Elshahed, 2012). Many different cultural media have been developed to germinate orchid seeds. Although many of these media have little difference in the composition, growth and development of the species may be significantly affected. The majority of research are cultured to study on nutritional requirements for orchid seed growth.

2.5 Murashige And Skoog Medium (MS)

Murashige and Skoog medium are plant growth mediums used in laboratories for plant cell culture cultivation. Media Murashige and Skoog are the first choice because they have a balanced composition against other media. Macro composition, micro nutrients, vitamins and organic is very suitable for most plant species (Aktar et al., 2008).

2.6 6-Benzylaminopurine (BAP)

BAP or 6-Benzylaminopurine (Figure 2.1) works as cytokinin in micropropagation to enhance the plant growth and development by stimulating cell division (Arab, Yadollahi, Shojaeiyan, Shokri, & Ghojah, 2014). BAP works as synthetic cytokinin which comes with auxins obtain plant growth and development. Cytokinin helps in shoot production while auxin helps in root development. In cultivar media, cytokinin proved to stimulate cell division, causing arrest the formation and germination of axillary beams and to prevent root formation. Cytokinines are compounds are relatively stable in culture media and can be stored hydrated at -20˚C. Cytokinin is often reported to be difficult to dissolve and sometimes some additions. The 1N HCl or 1N NaOH droplets simplify their dissolution (Saad & Elshahed, 2012). They are usually involved in cell growth and differentiation and give the effect to apical domination, axillary bud growth and leaf aging. Cytokinin assist to

slow the aging of plant organs by preventing breakdown of protein, activating synthesis of protein and collect nutrient from nearby tissues.

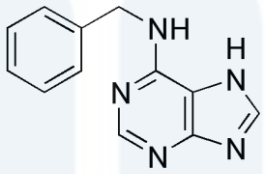

Figure 2.1 Molecular structure of 6-Benzylaminopurine

Sources: https://en.wikipedia.org/wiki/6-Benzylaminopurine (2018)

BAP also act as an inhibitor of respiratory kinase in plant, which can increase the postharvest to plant. Treatment using BAP can involve metabolically in the stimulation of germination and increasing the seedling. BAP also give significant effect on formation of callus (Keighobadi, Golabadi, & Mortazeenezhad, 2014). BAP assist in rising to embryos from the primary culture stage.

2.7 1-Naphthaleneacetic Acid (NAA)

1-Naphthaleneacetic acid (Figure 2.2) is a synthetic auxin. Auxins are primarilary synthesized in shoot apex and move in a polar, basipetal fashion through stems, and acropetally in roots with a transition to the basipital flow from the root tip, which is often described as streams like a fountain. Thus, the auxin effect on the shoot formation may be

species, tissue, and developmental specifics; and in some cases, it may not require the presence of cytokinin to get this developmental response. Auxin differs in their physiological activity and how much they translocate through tissue and metabolized. In tissue culture, auxins are usually used to stimulate the production of callus and cell growth, to start shoots and rooting, to promote somatic embryogenesis, to stimulate growth from apices shooting and shooting bar culture (Saad & Elshahed, 2012). BAP causes a sudden or horizontal growth reaction, while the NAA triggers a positive gravitropic growth (Novak, Luna, & Gamage, 2014). Chen (2005) also reported that NAA benefited the multiplication of PLB, and the multiplication of orchids. Auxin's ability to regulate the aspects of plant development has been characterized well in the model plant system. On the contrary, the orchids have received less attention, but the awareness that many endangered species of orchids have led to a culture-based dissemination study that has embarked on several functions for auxin in this system (Novak et al., 2014).

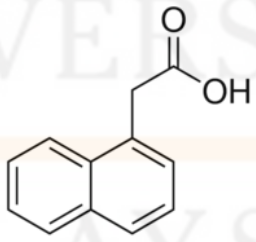

Figure 2.2 Molecular structure of 1-Naphthaleneacetic acid (NAA)

Sources: https://en.wikipedia.org/wiki/1-Naphthaleneacetic_acid (2018)

CHAPTER 3

MATERIALS AND METHOD

3.1 Plant Materials

The epiphytic orchid *Cymbidium finlaysonianum* was found on the oil palm tree (Figure 3.1). The yellowish and dried seed of wild orchid *Cymbidium finlaysonianum* were obtained from the Agropark in Universiti Malaysia Kelantan Jeli Campus (Figure 3.2).

3.2 Equipments

The materials that use in this study were ethanol, distilled water, sodium hypochlorite, agar, stock solution, vitamin, hormone for plant such as 6-benzylaminopurine (BAP) and 1-Naphthaleneacetic acid (NAA).

Figure 3.1 Epiphytic orchid, *Cymbidium finlaysonianum*

Figure 3.2 *Cymbidium finlaysonianum* seed

The equipment used in this research were measuring cylinder, 1000ml beaker, conical flask 300ml, test tube, jar, micropipette and tips, analytical balance, sterile petri dishes, spatula, hot plate stirrer, magnetic stirrer, pH meter, forceps, scalpel, sterile razor blades, parafilm, microwave, laminar air flow, hot heads sterilizer and spirit lamp.

Five chemicals were used to prepare macronutrient stock solution. There were ammonium nitrate ($NH_4 NO_3$), potassium nitrate (KNO_3), calcium chloride ($CaCl_2.2H_2O$), magnesium sulpahte ($MgSO_4.7H_2O$), and potassium phosphate monobasic (KH_2PO_4). Furthermore, nine chemicals needed to make micronutrient stock. There were boric acid (H_3BO_3), cobalt chloride hexahydrate ($CoCl_2.6H2O$), copper sulphate pentahydrate ($CuSO_4.5H_2O$), EDTA disodium salt dihydrate ($C_{10}H_{14}N_2Na_2O_8$), ferrous sulphate heptahydrate ($FeH_{14}O_{11}S$), manganese sulphate monohydrate ($MnSO_4.4H_2O$), molybdic acid ($MoO_3.H_2O$), potassium iodide (KI) and zinc sulphate heptahydrate ($ZnSO_4.7H_2O$). Vitamin also needed to make stock solution. The chemical that required were myo-inositol ($C_6H_{12}O_6$), nicotinic acid ($C_6H_5NO_2$), pyridoxine HCl ($C_8H_{12}CINO_3$) and thiamine hydrochloride ($HC_{12}H_{17}ON_4SCl_2$).

3.3 Preparation For Stock Solution

3.3.1 Dendrobium (Orchid) Seed Germination Medium (DSG)

For 500ml stock solution, the amount of macronutrient, micronutrient and vitamin were weighed according to the Dendrobium (Orchid) Seed Germination Medium (DSG)

calculation in Table 3.1. The amount that needed were dissolved with distilled water. The chemical was solubilized and the final volume of solution was calculated. The stock solution was kept in 4°C.

3.3.2 Murashige and Skoog Medium (MS)

For 500ml stock solution, the amount of macronutrient, micronutrient and vitamin were weighed according to the Murashige and Skoog Medium (MS) calculation in Table 3.2. The amount that needed were dissolved with distilled water. The chemical was solubilized and the final volume of solution was calculated. The stock solution was kept in 4°C.

3.4 Preparation of Media With Different Concentration Of BAP Hormone

3.4.1 Dendrobium (Orchid) Seed Germination Medium (DSG)

For the preparation of 1L Dendrobium (Orchid) Seed Germination Medium, 20ml from micronutrient, 100ml of macronutrinet and 20ml of vitamin were taken into beaker contain 200ml distilled water. 20g of sucrose was added into the beaker and stirred using

the magnetic stirrer. BAP stock solution was added into the media accordingly to the desired concentration tabulated in Table 3.3.

Distilled water was added until 1L. The pH was adjusted in the range 5.5 to 5.8. 8.0g of agar was added into solution and melted in the microwave for 10 minutes. The media was then poured into cultured jar and autoclave at 121°C for 15 minutes.

3.4.2 Murashige and Skoog Medium (MS)

For the preparation of 1L Murashige and Skoog Medium, 5ml from micronutrient, 50ml of macronutrinet, 5ml of ferum source and 2ml of vitamin were taken into beaker contain 200ml distilled water. 20g of sucrose was added into the beaker and stirred using the magnetic stirrer. BAP stock solution was added into the media accordingly to the desired concentration tabulated in Table 3.4.

Distilled water was added until 1L. The pH was adjusted in the range 5.5 to 5.8. 8.0g of agar was added into solution and melted in the microwave for 10 minutes. The media was then poured into cultured jar and autoclave at 121°C for 15 minutes.

After 6 weeks, the plantlet of orchid was subculture into MS media contain different concentration of NAA accordingly tabulated in Table 3.5.

Table 3.1. The chemical components of Dendrobium (Orchid) Seed Germination Medium (DSG).

Media components	**DSG**	
A. Macronutrient	**1X**	**10X (g/500ml)**
1. NH_4NO_3	0.4125	4.1250
2. KNO_3	0.4750	4.7500
3. $CaCl_2.2H_2O$	0.0830	0.8300
4. $MgSO_4.7H_2O$	0.0450	0.4500
5. KH_2PO_4	0.0450	0.4250
B. Micronutrient	**1X**	**10X (g/500ml)**
1. H_3BO_3	0.0008	0.0075
2. $CoCl_2$.6H2O	0.0001	0.0008
3. $CuSO_4.5H_2O$	0.0001	0.0008
4. $C_{10}H_{14}N_2Na_2O_8$	0.0466	0.4663
5. $FeH_{14}O_{11}S$	0.0348	0.3475
6. $MnSO_4.4H_2O$	0.0211	0.2113
7. $MoO_3.H_2O$	0.0003	0.0027
8. KI	0.0010	0.0104
9. $ZnSO_4.7H_2O$	0.0108	0.1075
C. Vitamin	1X	**50X (g/500ml)**
1. $C_6H_{12}O_6$	0.0250	1.2500
2. $C_6H_5NO_2$	0.0001	0.0063
3. $C_8H_{12}CINO_3$	0.0001	0.0063
4. $HC_{12}H_{17}ON_4SCl_2$	0.00003	0.0013

("Dendrobium (Orchid) Seed Germination Medium,"2010.)

Table 3.2. The chemical components of Murashige and Skoog Medium (MS).

Media components	**DSG**	
A. Macronutrient	**1X**	**20X (g/500ml)**
1. NH_4NO_3	0.8250	16.5000
2. KNO_3	0.9500	19.0000
3. $CaCl_2.2H_2O$	0.2200	4.4000
4. $MgSO_4.7H_2O$	0.1850	3.7000
5. KH_2PO_4	0.0850	1.7000
B. Micronutrient	**1X**	**200X (g/500ml)**
1. $MnSO_4.4H_2O$	0.0112	2.2300
2. $ZnSO_4.7H_2O$	0.0043	0.8600
3. H_3BO_3	0.0031	0.6200
4. KI	0.0004	0.0830
5. $NaMoO_4.2H_2O$	0.0001	0.0250
6. $CuSO_4.5H_2O$	0.00001	0.0025
7. $CoCl_2.6H_2O$	0.00001	0.0025
C. Vitamin	**1X**	**500X (g/125ml)**
1. Myo-inositol	0.0125	6.2500
2. Glycine	0.0003	0.1250
3. Thiamine-HCL	0.0001	0.0625
4. Nicotinic acid	0.00006	0.03125
5. Pyridoxine-HCL	0.00006	0.03125
D. Ferum Source	**1X**	**200X (g/500ml)**
1. $FeSO_4.7H_2O$	0.0139	2.7800
2. $Na_2EDTA.2H_2O$	0.0189	3.7800

Table 3.3. The volume of BAP stock solution used.

Media	Volume of BAP stock solution (ml)
DSG 0 (Control)	0
DSG + 1.0 mg/L BAP	0.25
DSG + 2.0 mg/L BAP	0.50
DSG + 3.0 mg/L BAP	0.75

(BAP stock concentration is 1 mg/ml)

Table 3.4. The volume of BAP stock solution used.

Media	Volume of BAP stock solution (ml)
MS 0 (Control)	0
MS + 1.0 mg/L BAP	0.25
MS + 2.0 mg/L BAP	0.50
MS + 3.0 mg/L BAP	0.75

(BAP stock concentration is 1 mg/ml)

Table 3.5. The volume of NAA stock solution used.

Media	Volume of NAA stock solution (ml)
MS 0 (Control)	0
MS + 1.0 mg/L NAA	0.25

(NAA stock concentration is 1 mg/ml)

3.5 Orchid Seed Sterilization

Seed pod of orchid was washed under running tap water together with detergent. The surface of pod was sterilize with 95% ethyl alcohol for 1 minutes, then 10% sodium hypochlorite for 10 minutes. Finally, the pod was sterilize by rinse it 5 to 6 times with sterilized distilled water.

3.6 Inoculation

Inoculation was carried out in laminar flow cabinet under sterile conditions. The floor of chamber was cleaned thoroughly with 95 % of ethanol. The laminar air flow cabinet was UV sterilized first before inoculation. The sterile orchid pod was cutting longitudinally by using forceps and razor blades. All equipment were sterilized using 95% by spraying it before use. The mouth of jar was flamed using spirit lamp to kill all microorganisms. Finally, seeds were inoculated on the DSG medium jar and sealed with parafilm. These cultures were kept in the primary growth room.

3.7 Culture Conditions

All culture were grown at 25 ± 2°C and the temperature were maintained by the air conditioners. All culture were kept under illumination of 16 hours photoperiod. Light intensity of 1600 lux was obtained by white cool fluorescent tube of 40 watts.

3.8 Experimental Design

Table 3.6. The experimental design between different DSG media samples.

		Number of samples		
Treatment	**Type of media**	R1	R2	R3
1	DSGO (Control)	3 sample	3 sample	3 sample
2	DSG + 1.0 mg/L BAP	3 sample	3 sample	3 sample
3	DSG + 2.0 mg/L BAP	3 sample	3 sample	3 sample
4	DSG + 3.0 mg/L	3 sample	3 sample	3 sample

Table 3.7. The experimental design between different MS media samples.

Treatment	Type of media	Number of samples		
		R1	R2	R3
1	MSO (Control)	3 sample	3 sample	3 sample
2	MS + 1.0 mg/L BAP	3 sample	3 sample	3 sample
3	MS + 2.0 mg/L BAP	3 sample	3 sample	3 sample
4	MS + 3.0 mg/L	3 sample	3 sample	3 sample

3.9 Statistical Analysis

This experiment were analyzed using completely randomized designs (CRD). DMRT- Ducan's multiply range test ($P \leq 0.05$) is one way analysis of variance (ANOVA) was applied on this experiment. SPSS (version 16, SPSS Inc., USA) were the program packages that help in statistical analysis. This statistic is used to provide an objective, non biased way to evaluate experiment. The probability of an event can be used to determine whether the effect can occur in this experiment. The result can help to determine whether the hypothesis can be accepted on not. Six data were collected in this experiment which were number of root, length of root, number of shoot and length of shoot.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Seed Germination

Under this study on a asymbiotic germination were tested with Dendrobium Seed Germination with 6-benzylaminopurine as plant growth regulator. The oval-shaped embryo starts to swell by absorbing water and nutrients from the media. Embryo swelling turns into a green circle and appears by breaking the seed coat (Figure 4.1). This stage is considered to be the beginning of seed germination. The time taken for embryonic swelling and percentage germination differ in different concentration of media. Haberlandt (1902) has considered that the appearance of protocorms to be a manifestation of embryogenesis because they represent a specialized stage in embryo development and derived directly from zygotic embryos. Among the media tested, DSG with 3.0 mg/L BAP was the most suitable for seed germination where 100% germination was recorded followed by DSG with 2.0 mg/L BAP (90%), DSG with 1.0 mg/L BAP (85%) and DSG with absence of BAP (83%) that act as control tabulated in Table 4.1. It showed that different concentration of

BAP with DSG media on *in vitro* seed germination influenced the number of germination of protocorms. Dendrobium Seed Germination Medium has been specially formulated for the *in vitro* germination orchid dendrobium species (Dendrobium (Orchid) Seed Germination Medium).

The usage of different concentration of BAP in the medium showed a significant respond in the germination of PLB (Figure 4.2). From all four treatment tested, DSG + 3.0 mg/L BAP has shown the best result in terms of germination of PLB compared to the other concentration with the mean value of 99.38. The second highest mean of the germination of PLB 77.50 had been observed for seed cultured in DSG media containing 2.0 mg/L BAP followed by 1.0 mg/L BAP with the mean value of 64.00. Lowest value of germination PLB observed was 47.17 for *Cymbidium finlaysonianum* seed orchid cultured in DSG with absence of BAP as PGR. The amount of DSG are half of amount number in MS medium. The research shows that DSG and MS medium give the same effect on seed germination (Shahinul et al., 2015).

Figure 4.1 Protocorm in DSG medium.

Table 4.1. The effect of different concentration of BAP on *in vitro* seed germination of *Cymbidium finlaysonianum.*

Treatment	**Type of media**	**Amount of seed perculture**	**Seed germination**	
			DAC	% germination
1	DSGO (Control)	150mg	49-56	83.0
2	DSG + 1.0 mg/L BAP	150mg	49-56	85.0
3	DSG + 2.0 mg/L BAP	150mg	49-56	90.0
4	DSG + 3.0 mg/L	150mg	49-56	100.0

DSG (Dendrobium Seed Germination)). Days after culture (DAC).

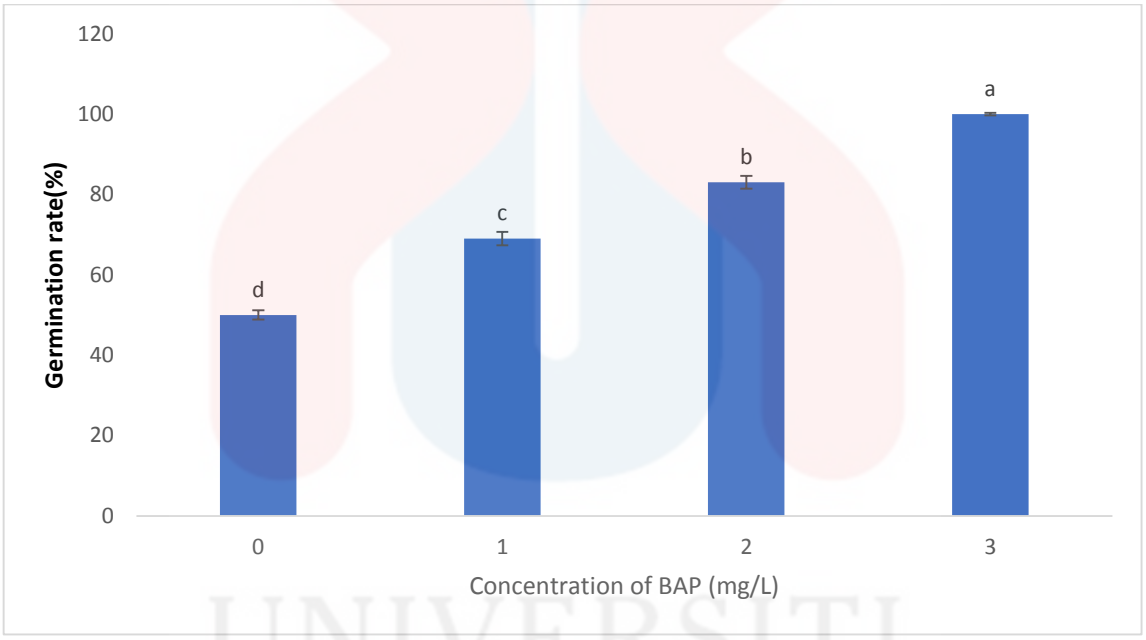

Figure 4.2. The effect of different concentration of BAP on germination rate in DSG media.

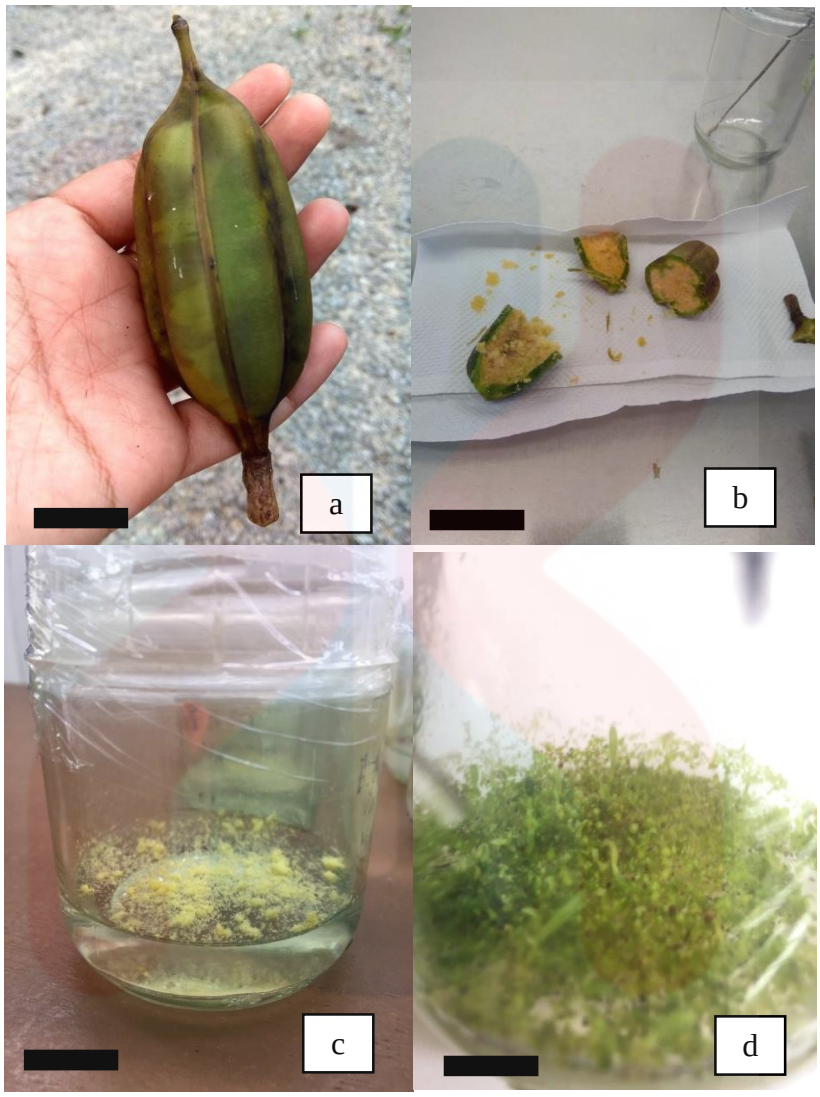

Figure 4.3. Axenic seed germination and protocorm development of *C. finlaysonianum*; a) Seed pod of *C. finlaysonianum*, b) Dissection of seed pod, c) *C. finlaysonianum* seed in DSG medium, d) Mass development of protocorm in DSG medium.
Bar; a = 1.7cm, b = 4.0cm, c = 1.4cm, d = 1.0cm

4.2 Plant Growth

The cultured seeds and protocorm were continuously been observed for data were collected on the 6 weeks of culture. All the figures and tables below showed the effect of different media and plant growth regulator concentration to the germination of protocorm, length of root, number of root, length of shoot and number of shoot. The analysis of variance showed that *Cymbidium finlaysonianum* orchid had a slightly effect on the growth as shown in Table 4.2. There were shows that every concentration have significance different value of BAP hormone. Islam et al. (2015) also reported that there are significant different between the concentration of BAP.

In this experiment, the axenic seed culture was established, which was similar to the previous report by (Islam et al., 2015). Arditti and Krikorian (1996) reported that orchid seeds have no endosperm or cotyledons in the embryo as primary storage material. Therefore, nutrients in the culture medium are needed for orchid germination.

Table 4.2. The effect of different concentration of BAP on length of shoot, number of shoot, length of root and number of root.

Type of media	**Length of shoot(cm)**	**Number of shoot**	**Length of root(cm)**	**Number of root**
	M ± S. E.	M ± S. E.	M ± S. E.	M ± S. E.
MS0	3.55 ± 0.27^{d}	3.50 ± 0.22^{c}	5.32 ± 0.21^{a}	4.67 ± 0.33^{a}
MS + 1 mg/L BAP	5.43 ± 0.16^{b}	4.00 ± 0.26^{bc}	1.52 ± 0.09^{b}	3.50 ± 0.22^{b}
MS + 2 mg/L BAP	7.02 ± 0.12^{a}	5.00 ± 0.37^{a}	0.83 ± 0.04^{c}	2.33 ± 0.21^{c}
MS + 3 mg/L BAP	4.38 ± 0.31^{c}	4.50 ± 0.22^{ab}	0.60 ± 0.07^{c}	1.50 ± 0.22^{d}

M (Mean) ± S. E. (Standard error). Value in a column with similar superscripts are not significantly different at $p \leq 0.05$ levels.

Different letter in column indicate significant different at $p < 0.05$ levels according to DMRT.

For multiple induction of shoot and elongation of shoot when BAP are used alone with different focus then show various responses. Based on the present investigation, the result showed the effect of different concentration of BAP in MS media on the length of shoot are also studied as shown in Figure 4.4. The amount of BAP concentration in present research also similar to previous research reported by Shahinul et al. (2015).The value started from lowest to highest. Based on the result obtained, MS media containing 2.0 mg/L BAP showed the highest mean length 7.02cm of shoot compared to other media. The second highest value was recorded for 1.0 mg/L BAP 5.43cm followed by other concentration. From the previous research by Islam et al. (2015) also reported that MS supplemented with 2.0 mg/L of BAP concentration was the high value for shoot elongation.

The effect of different concentration of BAP on number of shoot was shown in Figure 4.5. Based on the four treatment that tested, 2.0 mg/L of BAP has shown the most number of shoot with the mean 5.00 followed by 3 mg/L BAP 4.50. MSO has shown the lowest number of shoot with mean 3.5. There are study shown that BAP that contain cytokinin has known been to be important for shoot induction and elongation of many plant species *in vitro* (Shahinul Islam et al., 2015). Present result showed the multiple shoot formation frequency and shoot length was best in 2 mg/L of BAP and decrease when PGR was added similar to research by (Shahinul et al., 2015). It is because the PGR was denatured in high concentration of BAP hormone. Talukder, Nasiruddin, Yasmin, Hassan, and Begum (2003) reported that high concentration of BAP hormone in MS media will not facilitate the growth of shoot.

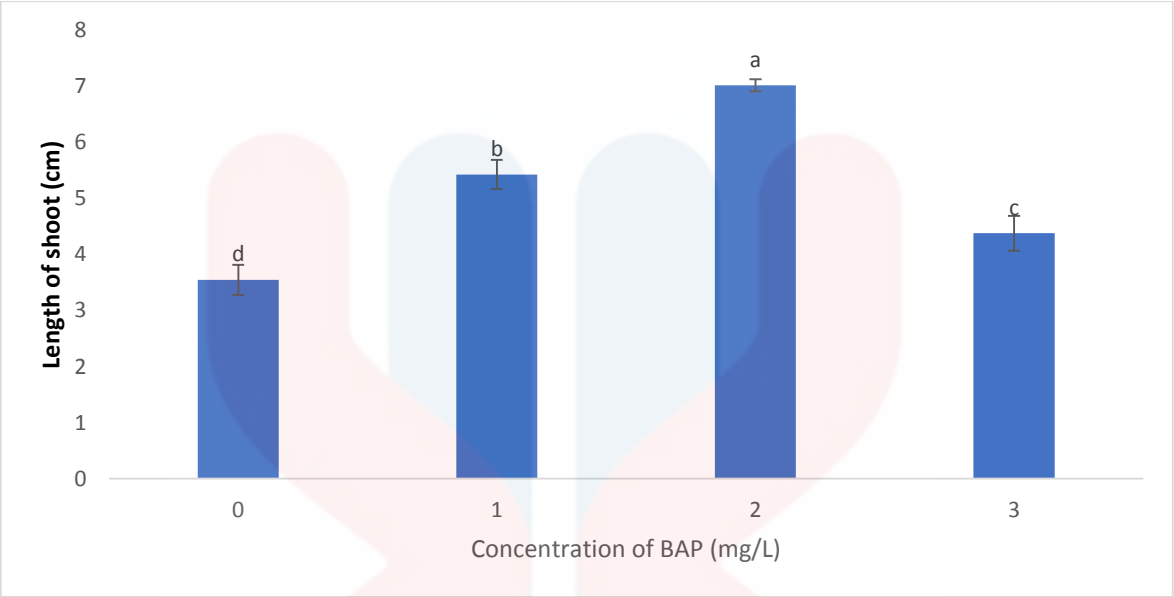

Figure 4.4 The effect of different concentration of BAP on length of shoot in MS media.

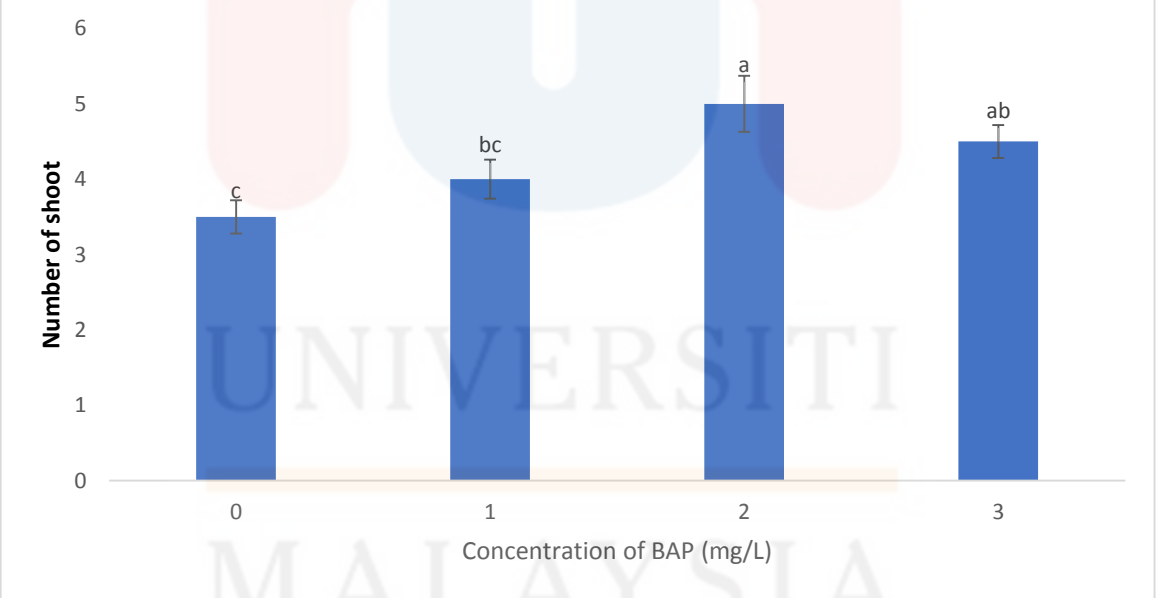

Figure 4.5. The effect of different concentration of BAP on number of shoot in MS media.

The efficiency of the rooting media was evaluated based on the increase in length and number roots developed per protocorm. The effect on root length of *Cymbidium finlaysonianum* plantlet of different combinations of PGRs with MS medium on elongation of individual shoots were shown in Figure 4.6. Every treatment gave different effect on length of root. The plantlet produced highest mean length was MSO (5.32cm). The second length appear on concentration 1 mg/L of BAP with 1.52cm followed by 2 mg/L of BAP (0.83cm) and the lastly was 3 mg/L BAP (0.6cm).

Highest number developed root was observed in Figure 4.7. The highest value was MSO (4.67). The second highest was 1 mg/L of BAP (3.50) followed by 2 mg/L of BAP (2.33) and 3 mg/L of BAP (1.50). MSO absence of BAP that have cytokinin. Cytokinin enhance shoot so it will not disturb the root formation (Chen Y, 2005; Shahinul Islam et al., 2015). However, high concentrations of auxin inhibit root elongation instead of enhance adventitious root formation (Chen Y, 2005). Similarly, cytokinin also cannot work properly in high concentration as cited in (Bangladesh Society for Conservation of Environment & Natural Resources., 2008; Bhadra and Hossain, 2003).

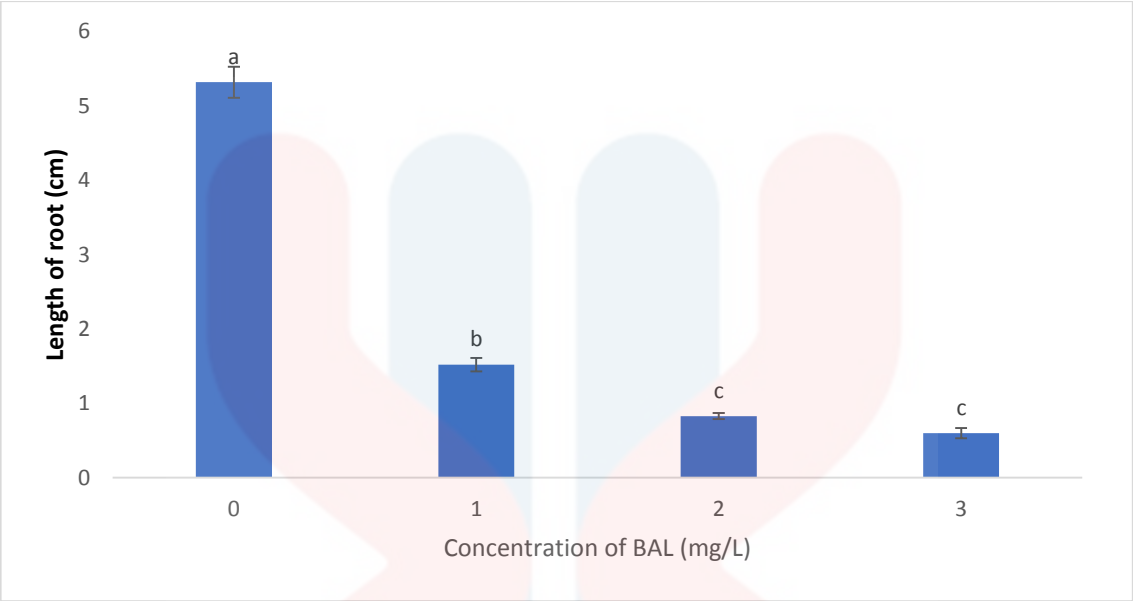

Figure 4.6.The effect of different concentration of BAP on length of root in MS media.

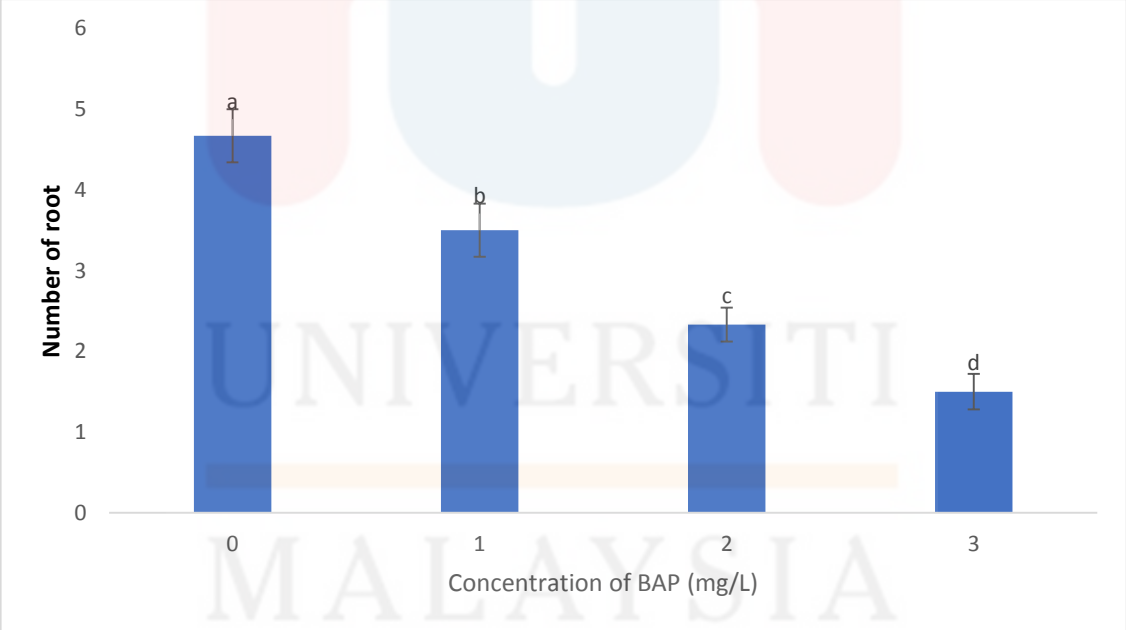

Figure 4.7.The effect of different concentration of BAP on number of root in MS media.

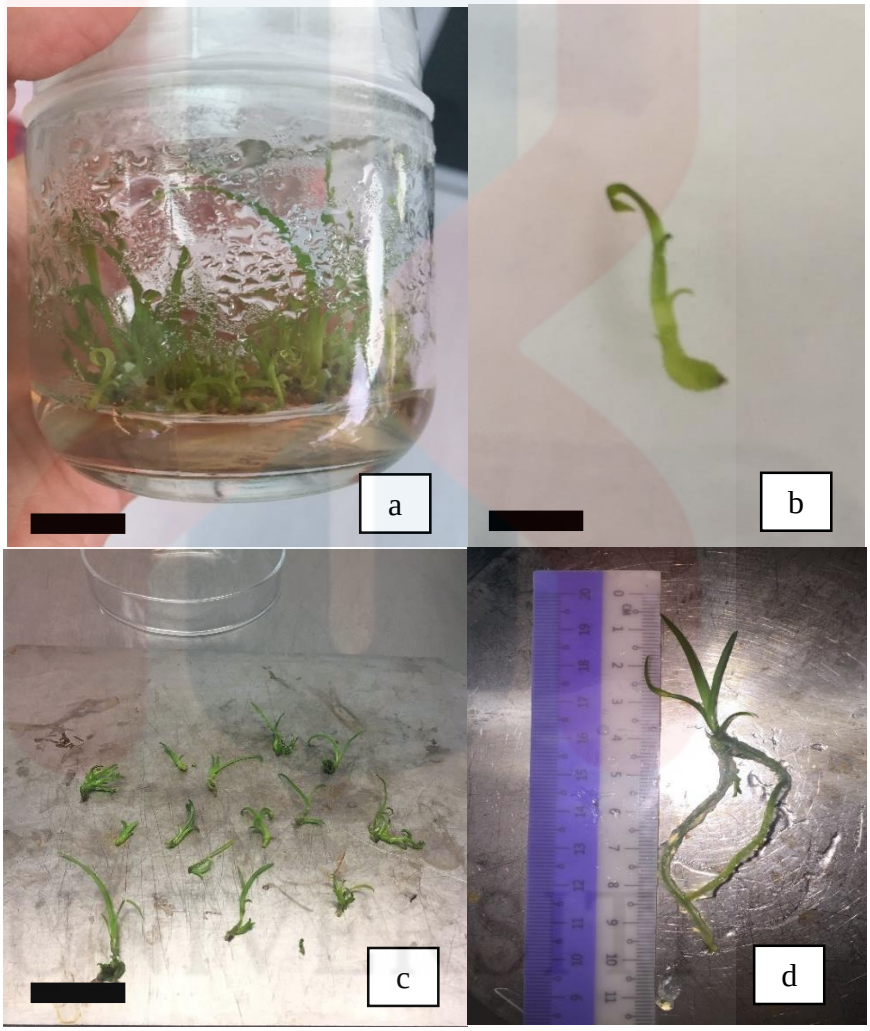

Figure 4.8. Growth of *C. finlaysonianum* orchid; a) Plantlets of *C. finlaysoninum* in medium MS, b) 2-3 leaves stage of protocorms in MS medium, c) Root and shoots initiation from protocorm in MS medium and d) Well developed plants in MS without plant growth regulator.
Bar; a = 1.4cm, b = 0.8cm, c = 2cm

High concentrations of cytokinin promote shoots growth, while auxin have high concentrations encourage root formation. Root induction is the property of plants that are controlled by endogenous hormones. Exogenous supply of stimulating root hormones such as auxin and dietary stress increases the process. The effect of different concentration of NAA on number of root and length of root were observed as shown in Table 4.3. There were significant difference between MS supplemented with different concentration of BAP and NAA. MS were supplemented with different concentration of BAP and NAA. The result shows MS + 1 mg/L BAP + 1 mg/L NAA have the highest value of mean (3.59) on number of root followed by MS + 2 m/L BAP + 1 mg/L NAA (2.75). The third highest was MS + 2 mg/L BAP + 0 mg/L NAA with the value number of mean 2.15 followed by MS + 0 mg/L BAP + 1 mg/L NAA (1.90), MS + 3 mg/L BAP + 1 mg/L NAA (1.76), MS + 3 mg/L BAP + 0 mg/L NAA (1.69), MS + 1 mg/L BAP + 0 mg/L NAA (1.16) and the poor of root induction was MS + 0 mg/L BAP + 0 mg/L NAA (0.20) (Figure 4.9). Similar result was also reported by (Hossain, Sharma, and Pathak, 2013) in some epiphytic orchids like dendrobium orchid. MSO medium was used and not supplemented with any plant growth regulators, rooting was delayed for root development. To generate a root system that facilitates adaptation is one of the major problems of growing plants *in vitro* to grow.

The different concentration of BAP and NAA affected the length of root. The highest value of mean recorded on concentration of MS + 1 mg/L BAP + 1 mg/L NAA (3.85cm) followed by MS + 2 mg/L BAP + 1 mg/L NAA (3.13cm), MS + 3 mg/L BAP + 1 mg/L NAA (2.76cm), MS + 0 mg/L BAP + 1 mg/L NAA (2.18cm), MS + 2 mg/L BAP + 0 mg/L NAA (1.72cm), MS + 3 mg/L BAP + 0 mg/L NAA (1.05cm), MS + 1 mg/L BAP + 0 mg/L NAA (0.83) and MS + 0 mg/L BAP + 0 mg/L NAA (0.25) (Figure 4.10). These result

was supported by Goswami, Yasmin, Nasiruddin, Khatun, and Akte (2016) who noted that the combination of NAA and BAP drives rooting at regeneration to produce a complete plant. Significant effects are seen on the effects of NAA and BAP that affect the length of the roots. This result is partly supported by Goswami et al. (2016) which shows that MS + 1 mg/L BAP + 1 mg/L NAA of each BAP and NAA perform better growth and orchid development. As a synthetic auxin, NAA is commonly used at relatively low dose to elicit auxin-type responses in cell growth, cell division, fruit setting, rooting (Srivastava,2002; Sun and Hong,2010). However, a high concentration of auxin prevents the elongation of the roots instead of increasing the aspiration of the roots.

Cytokinin also cannot function properly in high concentrations. The adventitious root production was increased rapidly at lower NAA concentration, while the number of roots was decreased at higher concentration same as research by Sung and Hong (2010). NAA caused a significant increase in rooting ability as compared with the control, and 1.0 mg/L was found to be the best at enhancing rooting ability, while higher doses of NAA caused a significant decrease in root formation. This verified Hentig and Gruber (1987) report that hormonal doses could induce the best rooting when being just below the toxic level. Similar results were obtained in Sun and Hong (2010) research, which found that lower concentrations of NAA (1.0 mg/l and 2.0 mg/l) in the root induction medium had a stronger effect on successive plant regeneration of the orchid *Dendrobium* than higher concentrations.

Table 4.3. The effect of different concentration of BAP and NAA on *in vitro* number of root and length of root of *Cymbidium finlaysonianum.*

Type of media	**Number of root**	**Length of root**
	M ± S. E.	M ± S. E.
MS + 0 mg/L BAP + 0 mg/L NAA	0.20 ± 0.03^{g}	0.25 ± 0.13^{g}
MS + 1 mg/L BAP + 0 mg/L NAA	1.16 ± 0.05^{f}	0.83 ± 0.09^{f}
MS + 2 mg/L BAP + 0 mg/L NAA	2.15 ± 0.06^{c}	1.72 ± 0.08^{e}
MS + 3 mg/L BAP + 0 mg/L NAA	1.69 ± 0.08^{e}	1.05 ± 0.05^{f}
MS + 0 mg/L BAP + 1 mg/L NAA	1.90 ± 0.05^{d}	2.18 ± 0.09^{d}
MS + 1 mg/L BAP + 1 mg/L NAA	3.59 ± 0.08^{a}	3.85 ± 0.06^{a}
MS + 2 mg/L BAP + 1 mg/L NAA	2.75 ± 0.06^{b}	3.13 ± 0.07^{b}
MS + 3 mg/L BAP + 1 mg/L NAA	1.76 ± 0.05^{de}	2.76 ± 0.10^{c}

M (Mean) ± S. E. (Standard error). Value in a column with similar superscripts are not significantly different at $p \leq 0.05$ levels. Different letter in column indicate significant different at $p < 0.05$ levels according to DMRT.

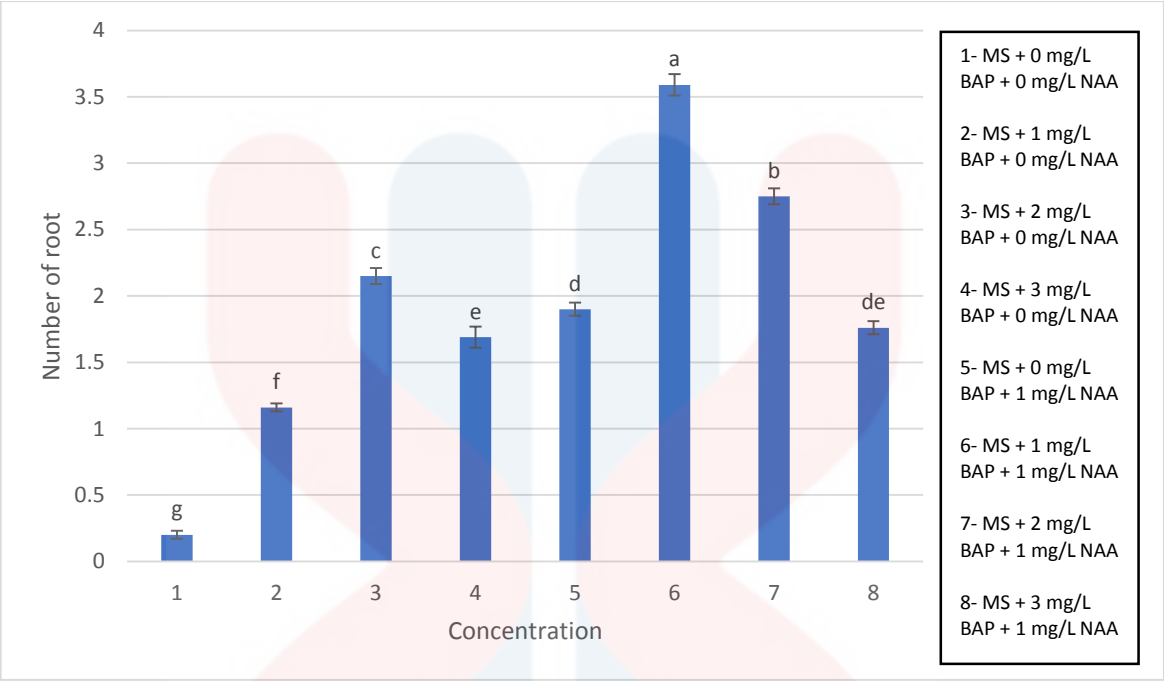

Figure 4.9.The effect of different concentration of BAP and NAA on number of root in MS media.

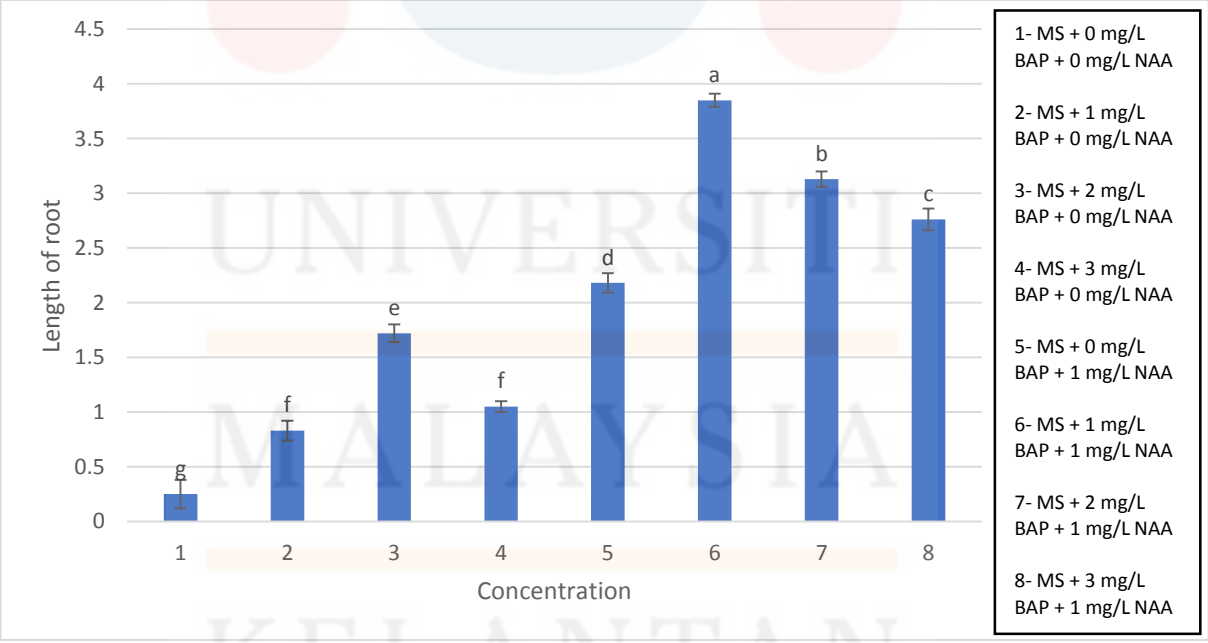

Figure 4.10.The effect of different concentration of BAP and NAA on length of root in MS media.

CHAPTER 5

CONCLUSION

5.1 Conclusion

For rapid production of *Cymbidium finlaysonianum* from using axenic seed culture protocol has been standardized. The results obtained in the experiment indicated that the exogenous plant growth regulators are important for callus induction in orchids. The concentration of DSG media supplemented with 3.0 mg/L of BAP was the optimum for effective protocorm development in *Cymbidium finlaysonianum.* Based on result obtained, a conclusion can be made where the best concentration on promote the large number of shoot and length of shoot was MS with 2.0 mg/L of BAP. For the root formation, MSO was the best media to promote the root. The combination of hormone between MS + 1 mg/L BAP + 1 mg/L NAA concentration showed the optimum value in rooting was 3.59 (number of root) and 3.85cm (length of root)This protocol is possible to produce viable, uniform and

healthy plants with the maximum survival rate that can be used for large-scale planting for orchid development.

5.2 Recommendation

Different focus on PGR were used and it was found that simple components are very important for multiplication and elongation are affected by the plantlet segment. This system will be set up again for an effective system for orchid seed schemes. Furthermore, the protocol may facilitate conservation of this orchid from extinction in the natural and also proven to be valuable for polyploidy and mutation breeding of the desired characters of *Cymbidium finlaysonianum*.

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APPENDICES

A. Duncan table of seed germination rate on different concentration of BAP.

Germination rate (%)

Duncan[a]

Concentration (mg/L)	N	Subset for alpha = 0.05			
		1	2	3	4
.00	6	47.1667			
1.00	6		64.0000		
2.00	6			77.5000	
3.00	6				99.3833
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

B. Duncan table of length of shoot on different concentration of BAP.

Length of shoot (cm)

Duncan[a]

Concentration (mg/L)	N	Subset for alpha = 0.05			
		1	2	3	4
.00	6	3.5500			
3.00	6		4.3750		
1.00	6			5.4300	
2.00	6				7.0200
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

C. Duncan table of number of shoot on different concentration of BAP

Number of shoot

Duncan[a]

Concentration (mg/L)	N	Subset for alpha = 0.05		
		1	2	3
.00	6	3.5000		
1.00	6	4.0000	4.0000	
3.00	6		4.5000	4.5000
2.00	6			5.0000
Sig.		.211	.211	.211

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

D. Duncan table of length of root on different concentration of BAP.

Length of root (cm)

Duncan[a]

Concentration (mg/L)	N	Subset for alpha = 0.05		
		1	2	3
3.00	6	.6000		
2.00	6	.8333		
1.00	6		1.5167	
.00	6			5.3167
Sig.		.194	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

E. Duncan table of number of root on different concentration of BAP.

Number of root

Duncan[a]

Concentration (mg/L)	N	Subset for alpha = 0.05			
		1	2	3	4
3.00	6	1.5000			
2.00	6		2.3333		
1.00	6			3.5000	
.00	6				4.6667
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

F. Duncan table of length of root of different concentration of NAA and BAP

Length of root

Duncan[a]

Concentration	N	Subset for alpha = 0.05						
		1	2	3	4	5	6	7
.00	3	.2500						
1.00	3		.8333					
3.00	3		1.0467					
2.00	3			1.7200				
4.00	3				2.1833			
7.00	3					2.7633		
6.00	3						3.1333	
5.00	3							3.8467
Sig.		1.000	.104	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

G. Duncan table of number of root of different concentration of NAA and BAP.

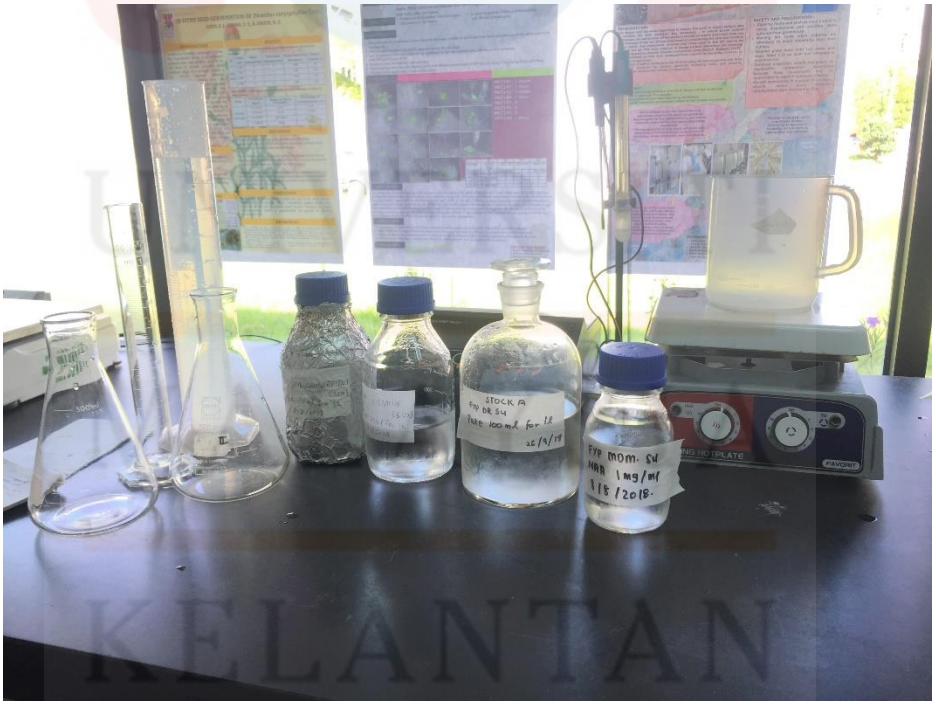
Number of root

Duncan[a]

Concentration	N	Subset for alpha = 0.05						
		1	2	3	4	5	6	7
.00	3	.2000						
1.00	3		1.1600					
3.00	3			1.6867				
7.00	3			1.7600	1.7600			
4.00	3				1.9033			
2.00	3					2.1467		
6.00	3						2.7467	
5.00	3							3.5867
Sig.		1.000	1.000	.394	.106	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.



H. The solutions and apparatus that used.

I. The plantlet after cultured in MS media with supplemented with 2 mg/L of BAP.