Callus Induction of *Vanilla planifolia* Orchid Using Different Concentration of Plant Growth Regulators

By

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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 "**Callus induction of *Vanilla planifolia* Orchid using different concentration of plant growth regulators**" by **Nurul Zakira binti Shamsul**, matric number **F14A0322** has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Agrotechnology) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

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Sincerely,

Nurul Zakira binti Shamsul

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ABSTRACT

Vanilla planifolia is famous as the original flavouring agent of vanilla. It belongs to Orchidaceae family. With the development of science technology, *Vanilla planifolia* has been propagated in tissue culture to increase the propagation of the species. The objective of this study is to induce callus in *Vanilla planifolia* by manipulation of plant growth regulator type and concentration. Callus is an unorganizing, unspecialized, growing and dividing mass of cells. Plant growth regulators (PGRs) help in stimulation of cell division and regulate the differentiation and growth of roots and shoots on explants and embryos in liquid or semisolid medium culture. Leaves explant of *Vanilla planifolia* was used to induce callus. Half strength of Murashige and Skoong (MS) medium used was supplemented with combination of BAP and NAA different concentration. Data on callus formation such as fresh weight of callus, colour of callus, appearance of callus, formation of shoot and hairy roots were collected and analysed by using One-way ANOVA and Tukey's HSD. From the result, the highest mean fresh weight was observed in T7 medium with combination of 2 mg/l of BAP and 3 mg/l of NAA have the highest mean fresh weight of callus formation by 1.57±4.58 g. There is significant different between concentration and mean fresh weight of callus P (<0.05). Colour of callus produced by observed calluses were white-green, creamish green, yellowish, yellowish green and green colour. In this study, all induced calluses observed as compact appearance.

Keywords: *Vanilla planifolia*, indirect organogenesis, callus, half strength MS medium, BAP, NAA, formation of shoot and hairy roots.

Induksi Kalus Terhadap Orkid *Vanilla planifolia* Menggunakan Kepekatan Pengawal Atur Pertumbuhan Tumbuhan Yang Berlainan

ABSTRAK

Vanilla planifolia merupakan keluarga Orchidaceae yang terkenal sebagai ejen perisa vanila aslinya. Dengan perkembangan sains dan teknologi, *Vanilla planifolia* telah ditanam dengan membudayakan kultur tisu bagi meningkatkan pertumbuhan spesis ini. Objektif kajian ini adalah untuk merangsang kalus dalam *Vanilla planifolia* dengan memanipulasi jenis dan kepekatan pengatur pertumbuhan tumbuhan. Dalam organogenesis tidak langsung, pembentukan kalus dihasilkan daripada sel tumbuhan. Kalus merupakan sel-sel yang tidak teratur, tidak khusus, berkembang dan membahagi kepada sel. Pengawal atur pertumbuhan (PGRs) membantu dalam merangsang pembahagian sel dan mengawal pembahagian dan pertumbuhan akar dan pucuk terhadap eksplan dan embrio dalam media kultur cecair dan separapepejal. Eksplan daun *Vanilla planifolia* digunakan untuk induksi kalus. Kepekatan separa medium Murashige dan Skoong (MS) digunakan bersama kombinasi BAP dan NAA dengan kepekatan yang berbeza. Data yang dikumpulkan adalah seperti pembentukan kalus, berat segar kalus, warna kalus, rupa kalus, dan pembentukan pucuk dan akar rerambut, dan dianalisis dengan menggunakan One-way ANOVA dan Tukey'HSD. Daripada hasil kajian, purata berat segar tertinggi diperhatikan dalam T7 yang mempunyai kombinasi 2 mg/l BAP dan 3 mg/l NAA dengan seberat 1.57±4.58 g. Terdapat perbezaan yang signifikan antara kepekatan dan min berat segar kalus P (<0.05). Warna-warna kalus yang dihasilkan oleh kalus adalah warna putih kehijauan, kekuningan, kuning kehijauan, dan hijau. Dalam kajian ini juga, semua kalus yang dilihat mempunyai struktur pepejal.

Kata kunci: *Vanilla planifolia*, organogenesis tidak langsung, kalus, MS medium separuh kepekatan, BAP, NAA, pembentukan pucuk dan akar berbulu.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATION AND SYMBOLS

BAP	6-benzyl amino purine
NAA	1-Naphthaleneacetic acid
MS	Murashige and Skoong
PGRs	Plant growth regulators
T	Treatment
R	Replicate
mg	Miligram
^{o}C	Degree Celcius
%	Percentage

CHAPTER 1

INTRODUCTION

1.1 Research background

Vanilla planifolia or *Vanilla* orchid are famous for the original flavouring agent known as vanilla. They belongs to Orchidaceae family (Bory, Grisoni, Duval, and Besse, 2008). There are many types of Vanilla species found by scientists, but only three types of Vanilla species are commercialized, which are *Vanilla planifolia* Andrews, *Vanilla pompona* Schiede, and *Vanilla tahitensis* J. W. Moore. Due to the unique flavour produced by *Vanilla planifolia*, this Vanilla species is widely cultivated throughout the world (Rao and Ravishankar, 2000).

With the development of science and technology, *Vanilla planifolia* has been propagated in tissue culture to improve the propagation of these species. In *Vanilla planifolia* tissue culture, two type of organogenesis had been used, they are indirect organogenesis (Davidonis and Knorr, 1991) and direct organogenesis (Philip and Nainar, 1986). Various types of explants that have been used to propagate *Vanilla planifolia* which were callus, protocorms, root tips, stem nodes, axillary bud, shoot tips, leaf segment and seed explants (Ravindra and Nataraja, 2007)

Mangesha *et al.* (2012) stated that the traditional cultivation through stem-cutting of *Vanilla planifolia* take longer time, labours and not sustainable. In addition, *Vanilla* fruits cannot sprout under natural condition without the presence of mycorrhiza as the food storage is inadequate (Porras-Alforo and Bayman, 2007). Therefore, to conserve the plant and for propagules requirement, rapid multiplication rate of *Vanilla planifolia* is required. The only technique that serve the way out is plant tissue culture which rapid multiplication rate as well as the *Vanilla planifolia* species conservation is ensured.

In this study, callus culture was conducted by using leaves explant of *Vanilla planifolia*. In organogenesis of *Vanilla planifolia*, leaves were reported as the most effective part for induction and calli proliferation by few scientists. The plant growth regulator used in this study was the combination of 6-benzyl amino purine (BAP) and 1-Naphthaleneacetic acid (NAA) in half strength of Murashige and Skoong (MS) medium. The BAP and NAA was chosen because it had been reported by Feng *et al.* (2009) that the BAP and NAA combination promotes almost 62% of callus induction on leaves explants. Saikia *et al.* (2013) reported that maximum callus biomass produced in half strength of MS medium which supplemented with BAP and NAA at 4% of sucrose concentration.

1.2 Problem statement

In traditional method, *Vanilla planifolia* was propagated by cutting method to produce another seedling which resulting in the death of mother plant which actually ready for flowering and produce seed pod. Other than that, the *in vitro* propagation of *Vanilla planifolia* rarely used the leaves explant because nodal segment is usually preferred as it induce direct organogenesis.

1.3 Hypothesis

Different concentration of plant growth regulator will affect the induction of callus in *Vanilla planifolia* leaf explant.

1.4 Objectives

The objective of this study is to induce callus of *Vanilla planifolia* by manipulation of plant growth regulators.

1.5 Scope of study

The scope of the study will involve the induction of callus from *Vanilla planifolia* leaf explant in half-strength concentration of Murashige and Skoog (MS) media supplemented with combination of BAP and NAA as plant growth regulators. The formation of callus on *Vanilla planifolia* leaf explants was observed after 8 weeks.

1.6 Significance of study

This study aims to identify the type and concentration of plant regulator for the efficient induction of callus on Vanilla explants. The establishment of an efficient protocol for production of callus in *Vanilla planifolia* will help in mass production of propagules by formation of somatic embryogenesis later on.

1.7 Limitation of study

The time restriction was one of the main problem in completing study in the given time period. The other main problem is contamination of sample which causes loss of culture.

CHAPTER 2

LITERATURE REVIEW

2.1 *Vanilla planifolia*

2.1.1 Background of *Vanilla planifolia*

Vanilla planifolia is a kind of creeping plant. *Vanilla planifolia* lives by creeping on others tree trunk by using supporters like its roots. *Vanilla planifolia* has greenish flowers and not all of the flowers produce aroma. *Vanilla* flowers bloom only once a year. This flower will appear if it experiences specific stimulus such as shoot cutting and environmental stress like long drought. Their flowers are able to last only for a day. To produce *Vanilla* fruit (seed pods), this flower must undergo a manual pollination process by using hand-pollination. This is because *Vanilla* flower's stigma is completely covered by the flower's petals. The Vanilla flower is incapable of self-pollination without the help of pollination agent to either transfer the pollen from the anther to the stigma or to lift the flap or rostellum to press the anther against stigma. The exact time to do the hand-pollination is when the flower opens during the morning. On the next day, if the flowers drop from the vine its mean that pollination occurs. *Vanilla* seed pod is capsule like structure, have short-stem, with pod length reaching 10 to 25 cm, it has diameter of 5 to 15 mm and has a smooth surface. In a seed pod, it contains thousands of *Vanilla* seeds. The seeds only contain protocom. Seed pods produced when vanilla plants mature which is generally larger than 10 feet height. After the seed pods

are mature, they are harvested and go through a curing process that ferments and then dries while retaining the maximum amount of essential oils (Dressler, 1993).

2.1.2 Taxonomy of *Vanilla planifolia*

Vanilla planifolia comes from the family of Orchidaceae which has more than 25000 species and 700 genera. *Vanilla* is an edible plant that contains aromas and distinctive flavour (Ranadive, 1994). *Vanilla* is an herbaceous tree whose perennial climbing plant over another plant trunk by using its roots for support. *Vanilla* seed pod is a valuable spice in the world as it takes a long time to grow and harvest (Mujar, Sidik, Sulong, Jaapar, & Othman, 2014).

Kingdom: Plantae

Subkingdom: Viridiplantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Asparagales

Family: Orchidaceae

Genus: Vanilla

Species: *Vanilla planifolia*

2.1.3 Origin of *Vanilla planifolia*

Vanilla planifolia is a tropical orchid that known to be indigenous in lowland areas in the forest of South East Mexico, Central America and Gautemala. Now, this *Vanilla* plant is cultivated in large scale in other tropical areas, especially in Indonesia, Comoro Island, and Malagasy Republic (Purseglove *et al*., 1981). Vanilla was introduced in India in the 18th century and there is no variability in Vanilla cultivation at this time. The India climatic regions are reflected in the wide diversity of its orchid flora which about 1600 species of orchids were recorded. Nearly 1150 orchid species reported in India (Kumar and Manilal, 1994). Biradar *et al.* (2016) reported that *Acampe, Aerides, Bulbophylum, Cymbidium, Diplocentrum, Eria Habenaric, Ipsea, Phalus, Rhyncostrylis, Vanilla* and many others can be found in Peninsular India. About 75 genera and 315 species were reported in peninsular region meanwhile about 151 genera and 876 species of orchids are found in Himalayan regions.

Vanilla planifolia is the most expensive spices second only to saffron because it's the only orchid of the tropics is valued for its cured fragrant beans. The *Vanilla* plant has been introduced to all parts of the tropics and grown extensively in Madagaskar, Reunion and Comoro Islands. One of the potential countries for *Vanilla* cultivation is India. Vanilla plant can be grown as an alternate crop in cardamom, area nut plantation, coffee plantation and as intercrop in coconut. Biradar *et al.* (2016) reported that vanilla is commercially propagated by stem cuttings which are reported as uneconomical as it involves sacrifices of the mother plant.

2.1.4 Food uses of *Vanilla planifolia*

Vanilla planifolia is the orchid that commercially produces the vanilla flavouring. The seed pod also known as *Vanilla* beans is the part that had been used to produce the vanilla flavour. It contains fragrance in the pods. *Vanilla* seed pods need to be harvested before they fully ripen. The most balanced, strong flavour and intensive vanilla founded by researcher at Madagascar. Meanwhile the fresher and softer flavour founded in Mexico which these *Vanilla* beans are lesser priced than other *Vanilla* beans. *Vanilla tahitensis* known as the most floral vanilla fragrance than other typres of *Vanilla* species which can be found in Tahititian, India are very special because it's availability so rare.

2.1.5 Propagation of *Vanilla planifolia*

Vanilla planifolia are tropical crops. Therefore, *Vanilla planifolia* prefers hot and humid climate to grow well. Purseglove *et al.* (1981) stated that *Vanilla planifolia* only favour temperature between 21°C to 23°C together with 175 cm to 225 cm annual rainfall which is evenly distributed for 10 months. However, to stimulate flowering, a dry weather for two months are required by vegetative growth limitation. The optimum altitude for *Vanilla planifolia* to grow up to 700 m at Mexico. *Vanilla* flower initiate after 3 to 4 years and subsequently flowers once a year. The *Vanilla* fruit or known as seed pods matured at 8 to 10 months before harvesting. Rao and Ravishankar (2000) stated that *Vanilla* fruit known as subject to the production of vanillin. Traditionally, cutting propagation had been applied to *Vanilla* commercially because *Vanilla* prefer with stem-cutting for asexual propagation. Purseglove *et al.* (1981) reported that to initiate flower earlier, the stem-cutting must be between 2 to 3.5 m long. Meanwhile, the stem-cutting that smaller than mentioned leads to

late initiation of flower and production of fruits but produce more plantation for longer period. Usually, for direct field plantation, longer stem-cutting are required meanwhile for nursery, the shorter stem-cutting are preferable. The plantation by seed is very rare practiced in the field.

2.2 In vitro propagation of *Vanilla planifolia*

Mengesha *et al.* (2012) stated that the traditional cultivation through stem-cutting of *Vanilla planifolia* take longer time, labours and not sustainable. In addition, Vanilla fruits cannot sprout under natural condition other than the presence of mycorrhiza which is because it supply of food storage inadequate (Porras-Alforo and Bayman, 2007). There are 10 viruses discovered globally had been contaminated *Vanilla planifolia* (Richard *et al*., 2009). In India, there are four viruses had been counted in Vanilla planifolia contamination, they are Bean common mosaic virus (BCMV), Bean yellow mosaic virus (BYMV), Cucumbur mosaic virus (CMV) and *Cymbidium* mosaic virus (CymMV) (Bhadramurthy, 2008). Therefore, to conserve the plant and for propagules requirement, rapid multiplication rate of *Vanilla planifolia* are needed. The only techniques that serve the way out is plant tissue culture which rapid multiplication rate as well as the *Vanilla planifolia* species conservation is ensured. Plant tissue culture only requires limited plant tissue supply as source of explant also it is known as season independent. Pierik (1990) stated that plant tissue culture help in disease-free plant production. Some scientist reported on in vitro mass generation by using different type of explant of *Vanilla planifolia*, which are nodal segment, shoot tips and callus culture (Gantait and Kundu, 2017).

The culture of different organs, tissues or any totipotent zone of the plant which is under aseptic condition also known as in vitro propagation. Harbage (2001) mentioned that in vitro propagation provide superiority more than traditional vegetative because of the rapid rate of multiplication as well as efficient in maintaining the uniformity of clonal of the species. Gantait *et al.,* (2016) stated that the effectiveness for in vitro to regenerate are depends on several physical and chemical factors such as source of the explant, composition of media and plant growth regulators (PGRs) as well as different condition of culture.

2.2.1 Organogenesis

Organogenesis is the formation of shoots, and the roots through the cells in the culture are the same as the adventitious roots and the formation of shoots in the form of cuttings. There are two types of organogenesis, namely direct organogenesis and indirect organogenesis. These two types depend on the combination of hormones used in media culture. Philip and Nainar (1986) have been reported that *Vanilla planifolia* has been propagated through direct organogenesis meanwhile Davidonis and Knoor (1991) have been reported that *Vanilla planifolia* also has been propagated through indirect organogenesis. In this research, organogenesis that has been used indirect organogenesis.

2.2.1.1 Direct organogenesis

Direct organogenesis or mostly known as the formation of somatic embryos does not need the callus formation unlike indirect organogenesis. In previous study on direct organogenesis, it had been reported that it directly form through cell that already in differentiated tissue (embryogenic cell). To promote the initiation of embryogenic cells, several conditions are required such as applying exogenous growth regulator therefore the expression of embryogenesis and the division of cell allowed. These cells tend to be more responsive than the cell involved in the formation of indirect organogenesis. In direct organogenesis, the auxins hormone does not required to initiate the division of cell because the application of plant growth regulator has finer role and the cell never leave the cell cycle. In previous study on direct organogenesis of *Vanilla planifolia*, it had been recorded that the combination of BAP (cytokinin) with Kin or NAA (auxins) showed the formation of multiple shoot from each explant (Abebe, Mengesha, Teressa and Tefera, 2009). Mushimiyamana *et al.* (2011) tested that at concentration 2.5 μM^{-1} of BA recorded that the maximum length of shoot and maximum number of shoot produced. The usage of silver nitrate in Murashige and Skoong (MS) media had been recorded by Giridhar *et al.* (2001) which resulted in maximum proliferation and length of shoot. There are some case study reported the important of plant growth regulator (PGR) in initiation of roots in *Vanilla planifolia*. Giridhar *et al.* (2001) had been recorded that the combination of IAA with IBA or NAA showed the initiation of numerous roots. Some scientist reported that by using only NAA successfully initiate the induction of root *Vanilla planifolia* (Morwal *et al.,* 2015). Activated charcoal had been reported successfully absorbed polyphenol through chemical process also for optimum rhizospheric atmosphere the luminosity elimination was required. Gantait *et al.* (2009) recorded that combination of activated charcoal and NAA has high efficiency in root initiation.

2.2.1.2 Indirect organogenesis

In indirect organogenesis, callus formation is produced from explant. Organ can be formed through this callus tissue. There are three main factors that affect the regeneration process. These factors are the source of explant, culture environment and nutrient media and constituent. For source of explant, the organ part used as an explant plays an important role in the formation of calluses. Not all organs can produce this callus formation. Among the organs that are always used for callus formation in *Vanilla planifolia* are leaves, seeds, shoots tips, and nodal segment. Physiology and ontogenic age of the explant organ also play a role in the formation of calluses. A healthy physiology and a young ontogenic age promote the formation of callus. Size of explant must be bigger because large size of explant contains more nutrient reserves and plant growth regulators to sustain the culture. Last but not least, the overall quality of the plant from which explants are taken. Explant should be obtained from a healthy tree mother. If the explant is obtained from an unhealthy plant, this will make it difficult for the explant growth in media culture. This causes high contamination due to the bacteria and diseases in the explant reproduce in the media culture.

In media culture, there are several types of physical form of the medium such as solid media, semi-solid media and liquid media. It shows that between these three media, solid media promote formation of callus. Other than that, the pH of the media also role in formation of callus. Light quality and quantity used in culture affect the formation of callus induction. In callus induction, the explant needs to be place in dark place for the initial induction. This is because dark place increase the effectiveness of callus induction (Davidonis, Knoor and Romagnoli, 1996). The temperature needed for induction of callus is exactly at 26°C.

2.3 Callus culture

Callus culture is an unorganizing, unspecialized, growing and dividing mass of cells. It formed when explants are cultures on solid medium with combination of auxin and cytokinin hormones in a correct condition. There are some degrees of dedifferentiation during the formation of callus in both metabolism and morphology which resulting in the loss of ability to photosynthesis (Manojsiddartha, 2014). Callus is a result of wound which naturally developed on plant which it can be left to develop or it can further divided. Basically, callus is non-organized tumour tissue. Some researchers stated that induction of callus was influenced by the combination of auxins and cytokinins. The use combination of NAA (auxins) and BAP (cytokinin) induced more proliferation of callus rather than single PGR (Divakaran et al., 2015). 2,4-dichlorophenoxy acetic acid (2,4-D) and NAA are effective callus inducer after combining with BAP. It also shows that 2,4-D are more effective than NAA since it generated more frequencies of callus induction and proliferation (Janarthanam and Seshadri, 2008). There are report on successfully obtained callus development which is 100% by using seeds as explant and placed the culture in dark incubation as well as using Murashige and Skoong (MS) medium supplemented with 2.27 μM of TDZ (Ramirez-Mosqueda and Iglesias-Andreu, 2015).

2.3.1 Morphology of callus

Morphology of callus can be identified in two main forms of texture and colour. Callus can be divided into two type of form, which is compact or friable. Compact callus has a compact form of aggregate cell. Compact callus on the other hand consist of tracheid-like cells, giant size of cells and closely packed cells it also may be in nodular form. Meanwhile,

friable callus has a friable form of associated cells and the callus can break apart so easily and it is softer than compact callus. Friable callus made of heterogenous mass of cells that having minimal contact. Friable callus often used for generating cell suspension cultures. Certainly, callus morphology are differ with different type of explant. In this case study, leaves explant were used in callus formation. Lavanya *et. al.* (2014) stated that callus that form from leaves explant were green in colour, friable in texture and slow growth in callus formation.

To identify the healthiness of callus, colour of callus often observed for identification. Usually, callus was observed in white or creamish yellow colour. Sometimes it pigmented. This pigmentation may be patchy or uniform. Green colour of callus also can be observed. This colouration change when white callus tissue which grown in the dark condition turns into green colour after exposed in light condition. Green colour of callus developed due to chloroplastid development in callus cell tissue. Meanwhile, yellow colour of callus cell may be due to the carotenoid synthesis pigment. It differ in cauliflower culture which the callus tissue is purple in colour which effect from anthocyanin accumulation in vacuoles or oxidized form of 3,4 dihydroxy phenylalanine (DoPA) produced. Frequently, brown colour was observed in callus tissue. These effects from phenolic substance excrete. A large number of phenolic compound and the polyphenol oxidase that contain in plant tissues separated the phenols spatially. When the explant is cut during aseptic technique, the phenols which come in contact with the enzyme then oxidized to quinones. The quinones polymerize then browning callus tissue formed. Generally, the phenols excretion inhibit in callus tissue growth.

2.3.2 Properties of callus

Callus culture does not need any photosynthesis because to initiate the callus induction, callus culture need to be grows in the dark condition with cold temperature. Usually, callus culture can be form from shoot during initial culturing. Callus culture also can be maintained easily. Callus culture can be used to isolate single cells with stem cell like properties or also known as totipotent.

2.3.3 Stage of callus culture

Callus cultures have three stages which are induction, proliferative stage, and morphogenesis stage. In induction stage, callus culture does not need photosynthesis which the culture were placed in dark condition with cold temperature. It is the stage where the explant starts to dedifferentiate and divide it cells. In proliferative stage, the divided cells are undergoes rapid cell division. Last but not least, the morphogenesis stage which in this stage it need photosynthesis to undergoes plant regeneration which the differentiation and organized structure will form in this process.

2.3.4 Habituation of callus

Induction of callus generally needs plant growth regulator in nutrient medium in order to maintain the growth through serial subcultures. But, callus tissue has been observed in some species of plant become habituated after prolonged culture which means that it able to continuously grow on basal medium or standard maintenance medium which growth hormones is devoid. This property also known as habituation of callus tissue.

Habituated callus tissue has been suggested to developed the capacity to synthesis sufficient amount of cytokinin and auxins which supplied hormones for their exogenously independency. In hormone requirement, both normal callus tissue and habituated callus tissue cannot be differing.

In plant tissue culture, plant tumour tissue can be isolated from explants aseptically. The tumour tissue like habituated callus, it capable to grow on simple basal medium such as no hormone supplement medium. Tumour tissue origin is differing from habituated callus. The virus remains and multiple in case of wound tumour within the cells and will be disappear after a long period in culture. In tissue culture, crown gall tumour tissues are made artificially which free from bacteria. Otherwise, the bacteria will grow in the cell culture. Secondary tumours can be directly cultured on medium. Bacterial DNA that presence in the crown gall tumour cells genome makes it possible as hormone-independent.

2.4 Source of explant

In plant tissue culture, the primary step is *in vitro* aseptic culture. Correct explant selection and the efficiency of disinfection procedure are factors of culture establishment depends on. According to Akin-Idowu *et al.* (2009), the most popular explant used for regeneration are axillary buds, shoot tips and root tips which can be found at merismetic ends of plants since they can accumulate the amount of PGR required and highly totipotent.

In *Vanilla planifolia*, the explant that efficiently used for in vitro direct organogenesis is nodal segment by several scientists. This is because nodal segments produced the highest number of shoots. Due to the axillary buds availability in nodal segments which actively break in opposition of the buds to other tissue that initiate adventitious buds before undergoes the proliferation (Gantait and Kundu, 2017). In organogenesis of *Vanilla planifolia*, there are only few scientist reported that the explant used for induction and calli proliferation by using leaf, shoot, immature seeds were successful. Leaf explant was reported has higher percentage of callus production than nodal explants which 60% and 35% respectively (Janarthanam and Seshadri, 2008). Due to somaclonal variation occurs in callus culture, this method is not a favoured option (Wang and Wang, 2012). But, the selection of different suitable attributes can be provided by callus (Krutovsky *et al.,* 2014) and related studies on cell suspension, genetic transformation as well as protoplast culture can be used.

In this case study, *Vanilla planifolia* leaf explant was used for callus induction. Leaf explant was reported has higher percentage of callus production than nodal explants which 60% and 35% respectively (Janarthanam and Seshadri, 2008). *Vanilla planifolia* leaf explant

was used as explant since the *in vitro* propagation of *Vanilla planifolial* rarely use the leaf explant, because nodal segment is usually preferred as it induce direct organogenesis.

2.5 Plant growth regulator

There are five major of PGR used which essential to add in the culture medium which are cytokinin, auxins, ethylene, abscissic acid and gibberellins. Usually, ethylene will be used when it involved in flower and abscission in plants but rarely used in plant tissue culture. The most widely used PGR is cytokinin and auxins. Manipulation of both PGR can lead to development of shoot (more cytokinin than auxin) or root (more auxin than cytokinin). Mohajer, Rosna, Khorasani and Jamilah (2012) stated that combination of BAP (cytokinin) and NAA (auxins) is more effective than BAP and IBA in formation of callus. The formation either directs organogenesis or indirect organogenesis is majorly depends on type and concentration of the PGR. BAP and NAA combination promotes almost 62% of callus induction on explants (Feng, Qu, Zhou, Xie and Xiang, 2009).

2.5.1 Combination of 6-benzyl amino purine (BAP) and 1-Naphthaleneacetic acid (NAA) in callus culture

6-benzyl amino purine or widely known as BAP is one of the plant growth regulator which is cytokinin type. It is synthetic cytokinin which the most widely used in initiate plant growth and development responses together with auxins. It is also most used in supplement to Murashige and Skoong (MS) medium. Azeem, Ullah, Ali, Khan and Bakht (2010) stated that BAP promoted cell division which clearly stated that it can initiate callus alone without

any combination with other. Maximum callus formation were observed on MS media supplemented with BAP (Rahaman *et. al.,* n.d.). BAP of plant growth regulator was proved and highly suggested to use it in callus induction. In plant tissue culture, BAP known to improve shoot proliferation and elongation (Glocke, Collins, and Sedgley, 2006).

1-Naphthaleneacetic acid or widely known as NAA is one of the plant growth regulator which is auxins type. It is the most popular synthetic auxins which widely used in initiate plant growth and development responses together with cytokinins. NAA promote in increasing cellulose fiber formation in plant when paired with gibberellic acid which is one of the phytohormone. In plant tissue culture, NAA promote induction of roots in various explants (Morikawa and Takahashi, 2004).

Ahmad and Spoor (1999) stated that both BAP and NAA are needed for initiation of callus and growth of callus. It is also stated that intermediate ratio or slightly high concentration of NAA than concentration of BAP result in good growth of callus. When cytokinin and auxins in balance ratio it showed more growth of callus. A similar result obtained in experiments which dealing with tissue culture in Brassica spp. (Sharma et al., 1990). Flick *et al.* (1983) recorded that low concentration of cytokinin and high concentration of auxins promotes formation of callus which abundant cell proliferation form. The combination of 17 percent of BAP and 95 percent of NAA promote in callus formation in cotyledon cultures (George and Rao, 1983). In plant tissue culture, organogenesis is produced by the balance ratio of concentration of auxins and cytokinin in Murashige and Skoong (MS) media cannot be applied to all type of explants. It is because of differences of explants material or environmental conditions or other growing factors (Skoog and Miller, 1957).

In the conclusion, cytokinin and auxins were both needed in initiation of callus culture. Balance ratio of cytikinin and auxins favoured in growth of callus while low concentration of cytokinin and high concentration of auxins promoted in formation of roots meanwhile high concentration of cytokinin and low concentration of auxins promoted in formation of shoots.

2.6 Murashige and Skoong (MS) medium for callus culture

Callus culture can be induced by using any type of nutrient medium, but the most widely used nutrient medium for callus culture is Murashige and Skoong (MS) media. The rate of callus induction in different concentration and types of hormonal treatment. Saikia *et al.* (2012) stated that the concentration of sucrose in nutrient medium play important role in callus induction. Saikia, Shrivastava and Singh (2013) reported that maximum callus biomass produced in MS medium which supplemented with BAP and NAA at 4% sucrose concentration. MS media was highly recommended to obtain high production percentage of callus with good quality of mass of callus.

CHAPTER 3

MATERIAL AND METHODOLOGY

3.1 Explant source

Vanilla planifolia plants that have been propagated *in vivo* will be collected from the Ayer Hitam, Johor in July, 2017. The plants will be used as the plant material for callus induction. The 1 year old of *Vanilla planifolia* orchid's leaves will be used as explant in this study.

Figure 3.1: *Vanilla planifolia* orchid in nursery

3.2 Media preparation

Explants will be placed on the surface of half strength of Murashige and Skoong (MS) basal medium supplemented with 30 grams of sucrose and 8 grams of agar. Plant growth regulators will be added (according to Table 3.2) prior to autoclaving according to the experimental objectives. The pH of the media was adjusted to 5.7 with 1N NaOH or HCl prior to autoclaving for 15 minutes at 121°C.

Table 3.1: Half-strength MS medium composition

Macronutrient	**1X (g/L)**
NH_4NO_3	1.65
KNO_3	1.90
$CaCl_2.2H_2O$	0.44
$MgSO_4.7H_2O$	0.37
KH_2PO_4	0.17
Micronutrient	**1X (g/L)**
$MnSO_4.4H_2O$	0.02230
$ZnSO_4.7H_2O$	0.00860
H_3BO_3	0.00620
KI	0.00083
$NaMoO_4.2H_2O$	0.00025
$CuSO_4.5H_2O$	0.000025
$CoCl_2.6H_2O$	0.000025
Vitamin	**1X (g/L)**
Myo-inositol	0.1
Glycine	0.002
Thiamine-HCL	0.001
Nicotinic acid	0.0005
Pyridoxine-HCL	0.0005
Ferum source	**1X (g/L)**
$FeSO_4.7H_2O$	0.0278
$Na_2EDTA.2H_2O$	0.0373

3.3 Experimental treatments

Each explant will be placed on half-strength Murashige and Skoong (MS) basal medium supplemented with different treatment (as in Table 3.2). Each treatment will have three replicate.

Table 3.2: Experimental treatment

Label	Treatments
T1	Control
T2	1 mg/l of BAP + 1 mg/l of NAA
T3	1 mg/l of BAP + 2 mg/l of NAA
T4	1 mg/l of BAP + 3 mg/l of NAA
T5	2 mg/l of BAP + 1 mg/l of NAA
T6	2 mg/l of BAP + 2 mg/l of NAA
T7	2 mg/l of BAP + 3 mg/l of NAA
T8	3 mg/l of BAP + 1 mg/l of NAA
T9	3 mg/l of BAP + 2 mg/l of NAA
T10	3 mg/l of BAP + 3 mg/l of NAA

3.4 Explant surface sterilization

The explants were washed thoroughly with running tap water for 10 minutes. The explants were washed with 5% detergent solution for 5 minutes. Then disinfected with 80% of Sodium Hypochloride for 15 to 20 minutes and were rinsed for 5 times using sterile distilled water to remove the sterilants.

3.5 Initiation of culture

Leaves segment and stem nodal segment explant were cut into 1.0 to 2.0 cm. Explants were cultured in glass bottle jars containing half-strength of Murashige and Skoong (MS) supplements with different concentration of plant growth regulators under 16 hours photoperiod at 28-36 µmol m^{-2} s^{-1} and 26°C.

3.5 Research design

For this study, randomized complete block design (RCBD) was used because it is suitable for experiments where the number of treatments is not large and the experimental site has a predictable productivity gradient.

3.6 Data collect

Parameters that were collected as presence of callus formation, fresh weight of callus, callus index, colour of callus, and appearance of callus. Then, calluses were observed the presence of shoot and hairy root formation. The data were presented as mean ± standard deviation.

3.7 Statistical analysis

The data were analysed by one way analysis of variance (One-way ANOVA) to detect the treatment effects. Post-Hoc test were conducted to analyse the significant of the data where the post-hoc test that was used in this experiment is Tukey's HSD test was used to compare the treatments efficient in induction of callus at $P \leq 0.05$. The data obtained were processed statistically with the IBM SPSS Statistics (version 21) software.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Formation of callus

Table 4.1: Effect of plant growth regulators and formation of callus

Treatment	Formation of callus
T1	Absent
T2	Present
T3	Present
T4	Present
T5	Present
T6	Present
T7	Present
T8	Present
T9	Present
T10	Present

According Table 4.1, MS medium that not supplemented with any plant growth regulator (T1) does not initiate any formation of callus. Therefore, the percentage of callus formation is 0%. According to Tan, Chin and Alderson (2011), there is no formation of callus on MS medium without supplemented of plant growth regulators. This statement clearly supported the result of this study. Ahmad and Spoor (1999) stated that both cytokinin and

auxin are needed for initiation of callus and the growth of callus. When the combination of cytokinin and auxin is balance in ratio it showed more growth of callus. The use combination of NAA (auxins) and BAP (cytokinin) induced more proliferation of callus (Divakaran *et al.,* 2015). This contrasts with studies that carried out by Judy *et al.* (2017) which the presence of PGRs was not important for induction of callus. This study also contrasts with studies that carried out by Gantait *et al.* (2012) which 32.9% of leaf explants were found able to induce callus without supplemented with any PGRs.

4.2 Fresh weight of callus

Table 4.2: Effect of different concentrations of plant growth regulator and fresh weight of callus

Treatment	Fresh weight of callus (g)
T1	0.0 ± 0.00^{d}
T2	1.46 ± 1.00^{c}
T3	1.25 ± 1.00^{c}
T4	1.52 ± 2.65^{b}
T5	1.10 ± 2.00^{b}
T6	1.27 ± 2.08^{b}
T7	1.57 ± 4.58^{a}
T8	1.42 ± 0.00^{d}
T9	0.80 ± 2.89^{b}
T10	1.08 ± 4.51^{a}

The effect of plant growth regulator towards callus induction is shown in Table 4.1. From the table we can differentiate the fresh weight of callus formation with different concentration of BAP and NAA. Table 4.1 clearly shows that T7 with combination of 2 mg/l of BAP and 3 mg/l of NAA have the highest fresh weight of callus formation by 1.57±4.58 g. Flick *et al.* (1983) stated that low concentration of cytokinin and high concentration of auxin promotes formation of callus with formation of abundant cell proliferation. This statement clearly supported this study which BAP (cytokinin) have lower concentration than NAA (auxin) promotes formation of abundant cell proliferation which result in highest average fresh weight of callus. Meanwhile, the lowest fresh weight of callus formation was showed on T9 with combination of 3 mg/l of BAP and 2 mg/l of NAA by 0.80±2.89 g. There are significant different between both treatments ($P<0.05$). In this study, the combination of 2 mg/l of BAP and 3 mg/l of NAA was found to be more effective for callus induction.

In the earlier studies, toward effect of PGRs on induction of embryogenic callus from leaf explants reported that the combination of higher concentration of auxin with low concentration of cytokinin in half-strength Murashige and Skoong (MS) medium was vital in promoting high survival percentages of explants and formation of embryogenic callus (Juddy, Jainol and Jualang, 2017). The highest fresh weight of callus was observed in medium T7 supplemented with 2 mg/l of BAP and 3 mg/l of NAA by 1.57±4.58 g and medium T4 supplemented with 1 mg/l of BAP and 3 mg/l of NAA by 1.52±2.65 g. There is no significant difference between both concentration since it higher than ($P>0.05$). In this study, cultured explant in half-strength MS medium without supplemented with PGRs was observed does not produced callus.

Gantait, Bustam and Sinniah (2012) stated that the proportions of PGRs have species-specific effect on induction of callus from leaf explants. BAP is synthetic cytokinin

plant growth regulator that ascendancy in producing callus from leaf explants was found in different type of orchid species including *Doritaenopsis* (Park, Yeung, Chakrabarty, & Paek, 2002), *Phalaenopsis* (Kuo, Chen and Chang, 2005) and *Oncidium* (Mayer, Stancato and Appezzato-Da-Gloria, 2010). Meanwhile, NAA is synthetic auxin type of plant growth regulator that widely used in in vitro propagation that help in promote induction of callus of orchid species including *Oncidium* (Mayer *et al.,* 2010), *Vanilla* (Tan, Chin and Alderson, 2011), *Phalaenopsis* (Khoddamzadeh *et al.,* 2011), *Dendrobium* (Mei, Danial, Mahmod, and Subramaniam, 2012), and *Renanthera* (Wu *et al.,* 2012).

4.3 Callus index

Table 4.3: Effect of different concentration of plant growth regulator and callus index

Treatment	Callus index (%)
T1	0 ± 0.0^{c}
T2	14.6 ± 1.0^{b}
T3	12.5 ± 1.0^{b}
T4	15.2 ± 1.0^{b}
T5	11.0 ± 1.0^{b}
T6	12.7 ± 1.0^{b}
T7	15.7 ± 1.0^{b}
T8	14.2 ± 9.5^{a}
T9	8.0 ± 1.0^{b}
T10	10.8 ± 1.0^{b}

The effect of plant growth regulator towards callus index is shown in Table 4.2. From the table we analysed that the callus index depends on the concentration of BAP and NAA which supplemented in half strength MS medium. Table 4.2 clearly shows that T7 with combination of 2 mg/l of BAP and 3 mg/l of NAA have the highest callus index by 15.7% ±1.0. The callus index was determined based on the fresh weight of callus. If the fresh weight of callus is higher, then the callus index also higher. Flick *et al.* (1983) reported that low concentration of cytokinin and high concentration of auxin promotes formation of callus with formation of abundant cell proliferation which this report clearly supported this study which the concentration of BAP (cytokinin) is lower than the concentration of NAA (auxin) produce the highest mean of callus induction which then produce the highest callus index. Meanwhile, the lowest callus index was shown on T9 with the combination of 3 mg/l of BAP and 2 mg/l of NAA by 8.0% ±1.0. There are significant different between both treatments supplemented which ($P<0.05$). In this study, T7 with the combination of 2 mg/l of BAP and 3 mg/l of NAA was found to be the highest callus index.

4.4 Colour and appearance of callus

Table 4.4: Effect of different plant growth regulator and colour and appearance of callus

Treatment	Colour of callus	Appearance of callus
T1	-	-
T2	Creamish green	Friable
T3	Creamish green	Friable
T4	Green	Friable
T5	Green	Friable
T6	White green	Friable
T7	Creamish green	Friable
T8	Yellowish green	Friable
T9	Yellowish green	Friable
T10	Green	Friable

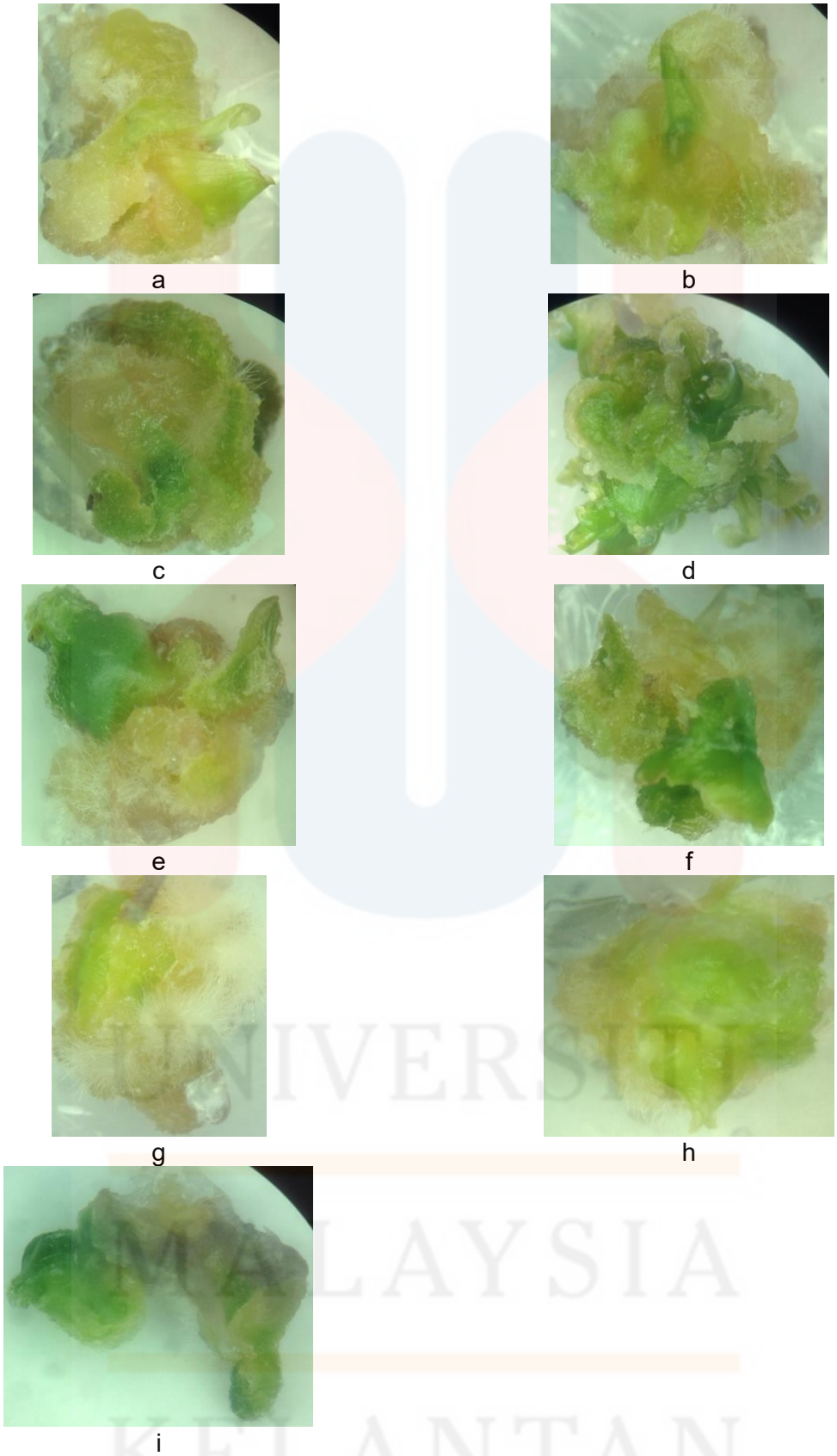

Figure 4.2: Callus observation. **a** T2: 1 mg/l of BAP + 1 mg/l of NAA. **b** T3: 1 mg/l of BAP + 2 mg/l of NAA. **c** T4: 1 mg/l of BAP + 3 mg/l of NAA. **d** T5: 2 mg/l of BAP + 1 mg/l of NAA. **e** T6: 2 mg/l of BAP + 2 mg/l of NAA. **f** T7: 2 mg/l of BAP + 3 mg/l of NAA. **g** T8: 3 mg/l of BAP + 1 mg/l of NAA **h** T9: 3 mg/l of BAP + 2 mg/l of NAA. **i** T10: 3 mg/l of BAP + 3 mg/l of NAA.

The initiation of *Vanilla planifolia* callus was observed after 8 weeks of culture from leaf explants with combination of hormone auxins and cytokinin which supplemented in half strength of Murashige & Skoong (MS) medium. When high concentration of auxin used in half strength of MS medium, the colour of callus was creamish green and yellowish green but when high concentration of cytokinin used in half strength of MS medium, the colour of callus was creamish green and green. The usual callus colour that was observed is white or creamish yellow in colour. In this study, majority of the callus almost turn in green colour. This green colour of callus developed due to chloroplastid development in callus cell tissue which the growth conditions trigger it. The green colour observed after the white callus was exposed in light condition. Yellowish green colour also can be observed in this study. The callus becomes yellowish in colour because of the carotenoid synthesis pigment. Overall, the healthiest callus is green in colour. This can be observed by their formation of colour. All of the calluses that have been observed have friable appearance which made of heterogeneous mass of cells that having minimal contact. Lavanya *et al.* (2014) stated that callus that form from leaves explant were green in colour, friable in texture and slow growth in callus formation.

4.5 Formation of shoot and hairy root

Table 4.5: Effect of different concentration of plant growth regulators and formation of shoot and hairy root

Treatment	Formation of shoot	Formation of hairy root
T1	Absent	Absent
T2	Present	Present
T3	Present	Present
T4	Present	Absent
T5	Present	Present
T6	Present	Present
T7	Present	Present
T8	Present	Present
T9	Present	Absent
T10	Present	Absent

In this study, the formation of callus was observed. There are some calluses form shoot and some calluses form hairy root also some form both shoot and hairy root. The observation then recorded in Table 4.5. The formation of shoot and hairy roots observed after 3 weeks when white calluses had been exposed into light condition. Light condition triggered chloroplastid development in callus cell tissue which developed in shoot formation and hairy roots formation.

Generally, the combination of BAP (cytokinin) and NAA (auxin) promote induction of shoot and hairy root formation from calluses which this has been found in previous studies on various monocotyledonous plant species such as *D. frimbriatum* (Roy and Banerjee,

2003), *Anthurium andreanum* (Vargas *et al.,* 2004) and *Curcuma amada* (Prakash *et al.,* 2004). The formation of shoot and hairy roots formed from *Vanilla planifolia* callus when both BAP and NAA was supplemented in the medium (Janarthanam and Seshadri, 2008). Baskaran and Jayabalan (2005) reported that BAP is one of the most effective plant growth regulators of cytokinins group for the induction of shoot in plant tissue culture. Several studies showed that BAP was more effective than other cytokinin group hormone in enhancing shoot for various plant species such as *Geoderum purpureum* (Mohapatra and Rout, 2005), *Rubus* (Wu *et al.,* 2009), *Crossandra infundibuliformis* (Girija *et al.,* 1999) and Curculigo orchioides (Nagesh, 2008). Janarthanam and Seshadri (2008) reported that the formation of roots from callus of *Vanilla planifolia* was observed when the concentration of NAA increased. Similar studies found in *D. candidum* (Zhou *et al.,* 2008) and *Striga hermonthica* (Ma *et al.,* 1998).

CHAPTER 5

CONCLUSION

5.1 Conclusion

The investigation was conducted for induction of callus for *Vanilla planifolia* by using leaves as explant source. From the result, T1 does not initiate callus since there is no plant growth regulator supplemented in the medium.

The highest mean fresh weight was observed in T7 with combination of 2 mg/l of BAP and 3 mg/l of NAA have the highest fresh weight of callus formation by 1.57±4.58 g meanwhile the lowest mean fresh weight was observed in T9 with combination of 3 mg/l of BAP and 2 mg/l of NAA by 0.80±2.89 g. There is significant different between concentration and mean fresh weight of callus ($P<0.05$). The highest callus index was observed in T7 with combination of 2 mg/l of BAP and 3 mg/l of NAA by 15.7% ±1.0 meanwhile the lowest callus index was observed in T9 with the combination of 3 mg/l of BAP and 2 mg/l of NAA by 8.0% ±1.0. There is significant different between concentration and callus index ($P<0.05$). The colour of callus produced by observed calluses were white-green, creamish green, yellowish green and green colour. In this study, all induced calluses observed as friable appearance. Majority of the callus form induce shoot and hairy root.

5.2 Recommendation

In this experiment by using leaves as explant, the growth of callus slows especially when supplemented with combination of NAA and BAP. Therefore, as recommendation to fasten the growth of callus and the good result on callus, TDZ plant growth regulators need to be used in this experiment. Also different parts of plants could be used as explant to produce callus. Next, study needs to carry out for callus induction using immature seeds of these orchid species and also to develop and standardise the protocol for *in vitro* regeneration and mass multiplication via callus formation.

Further studies in tissue culture and biological activities of this species are vital and should be carried out in the future, focusing more on the callus induction, formation of PLB, acclimatization and synthetic seed production.

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APPENDICES

APPENDIX A

Table A.1: Test of homogeneity of variances of fresh weight of callus

Levene Statistic	df1	df2	Sig.
2.853	9	20	.024

Table A.2: Anova table of fresh weight of callus

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	169.467	9	18.830	2.810	.026
Within Groups	134.000	20	6.700		
Total	303.467	29			

Table A.3: Homogenous subsets of fresh weight of callus

Concentration of PGRs	N	Subset for alpha = 0.05	
		1	2
Control	3	1.0000	
1 mg/l of BAP + 1 mg/l of NAA	3	3.0000	3.0000
1 mg/l of BAP + 3 mg/l of NAA	3	5.0000	5.0000
1 mg/l of BAP + 2 mg/l of NAA	3	6.0000	6.0000
3 mg/l of BAP + 3 mg/l of NAA	3	6.6667	6.6667
2 mg/l of BAP + 1 mg/l of NAA	3	7.0000	7.0000
2 mg/l of BAP + 2 mg/l of NAA	3	7.3333	7.3333
2 mg/l of BAP + 3 mg/l of NAA	3	8.0000	8.0000
3 mg/l of BAP + 2 mg/l of NAA	3	8.3333	8.3333
3 mg/l of BAP + 1 mg/l of NAA	3		9.0000
Sig.		.058	.189

APPENDIX B

Table B.1: Test of Homogeneity of variances of callus index

Levene Statistic	df1	df2	Sig.
10.587	9	20	.000

Table B.2: Anova table of callus index

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1710.000	9	190.000	19.192	.000
Within Groups	198.000	20	9.900		
Total	1908.000	29			

Table B.3: Homogenous subsets of callus index

Concentration of PGRs	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
Control	3	1.0000					
1 mg/l of BAP + 1 mg/l of NAA	3	3.0000	3.0000				
1 mg/l of BAP + 2 mg/l of NAA	3	6.0000	6.0000	6.0000			
1 mg/l of BAP + 3 mg/l of NAA	3	9.0000	9.0000	9.0000	9.0000		
3 mg/l of BAP + 1 mg/l of NAA	3	9.0000	9.0000	9.0000	9.0000		
2 mg/l of BAP + 1 mg/l of NAA	3		12.0000	12.0000	12.0000		
2 mg/l of BAP + 2 mg/l of NAA	3			15.0000	15.0000	15.0000	
2 mg/l of BAP + 3 mg/l of NAA	3				18.0000	18.0000	18.0000
3 mg/l of BAP + 2 mg/l of NAA	3					22.0000	22.0000
3 mg/l of BAP + 3 mg/l of NAA	3						25.0000
Sig.		.115	.054	.054	.054	.229	.229