EFFECT OF ORGANIC ADDITIVES ON *IN VITRO* SEED GERMINATION OF BORNEO'S ENDEMIC ORCHID, *Vanda dearei.*

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This project is submitted in partial fulfilment of requirements for the degree of Bachelor of Science with Honours in Resource Biotechnology

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DECLARATION

I hereby declare that this thesis is based on my original work except for quotation and citation, which have been duty acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institution.

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TABLE OF CONTENTS

LIST OF ABBREVIATION

2,4-D	2,4-Dichlorophenoxyacetic acid
½MS	Half strength Murashige and Skoog medium, 1962
ANOVA	Analysis of variance
DAC	Day after culture
$Ca(OCl)_2$	Calcium hypochlorite
DMRT	Duncan's Multiple Range Test
HCl	Hydrogen chloride
KC	Knudson C medium, 1946
MS	Murashige and Skoog medium, 1962
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
PLBs	Protocorms-like-bodies
p.s.i	Pound per Square Inch
RT	Raghavan and Torrey, 1964
SPSS	Statistical Package for Social Science
VW	Vacin and Went, 1949

LIST OF TABLES

LIST OF FIGURES

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ABSTRACT

The effect of different organic additives on *in vitro* seed germination of *Vanda dearei*, an endemic species of orchid to Borneo was investigated. The seeds from about 6-month old seed pod of *V. dearei* was used as the plant material and allowed to grow on ½ MS medium supplemented with four different concentrations of banana homogenate (1 g/L, 2 g/L, 3 g/L and 4 g/L, w/v), coconut water (5%, 10%, 15% and 20%, v/v), potato homogenate (1 g/L, 2 g/L, 4 g/L and 6 g/L, w/v) and tomato homogenate (1 g/L, 2 g/L, 4 g/L and 6 g/L, w/v). The culture condition was maintained at a 16 light/8 dark photoperiod at room temperature of 25±2 ℃. The seeds started to germinate after approximately one month of culture. The maximum germination percentage was recorded from ½ MS medium supplemented with 4 g/L (w/v) potato homogenate with 90.11±4.79% after 80 days of culture followed by 10% coconut water (v/v) (83.78±3.32%). The addition of organic additives has significantly enhanced the seed germination rate of *V. dearei* cultured *in vitro*. However, higher concentrations of coconut water at 15% and 20% (v/v) were found to be inhibitory for seed germination of *V. dearei*.

Key words: *Vanda dearei*, *in vitro* asymbiotic seed germination, organic additives, protocorm, ½ MS medium

ABSTRAK

Kesan penambahan organik aditif yang berlainan dikaji ke atas percambahan biji benih Vanda dearei, orkid endemik di Borneo. Biji benih V. dearei yang diperolehi dari kapsul orkid yang berusia 6 bulan digunakan sebagai bahan tumbuhan kajian dan dibiarkan tumbuh pada ½ MS medium yang ditambahkan dengan homogenat pisang, air kelapa, homogenat kentang dan homogenat tomato pada empat kepekatan yang berbeza. Keadaan kultur dikekalkan pada fotokala selama 16 jam cahaya/ 8 jam gelap dan suhu persekitaran pada 25±2 ℃. Benih mula bercambah selepas satu bulan pengkulturan. Peratus percambahan maksimum direkodkan pada media ½ MS yang ditambahkan dengan 4 g/L (w/v) homogenat kentang yang sebanyak 90.11±4.79% selepas 80 hari pengkulturan. Media ½ MS yang ditambahkan dengan 10% (v/v) air kelapa dicatatkan dengan 83.78±3.32%. Penambahan bahan aditif organic didapati dapat meningkatkan kadar percambahan benih V. dearei secara in vitro. Walaupun, kepekatan air kelapa yang tinggi pada 15% dan 20% (w/v) yang ditambah ke dalam media ½ MS telah didapati mengalami perencatan terhadap percambahan biji benih V. dearei.

Kata kunci: *Vanda dearei, percambahan biji benih asymbiotik in vitro, bahan tambahan organik, protocorm, medium ½ MS*

CHAPTER I

INTRODUCTION

Orchidaceae is considered as one of the largest flowering plant families which comprised of over 880 genera and approximately 25,000 to 30,000 species worldwide (Paek *et al.*, 2011; Bektas *et al.*, 2013). Chan *et al.* (1994) stated that approximately 10% of orchids in the world were found in Borneo, which is around 2500-3000 species. Among these species, about 30-40% are said to be endemic species to Borneo where Borneo is denoted as "Orchid Island" (Chan *et al*., 1994).

According to Beaman *et al.* (2001), *Vanda* is a monopodial genus of orchids that produce attractive flowers which are generally found at hill-forests or tropical lowlands. The magnificent flowers produced have been the reason for the high horticultural value of *Vanda* in which this genus is among the top five popular genera in horticultural industry. *Vanda* has been developed as potted plants and cut-flowers which also being subjected into producing hybrids (Teixeira da Silva, 2013). As stated by Beaman *et al.* (2001), *Vanda* is especially well presented in Philippines and Thailand where only five species are documented from Borneo like *V. hastifera, V. lamellata* and *V. scandens*. Among these five species, four of them are collected in Sarawak whereby *Vanda dearei* was believed to have spread widely on riverside trees formerly especially in Sarawak (Chan *et al.*, 1994).

Over-collection of orchids has endangered some of the species which have high commercial value in a variety of industries such as medical, horticultural and ornamental industries. Natural habitat of orchid has been destructed and disturbed by human activities, making the survival of wild orchid even tougher (Jawan *et al.*, 2010). According to Manners *et al.* (2011), the germination rate of orchid seed in nature is less than 5% mainly

due to the mycorrhizal association needed for seed germination. The survival rate of orchid in wild habitat is very low which also presses the need to perform *in vitro* seed germination. Based on the study that is fulfilled by Beaman *et al*. (2001), poorly preserved herbarium collections and insufficient information about the flower species of genus *Vanda* have made it substantial to practise cultivation upon the species.

The significance in performing *in vitro* seed germination of *V. dearei* is to optimize the seed germination process under *in vitro* condition in which the conditions and requirements of seed germination can be well-controlled. This can prevent the waste of resources and conserve the process of seed germination in making sure of the efficacy of the process. Furthermore, the addition of organic additives to the growth media in appropriate concentration and under properly maintained circumstance are able to enhance the effectiveness of seed germination carried out *in vitro* with relatively lower costs.

The objectives of this study were:

i. To investigate the effect of organic additives at different concentrations on *in vitro* seed germination of *Vanda dearei*;
ii. To determine the best organic additive for *in vitro* seed germination of *Vanda dearei*, and
iii. To develop an appropriate and cost-effective protocol for *in vitro* seed germination of *Vanda dearei*.

CHAPTER II

LITERATURE REVIEW

2.1 Orchidaceae

As one of the most diverse flowering families, orchid has an extensive number of species in which all of them displayed different functions and benefits toward the environment, ecology as well as economy. According to Cribb *et al.* (2003), the population of orchids exhibit an uneven distribution and noticeably more abundant at tropics area in relation to different continents. Despite of the fact that orchids are among the most widespread angiosperm families, many of the species are threatened with extinction mainly due to their propagation can only take place in some confined areas (Cribb *et al.*, 2003).

Orchids are growing as epiphytes, lithophytes, climbing herbs or terrestrial plants (Weston *et al.*, 2005). From ecological point of view, there are species of orchids that commonly maintaining a commensalism relationship with the plant that they are growing on like epiphytic orchids. There are three common types of epiphytic life-form that commonly exhibited by orchid in Borneo which are the trunk epiphytes, twig epiphytes and pendent epiphytes (Wood *et al.*, 1993). They obtain nutrient like nitrogen circulated by the trees as well as getting protection and sunlight from the taller trees. At higher altitude, pollination of the orchids is more likely to achieve.

Besides the horticultural value of the beautiful orchid flowers, medicinal value of orchids has also been widely documented. Orchids have been extensively used in the field of medicine traditionally. Various parts of orchids are used for medicinal purposes auch as the bulb, rhizome, root, tuber and pseudobulb (Singh & Dunggal, 2009). *V. testacea*, *V. tessellata* and *V. roxburghii* are among the examples of medicinally important orchids. There are a variety of phytochemicals reported to be successfully isolated from different

orchids (Singh & Dunggal, 2009). Among these are like aeridin, gigantol, oxoflaccidin, agrostophyllinol and so on. Singh and Dunggal (2009) also claimed that alkaloids are commonly found in medicinal orchids.

Furthermore, more and more ecotourists would like to explore the world of plants. Orchid, in particular has attracted a lot of the attention of the tourists. Ecotourism has developed into a revenue-generating industry as many would like to actually experience and appreciate the beauty of the wildlife reserves of flora and fauna.

2.2 Propagation of orchids

The seed of orchid is very fine and delicate. The seeds of orchid normally produced in a large quantity in which Arditti (1967) indicated that a capsule may contain about 1,300 to 4,000,000 seeds. The orchid embryo usually maintains its globular or spherical shape in contrast to the great variety of the shape of the seed coat which may be in globular, elliptical, rounded, butterfly shaped or fusiform (Arditti, 1967). Protocorm is formed when the swelling embryo ruptured the seed coat in which the protocorm is an undifferentiated mass of cells (McKendrick, 2000). Subsequently, the first leaf primordium will be projected out of the upper flat surface. The protocorm then starts growing and the absorbing hairs will start to emerge at the periphery of the lower surface. Next, the first minute leaf is produced (Arditti, 1967). Soon after this, the first root will be formed. The development continues until a small plant takes it shape.

2.2.1 Conventional vegetative propagation of orchid

As all the cells of a plant as believed to be carrying the genetic information required for the regeneration of an entire plant, the vegetative parts can be propagated asexually by vegetative propagation. It may involve cutting, grafting or budding (Sandor, 2007). Vegetative propagation of orchid can be carried out by using various part of the orchid such as keiki, pseudobulbs, leaf, PLBs and rhizomes. For example, keiki or knowing as additional offshoot is preferable to be used in vegetative propagation of orchids (Zotz, 1999). It was described that keikis resembled small shoots of orchid which were found growing on flower stalk or vegetative stems of some orchid genera. DeYoung *et al.* (2011) said that the plant produced from vegetative propagation will behave similarly to the parent plant since they are genetically identical.

The most commonly used method in vegetative propagation is division (DeYoung *et al.*, 2011). Parent plant is divided into parts that every new section is ensured to contain at least one new vegetative bud or shoot. For instance, the old pseudobulbs referred to as the backbulb could be propagated by cutting it off the parent plant. However, DeYoung *et al.* (2011) also stated that this could pressurize the parent plant and it may require several years for the propagated plant to flower. This technique has been widely used on several orchid genera which include *Cattleya, Cymbidium, Brassia* and *Paphiopedilum* (Fitch, 2006).

Besides, cutting propagation is also an important technique that generally contributes to the clonal regeneration of many horticultural and ornamental plants. However, important limitations of this technique were described by Högberg (2003) in diminishing the rooting ability and induce plagiotropic growth of rooted cutting plants.

By using conventional methods to cultivate orchid, the propagation rate is lower as compare to *in vitro* tissue culture method (Teixeira da Silva, 2013).

2.2.2 *In vitro* germination of orchid

As one of the most prevalent monopodial ornamental orchid genera, *Vanda* orchids are widely propagated by using *in vitro* culture techniques. *In vitro* methods are used to improve and assist the development of plants that are vulnerable to grow in nature as all of the essential conditions for growth can be manipulated and optimized (Fay, 1992). The development of the seeds cultured *in vitro* is quantified by looking at the stages of the embryo growth. The embryo will swell and occupy the seed coat. During this process, the colour and shape of the seed undergo changes. Prakash *et al.* (2012) discussed that the seeds of *V. tessellata* go through yellow colour to creamy structure when the embryo

ruptured the seed coat. It is then becoming a green colour spherule which is recognized as the protocorm stage.

Figure 2.1 showed the visual of seed development of *Grammatophylum speciosum* cultured *in vitro* by using different basal salt media with or without the supplementation of substances like activated charcoal, thidiazuron (TDZ) and N6-benzyladenine (BA) done by Khampa *et al.* (2010).

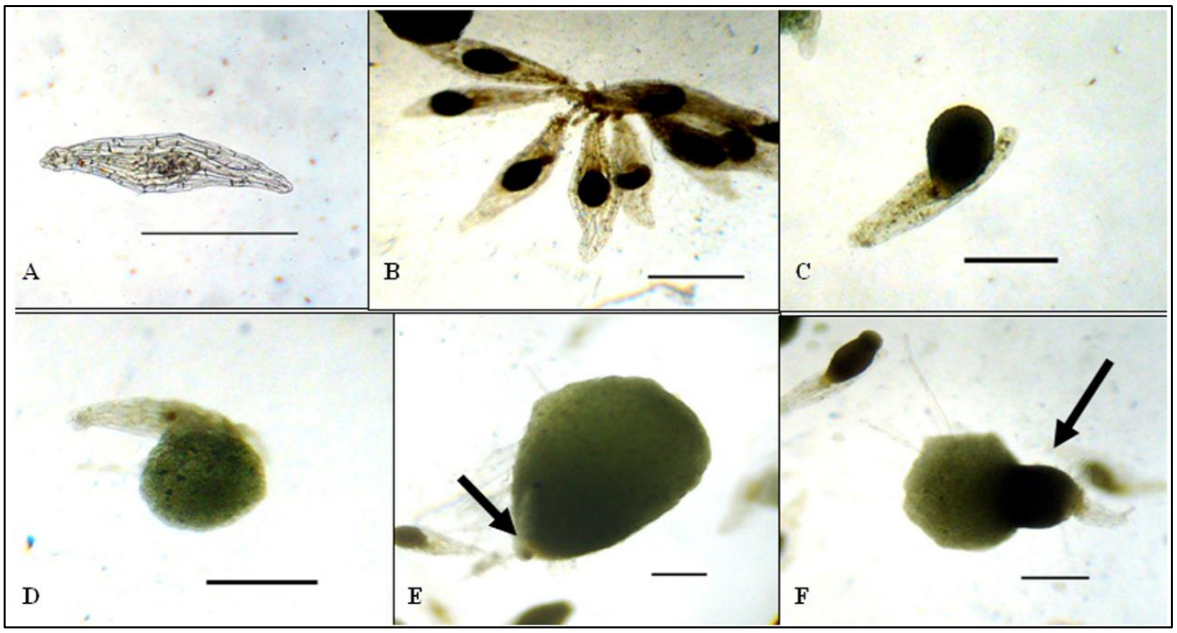

Figure 2.1: Stages of development of the seeds cultured in vitro of *Grammatophylum speciosum* (Khampa *et al*., 2010). In A, the seed has only started to grow; B, the embryos are visible and start to interrupt with the seed coat during germination process; C, the embryo has emerged out of the testa and formed protocorm; D, a small size protocorm; E, the protomeristem has emerged indicated by an arrow; F, a long protomeristem protocorm (Khampa *et al*., 2010).

The symbiotic and asymbiotic *in vitro* germination techniques have been used for the seed germination of numerous orchid species (Fay, 1992).

a. Symbiotic seed germination

In relation to orchid seed germination and development in nature, fungus infection is seemed to be a substantial element for the growth of certain tropical epiphytic orchids as the seeds are insufficient with the carbohydrate reserves as they have no endosperm

(Manners *et al*., 2011). The young plant requires the supply of nutrients, sugar and organic material from the mycorrhizal fungus until the plant is capable of producing its own food (McKendrick, 2000). This symbiotic relationship that assist in seed germination may persist into adulthood or remain in the tissue of orchid for its entire lifespan (Zettler *et al.*, 2003). Zettler *et al.* (2003) stated that even though there are a lot of new techniques evolve for the propagation of orchids without the seemingly require the present of mycorrhizal fungi, the absent of these mycobionts still resulting in extinction of orchids by some means.

The food reserves found in the orchid embryo mainly comprise of lipid and protein bodies. Once the fungus is penetrated into the seed, it is functioning as an exogenous carbohydrate for the growing embryo upon the digestion of the fungal hyphae (Kauth, 2005). Moreover, fungi may be treated as a water supply since germination is started by imbibition (Kauth, 2005). Fungi association is necessary for the development of seedling as the reserves in the seeds only sustainable until protocorm is formed. Fungi supports the growth of protocorm by supplying simple sugar, carbohydrates, mineral nutrients and amino acids needed for early metabolism such as initiation of gluconeogenesis and utilization of lipid reserves (Zettler *et al.*, 2003). Even after successful germination and seedling production *in vitro*, the establishment of the seedling on soil will still be a conundrum if this symbiotic relationship with fungi is unavailable.

For the seeds that are to be germinated symbiotically, sowing is performed with a piece of selected mycorrhizal fungus. Symbiotic relationship is established when the fungus propagates and colonized the seed germination media (Mckendrick, 2000). Before the plant is capable of making its own food, the fungus is believed to be sustaining the protocorm. Nonetheless, the proper strain of mycorrhizal fungus is required or else it might lead to seedling death as the fungus strain becomes parasitic (McKendrick, 2000).

Proliferation of temperate terrestrial orchids is suitable to apply with this technique and it is greatly affected by the temperature and light conditions (Teixeira da Silva, 2013).

Aggarwal *et al.* (2012) reported that *in vitro* symbiotic seed germination of *V. coerulea* seeds with fungal inoculation have the germination started within 3 weeks. It was was two weeks earlier than the control which did not inoculate with mycorrhizal fungus. Furthermore, seeds without fungal inoculation have failed to grow further after inoculation of three months. Conversely, about 80% of seeds inoculated with endophytic fungus have successfully developed with leaf and root formation within 9 months (Aggarwal *et al.*, 2012). This has presented the favourable effect of symbiotic association of mycorrhizal fungus for *in vitro* seed germination of orchid although it may possess a certain degree of risks.

Ospina and Bayman (2009) had compared the effect between symbiotic and asymbiotic techniques on seed germination of tropical epiphytic orchid, *Tolumnia variegate*. Based on their research, they had found out that the presence of fungi isolated from the root of *T. variegate* plant in the culture media better encouraged the germination rate than in commercial Knudson C medium. Moreover, symbiotic seed germination could have constrained the invasion of other contaminating fungi and bacteria (Ospina & Bayman, 2009). The basal media used in asymbiotic germination that rich in nutrients, minerals and sugar could encourage the contamination by pathogenic fungi. Meanwhile, it also requires relatively longer time to achieve germination which renders the seeds to be susceptible to contamination.

However, the use of mycorrhizal fungi for orchid cultivation could be highly specific. Ospina and Bayman (2009) found that the use of the same strain of mycorrhizal

fungi isolated from *T. variegate* has no beneficial effect for the seed germination of other epiphytic orchid species. Hence, this confounds the process to be commercialized.

b. Asymbiotic seed germination

Tropical orchids are easier to grow as compared to temperate terrestrial orchids. Thus, asymbiotic germination method is normally applied for the *in vitro* germination of tropical orchids. The media used is more intricate than those used in symbiotic germination (McKendrick, 2000). Yet, the simple cultivation technique that is able to achieve high rate of development and production of healthy seedling has make *in vitro* asymbiotic culture more favourable over symbiotic method (Ponert *et al.*, 2013). Without the mycorrhizal fungus, the nutrients required for proper germination have to be fully supplied in the medium.

There are various examples of simple media which are used for the seed germination of orchid which include Murashige and Skoog (MS), Vacin and Went (VW), Hyponex and Knudson C (KC) media (Paek *et al*., 2011). Without using the mycorrhizal fungus as a symbiotic element, Knudson has achieved successful seed germination for several epiphytic orchid genera which lead to the development of Knudson Solution B (Kauth, 2005). After that, Knudson substituted ferric phosphate with ferrous sulphate and supplemented manganese into the medium in order to develop a more complex Knudson C medium that enable the *in vitro* seed germination and plant tissue culture suitable for more species (refer to Appendix A) (Kauth, 2005). KC medium is a relatively simple basal medium commonly used for orchid culture that lack vitamin and a few other minerals as compared to MS medium. However, some of the orchid species only needed the simple composition of media to achieve a high rate of germination.

Occasionally, low concentration of plant growth regulators like auxin and cytokinin are supplemented into the media in the early stage of protocorm proliferation for certain species of orchids (Paek *et al*., 2011). The incorporation of auxin and cytokinin for *in vitro* orchid culture has presented enhancing effect. Manners *et al.* (2011) stated that the presence of plant growth regulators in orchid culture media may perform the similar function as endophytic fungi for the developing protocorms. The commonly added plant hormones are like 1-naphthaleneacetic acid (NAA), indole3-acetic acid (IAA) and 6-benzyl aminopurine (BAP).

Naha *et al.* (2013) reported that NAA is effective in improving seedling growth of *V. testacea* and promote the formation of leaf primodia. Except from that, Manners *et al.* (2011) also applied BAP and IAA separately in the MS medium to observe their effect on seed germination of *V. coerulea* which they found out that both BAP and IAA have positive effects in stimulating seed germination and seedling growth of the species. However, the appropriate concentration of the hormone has to be used to get the optimum result because too high or too low the concentration can lead to inhibitory effect.

2.3 Factors affecting *in vitro* seed germination of orchid

2.3.1 Maturity of capsule and seeds of orchid

Germination of seed can be affected by seed maturity. According to Arditti (1967), orchid embryos are viable and able to undergo normal growth even before it is fully ripe. By using asymbiotic *in vitro* seed germination technique, immature seed has found to be more effective than mature seed to germinate (Yamazaki & Miyoshi, 2006). Claiming that the embryos have developed completely but not yet enter the dormant stage, the seeds harvested at 43 to 58 days after pollination is ideal for *in vitro* seed germination of orchid (Fay, 1992).

As studies by Vejsadová (2006), by performing the surface sterilization properly to wash the inhibiting substances from the mature seed away, embryo germination and protocorm formation can be induced and increased as the inhibiting substances are washed away from the mature seed. Vejsadová (2006) mentioned that the orchid seed will swell and germinate only if it is in the color of ivory or white. There are two types of commonly used sterilization materials which are the calcium hypochlorite filtrate, $Ca(OCl)_2$ and sodium hypochlorite solution, NaOCl. The time of exposure of seeds to these two substances could actually decolorize the seed from brown to white color (Vejsadová, 2006). Furthermore, pre-treatment with the use of 70% ethanol along with $Ca(OCl)_2$ could have stimulate the protocorms formation. Vejsadová (2006) reported that $Ca(OCl)_2$ has the ability to encourage the protocorms development in many species while NaOCl on the other hand has the inhibiting effect.