IN VITRO SEED GERMINATION AND PROPAGATION OF ENDEMIC SCENTED ORCHID - *Vanda hastifera*

BONG CHUNG SHYAN

SCHOOL OF SCIENCE AND TECHNOLOGY
UNIVERSITI MALAYSIA SABAH
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ABSRACT

IN VITRO SEED GERMINATION AND PROPAGATION OF ENDEMIC SCENTED ORCHID - *Vanda hastifera*

Vanda hastifera is listed under endangered orchid species of Appendix II in The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) 2002. It is an endemic orchid to Borneo which has large and sweetly scented flowers. The objective of this study was to develop protocols for *in vitro* seed germination and micropropagation of *V. hastifera*. The techniques for seed germination, protocorm proliferation, protocorm development, and Protocorm-like body (PLB) induction were established by manipulating various factors such as basal media (Knudson (KC), Mitra, Murashige and Skoog (MS) and Vacin and Went (VW)), complex additives (coconut water, tomato juice, banana pulp, potato homogenate, peptone and yeast extract), plant growth regulators (PGRs) (6-benzylaminopurine (BAP), Kinetin, α-Naphtaleneacetic acid (NAA), Indole-3-butyric acid (IBA), and Indole-3-acetic acid (IAA)) and sugars (sucrose, glucose, fructose and galactose). Results showed that KC medium promoted seed germination up to 77.84% (obtained after 150 days of culture) followed by MS medium at 30.75%, and no germination was observed in VW medium. However, both basal media (KC and MS) delayed seed germination and also produced dead protocorms indicating the insufficiency of nutrient contents to support seed germination. Therefore, complex additives were added in the basal medium to increase the percentage of seed germination and shorten the time for seed germination. The fastest seed germination was observed in 10% (v/v) potato homogenate (19 days), followed by 0.2% (w/v) yeast extract and 10% (v/v) banana pulp (22 days), 10% (v/v) tomato juice (26 days), 10% (v/v) coconut water (28 days), and 0.2% (w/v) peptone (29 days). Observation after 150 days showed that seed germination increased up to 99.21% and 98.74% in medium supplemented with potato homogenate and tomato juice, respectively. Meanwhile, germinated protocorms in 0.2% (w/v) yeast extract medium failed to survive. The use of sucrose in the medium was found to promote higher seed germination as compared to glucose and fructose. The highest percentage of seed germination (86.13±1.90%, growth index value 281.75) was observed in 1% (w/v) sucrose. However, addition of galactose inhibited seed germination. In protocorm proliferation study, treatments with NAA and BAP combinations were more effective in promoting protocorm proliferation compared to single PGRs. Addition of 0.5 mg/l NAA+2.0 mg/l BAP had intensely induced protocorm proliferation up to 91.19±7.07% and induced 9.20±0.56 new protocorms after 150 days of culture. Meanwhile, single ctyokinins (BAP and kinetin) promoted protocorm proliferation while auxins (NAA, IAA, and IBA) promoted shoot and root rather than multiplication. The highest number of protocorm proliferation using single PGRs was obtained in 1.0 mg/l BAP (80.00% protocorms proliferated and produced 7.36±1.52 new protocorms). In the study of nutrients strength, KC medium at various nutrient strengths (¼X, ½X, 1X) was found to support protocorm proliferation better than Mitra (¼X, ½X, 1X) and MS (¼X, ½X, 1X, 2X) media. Of all of these, full strength KC medium was found to be the best medium strength (88.00% protocorm proliferated and produced 9.79±1.05 new protocorms), while, MS medium at all nutrient strengths was not suitable for the protocorm proliferation. Sucrose had been identified as the best sugar to support

protocorm proliferation followed by glucose and fructose. The highest number of proliferated protocorm (79%) and new protocorm (10.50±1.86) was obtained on medium treated with 2% (w/v) sucrose. In protocorm development study, complex additives were able to shorten the time to induce protocorm development as compared to control. The fastest protocorm development was observed in media treated with peptone (70 days), followed by tomato juice, coconut water, potato homogenate, banana pulp and yeast extract. After 120 days, all the protocorms cultured in 0.2% (w/v) peptone developed into seedlings. This treatment induced the highest number of leaves (4.48±0.10) and roots (3.43±0.17). Treatments with banana pulp and yeast extracts were not suitable to support protocorm development of *V. hastifera.* Sucrose had been recognized as the best sugar to support protocorm development followed by glucose and fructose. Addition of 2% (w/v) sucrose enhanced the development of all the protocorms into seedlings after 70 days, and induced the highest number of leaves (4.56±0.44) and roots (3.24±0.34) formation. In PLBs induction study, PLBs were induced at the cut-end site of the leaf explants, they were not formed on the surface or the tip of the explants. Leaf base explant was found to produce more PLBs compared to leaf tip explants. Treatment with PGRs induced PLBs as early as 37 days after culture in medium containing 4.0 mg/l NAA+4.0 mg/l BAP (64.00% leaf base explants produced 10.45±2.47 PLBs) after 60 days of culture. Meanwhile, for single PGRs treatment, BAP had superior effect than kinetin, NAA, IAA and IBA. The highest number of PLB induction was observed in 2 mg/l of BAP (induced 32.00% leaf base explants, 3.15±2.98 PLBs) after 49 days of culture. The addition of IAA and IBA into the basal medium caused inhibited on PLBs induction.

ABSTRAK

Vanda hastifera dikelaskan di bawah orkid terancam dalam Appendix II CITES (2002) (The Convention on International Trade in Endangered Species of Wild Fauna and Flora). Orkid endemik ini mempunyai bunga yang besar dan beraroma manis. Objektif kajian ini adalah untuk membangunkan protokol bagi percambahan biji bernih secara in vitro dan mikropropagasi untuk V. hastifera. Teknik untuk percambahan biji benih, proliferasi protokorm, pertumbuhan dan perkembangan protokorm, dan pengaruhan jasad seperti protocorm (JSP) telah dijalankan dengan memanipulasi pelbagai jenis faktor seperti media asas (KC, Mitra, MS, dan VW), kompleks aditif (air kelapa, jus tomato, pulpa pisang, homogenat ubi kentang, pepton dan ekstrak yis), pengawalatur pertumbuhan (BAP, Kinetin, NAA , IAA, dan IBA), dan gula (sukrosa, glukosa, fruktosa, dan galaktosa). Keputusan kajian menunjukkan 77.84% biji benih bercambah pada media asas KC (dicerap selepas 150 hari pengkulturan) diikuti oleh 30.75% pada media asas MS, manakala, percambahan tidak berlaku pada media asas VW. Namun demikian, kedua-dua media asas (KC dan MS) memperlahankan percambahan biji benih dan juga menyebabkan protokorm berubah menjadi hitam (mati). Ini menunjukkan bahawa media asas KC dan MS kurang sesuai untuk menyokong percambahan biji benih. Oleh itu, kompleks aditif ditambah dalam media asas untuk mempercepatkan kadar dan memendekkan masa bagi percambahan biji benih. Percambahan biji benih terpantas diperhatikan pada 10% (v/v) homogenat kentang (19 hari), diikuti oleh 0.2% (w/v) ekstrak yis dan 10% (v/v) pisang pulpa (22 hari), 10%(v/v) jus tomato (26 hari), 10% (v/v) air kelapa (28 hari), dan 0.2% (w/v) peptone (29 hari). Pencerapan selepas 150 hari pengkulturan menunjukkan percambahan biji benih meningkat sehingga 99.21% dan 98.74% bagi media yang ditambah dengan homogenat kentang dan jus tomato. Walaubagaimanapun, biji bernih yang bercambah gagal untuk terus hidup pada media yang ditambah dengan 0.2% (w/v) ekstrak yis. Penggunaan sukrosa dalam media didapati dapat menyokong percambahan biji benih yang lebih tinggi berbanding dengan penggunaan glukosa dan fruktosa. Peratusan percambahan biji benih tertinggi diperhatikan pada 1% (w/v) sukrosa (86.13±1.90%, GI value of 281.75). Penggunaan galaktosa pula didapati merencat percambahan biji benih. Dalam kajian proliferasi protokorm, rawatan kombinasi NAA dan BAP adalah lebih berkesan dalam menggalakkan proliferasi protokorm berbanding dengan pengawalatur pertumbuhan tunggal. Kajian menunjukkan bahawa 91.19±7.07% protokorm berproliferasi dan menghasilkan 9.20±0.56 protokorm pada media asas yang dirawat dengan 0.5 mg/l NAA+2.0 mg/l BAP selepas 150 hari pengkulturan. Sementara itu, rawatan sitokinin (BAP dan kinetin) didapati menggalakkan proliferasi protokorm manakala auksin (NAA, IAA dan IBA) lebih cenderung untuk menggalakkan pembentukan pucuk dan akar dan bukannya proliferasi. Bilangan tertinggi protokorm berproliferasi dicerap pada 1.0 mg/l BAP (80.00% protokorm berpoliferasi dengan menghasilkan 7.36± 1.52 protokorm baru). Dalam kajian kekuatan nutrien, media asas KC (¼X, ½X, 1X) adalah lebih baik untuk menyokong proliferasi protokorm berbanding dengan Mitra (¼X, ½X, 1X) dan MS (¼X, ½X, 1X, 2X). KC (1X) memberikan kekuatan nutrient yang terbaik iaitu memberikan 88.00% protokorm berproliferasi dan menghasilkan 9.79±1.05 protokorm baru. Media asas MS pada semua kekuatan nutrien pula tidak sesuai untuk proliferasi protokorm. Sukrosa telah dikenal pasti sebagai sumber gula yang terbaik untuk menyokong proliferasi protokorm diikuti dengan glukosa dan

fruktosa. Peratusan tertinggi protocorm berproliferasi (79%) dan bilangan protokorm baru (10.50±1.86) yang terbentuk dicerap pada medium yang dirawat dengan 2% (w/v) sukrosa. Dalam kajian perkembangan protokorm, kompleks aditif didapati berupaya memendekkan masa untuk protokorm berkembang berbanding dengan rawatan kawalan. Perkembangan protokorm terpantas diperhatikan pada media yang ditambah dengan pepton (70 hari), diikuti dengan jus tomato, air kelapa, homogenat kentang, pulpa pisang dan ekstrak yis. Selepas 120 hari, semua protokorm pada 0.2% (w/v) pepton telah berkembang menjadi anak pokok. Rawatan ini menghasilkan bilangan daun (4.48±0.10) dan akar (3.43±0.17) tertinggi. Media yang ditambah dengan pulpa pisang dan ekstrak yis tidak sesuai untuk membantu perkembangan protokorm V. hastifera. Sukrosa dikenalpasti sebagai gula yang terbaik untuk menyokong perkembangan protokorm diikuti dengan glukosa dan fruktosa. Penambahan 2% (w/v) sukrosa mempercepatkan perkembangan protokorm menjadi anak pokok selepas 70 hari, dan menghasilkan bilangan daun tertinggi (4.56±0.44) dan akar (3.24±0.34). Pembentukan JSP hanya diperhatikan pada bahagian yang dipotong pada eksplan daun dan bukannya terbentuk di atas permukaan atau hujung eksplan. Eksplan pangkal daun didapati lebih banyak menghasil JSP berbanding dengan eksplan hujung daun. Rawatan dengan pengawalatur pertumbuhan berjaya mengaruh JSP seawal 37 hari pengkulturan dalam media asas yang mengandungi 4.0 mg/l NAA+4.0 mg/l BAP. Rawatan ini mengaruh 64.00% daun pangkal menghasilkan 10.45±2.47 JSP selepas 60 hari pengkulturan. Sementara itu, rawatan tunggal, BAP didapati memberi kesan yang lebih baik berbanding kinetin, NAA, IAA dan IBA. Bilangan tertinggi JSP yang terhasil diperhatikan pada 2 mg/l BAP (32.00% dari pangkal daun dengan menghasilkan 3.15±2.98 JSP) selepas 49 hari pengkulturan. Sementara itu, rawatan IAA dan IBA didapati merencat pengaruhan JSP.

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LIST OF ABBREVIATIONS AND SYMBOLS

+	Add
<	Less than
±	Plus minus
¼X	Quarter concentration
½X	Half concentration
2X	Double concentration
B	Boron
BAP	6-benzylaminopurine
Ca	Calcium
cm	Centimeter
Co	Cobalt
Cu	Copper
DMRT	Duncan Multiple Range Test
Fe	Ferum (Iron)
GI	Growth Index
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
K	Potassium
KC	Knudson C medium
Mg	Magnesium
Mn	Manganese
Mo	Molybdate
MS	Murashige and Skoog medium
N	Nitrogen
NAA	α-Naphtaleneacetic acid
NaOH	Sodium hydroxide
P	Phosphorus
PGRs	Plant growth regulators
PLBs	Protocorm like bodies
S	Sulfur
SD	Standard deviation
SPSS	Statistical Package for social science
VW	Vacin and Went medium
Zn	Zinc

CHAPTER 1

INTRODUCTION

Orchid is the largest and most diverse flowering plant comprises of approximately 700-800 genera and 20,000-30,000 native species (Arditti, 1967). Apart from that, more than 30,000 orchid hybrids which resulted from interbreeding have been produced and registered, many of which are multi-generic (Wong and Phillipps, 1996; Hew and Yong, 2004). Orchids flower have great variety of shapes, sizes, colors and fragrances. Some of the orchid genus such as *Vanda, Phalaenopsis, Dendrobium, Oncidium* has colorful and elegant flowers. The flowers can remain attach to flower stalk for long shelf time and they can adapt to room conditions. Malaysia, Singapore, Philippines, Taiwan and Thailand export orchids to Europe, USA and Japan regularly and earned large amount of foreign currency (Hew, 1994). Orchids are marketed as plant and cut flower. The prospect for cut flower industry in Malaysia is very bright due to the growth in domestic and export markets. Malaysian orchids have several advantages include wider product range, longer shelf-life, ability to export flowers year round and have potential to exploit its biodiversity for product development. Orchid floriculture is an important industry in Malaysia with the production value of RM150 million annually (Ooi, 2005). In the 3rd National Agriculture Policy (1992-2010), cut flowers has been identified as the priority group of crops with good potential to meet the growth of domestic and international demand and generate higher income for producers (Latiffah *et al.,* 2010).

Vanda hastifera is an endangered species which is listed in Appendix II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2002). It is endemic to Borneo and is a tropical epiphytic orchid. It can be found in Mt. Kinabalu area and Tambunan Distinct in Sabah. Flowers of *V. hastifera* are huge, sweetly scented, long-lasting and bloom throughout the year (Chan *et al.,* 1994). It can be used as a parent in producing hybrid plants for cutting flower. Despite of legal protection under National Forestry Act 1984 (Laws of Malaysia,

2006); *Vanda* orchids with high horticultural value are found to be threatened by illegal collection (Buyun *et al.,* 2004; Vyas *et al.,* 2009; Devina *et al.,* 2010). In addition to the fast development of industrialization in Malaysia, large tracts of rainforest are lost due to human activities such as farming, development of factories, and logging (Seeni and Latha, 1992; Batty *et al.,* 2002; Devina *et al.,* 2010).

Effective approach is crucial for conservation as well as horticultural exploitation (Arditti, 1967; Batty *et al.,* 2002). Plant tissue culture technologies (asymbiotic germination and propagation) can increase the reproduction rate and reintroduce orchids back to their natural habitat. *In vitro* seed germination produces large number of seedlings in a relatively shorter time (1.5 to 2 years). Plants which regenerated from seeds have a broader genetic background than those developed by clonal propagation methods. The use of *in vitro* protocol has also been a successful approach for ex-situ conservation and reintroduction of endangered orchids (Teng *et al.,* 1997; Chugh *et al.,* 2009; Godo *et al.,* 2010). The advantages of using *in vitro* protocol are plant materials are maintained in control condition and production can be carried out throughout the year, and does not depend on season and weather condition. These seedlings can be exported since they are kept in sterile bottles and excluded from the usual CITES and phyto-sanitary regulations (Christopher, 1992).

In vitro seed germination and propagation technique for genus of *Vanda* has been established for a long time. Previous research showed that *in vitro* seed germination were studied on *Vanda dearei, V. John Club, V.* tessellate and *V. helvola* (Bhaskar and Rajeevan, 1996; Roy and Banerjee, 2002; Roslina *et al.,* 2010; Devina and Jualang, 2010). Numerous studies have attempted to use protocorm as explant for proliferation and development in orchids such as *Vanda dearei, V. helvola, Phalaenopsis gigantea* and *Paphiopedilum* orchid (Rosmah *et al.,* 2006; Ng and Norihan, 2010; Devina *et al.,* 2010; Roslina and Jualang, 2010). Meanwhile, PLBs induction using leaf explant was demonstrated in *Vanda cristata* and *V. testacea*, *V. coerulea*, *Aerides maculosum*, *V. dearei*, and *Vanda helvola* (Vij and Pathak, 1990; Seeni and Latha, 2000; Murthy and Pyati, 2001; Roslina *et al.,* 2010;

Devina *et al.,* 2010). To date, there is no documented publication on *in vitro* seed germination and micropropagation on *V. hastifera*. Like other orchids, seeds of *V. hastifera* are very difficult to germinate in nature. Hence, by using *in vitro* germination method, large number of plants can be produced. Therefore, the aims of this study were:

a) To study the effect of different basal media, complex additives and sugars on *in vitro* seed germination of *Vanda hastifera*
b) To study the effect of different plant growth regulators (PGRs) and sugars on protocorm proliferations of *Vanda hastifera*
c) To study the effect of different complex additives and sugars on protocorm development of *Vanda hastifera*
d) To study the effect of different PGRs on Protocorm-Like Bodies (PLBs) induction from *in vitro* leaf segment of *Vanda hastifera*.

CHAPTER 2

LITERATURE REVIEW

2.1 Distribution of Orchid

Orchid is most widely distributed plants in the globe with a greater concentration in tropical and subtropical regions of high humidity. Around the world there are about 700-800 orchid genera and 20,000-30,000 native species (Arditti, 1967; Wong and Phillipps, 1996; Hew and Yong, 2004). In Malaysia, rare and endangered orchid species are found in the mountains, forest, along rivers and also swampy areas (Leipzig, 1996). Borneo is the centre of diversity of many orchids' genera and it is known as "Orchid Island" (Chan *et al.,* 1994). Wong and Phillips (1996) has estimated about 2500-3000 orchid species in 150 genera are found in Borneo, which is also equivalent to 10% of world's orchids and 75% of the Malesian orchid flora. In addition, 30-40% of all Borneo's orchids are thought to be endemic (Chan *et al.,* 1994).

2.2 *Vanda*

The name *"Vanda"* is derived from the Sanskrit name which refers to sacred mistletoe (Hew *et al.,* 2002). The genus comprises about 50 species distributed mainly in the tropical Asian regions which span from Sri Lanka and India north to south part of China, south to Indonesia, and eastwards to northern Australia, New Guinea and the Solomon islands (Wood and Cribb, 1994). *Vanda* is a monopodial orchid and is mostly epiphytic. It can be found on trunks and branches of trees in the jungle. Sometime they grow as a lithophyte on rocks (Chan *et al.,* 1994). All *Vanda* enjoy light, and with sufficient sunlight they may bloom two or three times a year. *Vanda* has leaves that are highly variable according to habitat. Some of the species have flat, broad, and strap-leaves, while others have cylindrical, fleshy leaves which adapted to dry periods. The stems of this orchid vary considerably in size; there are miniature plants and plants with a length of several meters. *Vanda* usually blooms every few months and the flowers will last for two to three weeks (Arditti and Ernst, 1993; Hew *et al.,* 2002; Darmono, 2005).