In vitro PROPAGATION OF *Dimorphorchis rossii,* AN ENDEMIC AND ENDANGERED SABAH ORCHID

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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excepts, equations, summaries and references, which have been duly acknowledged.

01 October 2016

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ABSTRACT

Dimorphorchis rossii is a threatened orchid species which is endemic to Borneo. Forest clearance and fires on its natural habitat and illegal collection by local people contributed to the extinction of this orchid. Application of plant tissue culture acts as an alternative for conservation and agronomic utilization of this precious species. The effect of capsule age (150, 180 and 210 days), effect of basal medium (KC, VW and MS), complex additives (coconut water, tomato juice, potato homogenate, peptone and yeast extract), plant growth regulators (BAP, KIN, IAA and NAA), carbon source (sucrose, fructose, glucose) and light condition (16 hour light, 24 hour light, 24 hour dark) on *in vitro* seed germination, protocorm proliferation, protocorm growth and development, protocorm-like bodies (PLB) induction from leaf segments were studied. Data was analyzed using ANOVA, t-test and Duncan test. Study on capsule maturity showed that capsule age from 210 days after pollination showed high germination rate (31.31±3.42%) after 30 days of cultured compare to 150 and 180 days. Thus, capsule was taken at 210 days for subsequent study on seed germination. MS basal media gave the highest percentage of germination (78.35±7.22%) after 150 days of cultured and addition of different types and concentrations of carbon sources showed that MS medium contained 2% (w/v) sucrose was an excellent carbon source (69.43±12.25%). For study of complex additives, 10% (v/v) coconut water showed the highest percentage of germination (95.43±2.00%) followed by 10% and 20% (w/v) tomato juice (94.09±1.19% and 93.78±2.70%, respectively). Besides that, the culture also showed optimum germination response (92.30±3.66%) when kept in growth chamber with 16 hour light in MS medium supplemented with 10% (v/v) coconut water and 2% (w/v) sucrose. In protocorms proliferation and shoots development, protocorms within 1.0 mm-2.0 mm size were used as source of explants. In this study, MS basal medium showed the highest protocorm proliferation (20.00±0.41%) and number of protocorm with leaf (25.00±0.44%). These values was enhanced by addition of 0.2% or 0.3% (w/v) yeast extracts for protocorms proliferation (25.00±0.44%) or with 10% (v/v) coconut water for shoots induction (75.00±0.44%) and rooting (65.00±0.49%). For study involved plant growth regulator, 0.5 mg/l KIN showed the highest percentage of protocorm proliferation (26.70±0.45%) while 2.0 mg/l BAP proved to be excellent hormone for inducing shoot development (63.33±0.49%) and 3.0 mg/l IAA for rooting (36.67±0.49%). Acclimatization of *D. rossii* was also done to observe the survivability of the orchid when planted outside and after about 60 days, 52.00±0.50% plantlets manage to survive. Leaf segments excised from *in vitro* seedling of *D. rossii* was used as source of explant for PLBs induction and shoot development. Treatment of 0.2% (w/v), 0.3% (w/v) yeast extract and 10% (w/v) tomato juice gave the highest respond on PLB induction (13.33±0.46%) in which 0.2% (w/v) yeast extract showed the highest mean number of PLB with value of 30.7±12.92. Explant that was able to formed shoot was observed in MS basal media supplemented with 10% (w/v), 20% (w/v) tomato juice and 10% (w/v), 20% (w/v) potato homogenate with the highest percentage about 13.33±0.45%. On the other hand, 2.0 mg/L BAP showed the highest induction of PLBs (13.3±0.46%) while high mean number of new PLBs was 25.00±4.54 in 2.0 mg/l KIN. For shoot developments, only 2.0 mg/l KIN, 3.0 mg/l IAA, 0.5 mg/l NAA and 2.0 mg/l NAA showed response with

percentage of 3.33±0.18%. In conclusion, propagation using complex additive showed promising result compare to plant growth regulators based on result obtained.

ABSTRAK

TEKNIK PROPAGASI Dimorphorchis rossii SECARA IN VITRO, ORKID ENDEMIK DAN TERANCAM DI SABAH

Dimorphorchis rossii merupakan salah satu spesies orkid yang terancam dan endemik di Sabah. Pembakaran hutan dan kemusnahan habitat semulajadi serta pengumpulan orkid secara tidak terkawal oleh masyarakat setempat telah menyumbang kepada kepupusan orkid ini. Aplikasi tisu kultur tumbuhan menjadi alternatif untuk membendung masalah ini. Kajian melibatkan kesan kematangan umur kapsul (150, 180 dan 210 hari), kesan media asas (KC, VW dan MS), kompleks aditif (air kelapa, pepton, ekstrak yis, homogenat ubi kentang dan jus tomato), pengawalatur tumbuhan (BAP, KIN, NAA dan IAA), sumber karbon (sukrosa, fruktosa dan glukosa), keadaan cahaya (16 jam cahaya, 24 jam cahaya, 24 jam gelap) terhadap percambahan biji benih, proliferasi dan perkembangan protokorm dan jasad seperti protokorm (JSP) serta pucuk daripada keratan daun Dimorphorchis rossii. Data dianalisis menggunakan ANOVA, t-test dan Duncan test. Kajian melibatkan kematangan kapsul menunjukkan umur kapsul 210 hari menghasilkan peratus percambahan biji benih yang tertinggi (31.31±3.42%) berbanding hari lain iaitu 150 dan 180 hari. Oleh itu, untuk kajian seterusnya, biji benih diambil daripada umur kapsul 210 hari. Biji benih bercambah paling banyak apabila dikultur di atas media MS (78.35±7.22%) selepas 150 hari pengkulturan dan penambahan sumber karbon yang berbeza menunjukkan 2% (w/v) sukrosa (69.43±12.25%) merupakan sumber karbon yang terbaik. Untuk kompleks additif, 10% (v/v) air kelapa menunjukkan peratus percambahan yang paling tinggi (95.43±2.00%) diikuti oleh 10% dan 20% (w/v) jus tomato (94.09±1.19% dan 93.78±2.70%). Selain itu, biji benih turut menunjukkan percambahan paling optimum apabila dilkultur di dalam media MS dengan 10% (v/v) air kelapa dan 2% (w/v) sukrosa (92.30±3.66%) serta disimpan di bawah 16 jam cahaya. Dalam kajian proliferasi dan perkembangan protokorm, protokorm pada saiz 1.0-2.0mm digunakan sebagai sumber eksplan. Media asas MS menunjukkan peratus proliferasi protokorm (20.00±0.41%) dan protokorm membentuk daun (25.00±0.44%) yang tertinggi berbanding media asas lain. Nilai peratus protokorm proliferasi dan membentuk daun (25.00±0.44%) semakin bertambah apabila 0.2% atau 0.3% (w/v) ekstrak yis ditambah manakala 10% (v/v) air kelapa mencatatkan peratus tertinggi bagi pertumbuhan daun (75.00±0.44%) dan akar (65.00±0.49%). Bagi kajian melibatkan pengawalatur tumbuhan, 0.5 mg/l KIN menunjukkan peratus proliferasi protokorm tertinggi (26.70±0.45%) dan 2.0 mg/l BAP untuk peratus pertumbuhan daun terbanyak (63.33±0.49%) manakala 3.0 mg/l IAA untuk pertumbuhan akar (63.33±0.49%). Penyesuaian anak pokok D. rossii turut dijalankan untuk memerhati keadaan anak pokok yang hidup apabila dipindahkan ke luar dan sebanyak 52.00±0.50% anak pokok berjaya hidup selepas 60 hari. Keratan segmen daun daripada anak pokok D. rossii digunakan sebagai sumber eksplan untuk pengaruhan JSP dan pucuk. Rawatan 0.2% (w/v), 0.3% (w/v) ekstrak yis dan 10% (w/v), 20% (w/v) jus tomato mengaruh peratus JSP yang tertinggi sebanyak 13.33±0.46% dengan purata JSP sebanyak 30.7±12.92 pada 0.2% (w/v) ekstrak yis. Eksplan yang mampu mengaruh pertumbuhan pucuk adalah rawatan media asas MS dengan 10% (w/v), 20% (w/v) jus tomato da 10%

(w/v), 20% (w/v) homogenat ubi kentang (13.33±0.45%). Selain itu, 2.0 mg/l BAP menunjukkan peratus pengaruhan JSP sebanyak 13.3±0.46% manakalan 2.0 mg/l mengaruh purata JSP baru sebanyak 25.00±4.54. Bagi pengaruhan pucuk, hanya rawatan 2.0 mg/l KIN, 3.0 mg/l IAA, 0.5 mg/l NAA dan 2.0 mg/l NAA memberikan respon (3.33±0.18%). Secara keseluruhan, propagasi menggunakan kompleks aditif menunjukkan kesan yang lebih baik berbanding menggunakan pengawalatur tumbuhan.

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LIST OF ABBREVIATIONS AND SYMBOLS

-	-	To
%	-	Percentage
±	-	Plus minus
°C	-	Degree celsius
ANOVA	-	Analysis of variance
BAP	-	Benzylamino purine
HCl	-	Hydrochloric acid
Hr	-	Hour
IAA	-	Indoleacetic acid
KIN	-	Kinetin
KC	-	Knudson C medium
Mg	-	Milligram
mg/l	-	Milligram per litre
Mm	-	Milimeter
MS	-	Murashige and Skoog medium
NAA	-	Napthaleneacetic acid
NaOH	-	Sodium hydroxide
pH	-	Power of hydrogens
PLB	-	Protorm-like bodies
PGR	-	Plant growth regulators
Psi	-	Pounds per square inch
Rpm	-	Revolution per minute
v/v	-	Volume per volume
VW	-	Vacin and Went medium
w/v	-	Weight per volume

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CHAPTER 1

INTRODUCTION

Orchidaceae is one of the largest families of flowering plant and comprises about 20,000 to 30,000 species (Chugh *et al.,* 2009). The uniqueness of orchid is notable by its exquisite and beautiful flowers that come in various shapes and colours. The size of orchid plants range from as small as 3-4 mm to several meters while the flowers are between 2-3 mm to 15-20 cm (Arditti, 1967). Borneo, as the third largest island in the world is the centre of diversity for many different types of orchids which comprise of about 12-16% of the flora or 3,000-4,000 species (Chan *et al.,* 1994). Orchids are important as floricultural (cut-flowers) and ornamental (pot and garden plants) and some have medicinal properties (Teixeira da Silva, 2013). Some orchids have medicinal properties, such as *Eulophia nuda* which is used as herbal medicine (Panwar *et al.,* 2012), *Satyrium nepalense* as energizing tonic (Mahendran and Bai, 2009) and *Dendrobium* as an Indian medicine to cure cough or cold (Misra, 2007).

Borneo is known for its rain forest and home to some extremely rare orchids that are valued for their exotic aromas and appealing beauty, such as *Dimorphorchis rossii*. This orchid is endemic to Borneo, specifically Sabah and only distributed on Mount Kinabalu (Wood *et al.,* 2011). The uniqueness of this orchid lies in its flower whereby the flowers are dimorphic, which mean that they have different flowers (basal flowers have sepals and petals that yellow or orange-yellow while the apical flowers have yellow or white colour) on the same inflorescence (Wood *et al*., 2011). Due to its beautiful and unique flower, this orchid is highly cherished by orchid growers and this causing the declining of this species in the wild as the orchid become difficult to locate. In addition to that, drought-induced fires that occurred on ultramafic forest from Hempuen Hill to Telupid also affected the orchid population especially *D. rossii* (Chan *et al.,* 1994; Wood *et al.,* 2011).

An efficient strategy need to be developed not only to save this orchid from vanishing but also to fulfill the increasing demand of orchids. Application of tissue culture through various methods can serve as an alternative to propagate *D. rossii.* Numerous studies on efficient *in vitro* propagation of orchid plants have been published and suggested that method is species-specific oriented (Arditti, 1967; Zeng *et al.,* 2013). In natural condition, due to its dependency on fungus (symbiotic relationship) to be able to germinate, raising orchids from seeds are quite difficult (Salifah *et al.,* 2011). Besides that, the germination rate are also very low where only about 2 to 5% seeds can be germinated in nature (Luan *et al.,* 2006; Dutta *et al.,* 2011 and Birhalawati *et al.,* 2014). Thus, conventional propagation methods cannot be applies effectively and plant tissue culture becomes an excellent alternative to propagate *D. rossii* for conservation purpose.

In vitro asymbiotic seed germination serves as the most reliable and effective method for conservation and mass multiplication of endangered orchid species (Parthiban *et al.,* 2012). *In vitro* seed germination followed by protocorm development was significantly influenced by capsule maturity, medium composition and culture conditions (Chen *et al.,* 2004b; Long *et al.,* 2010; Partibhan *et al.,* 2012 and Zhang *et al.,* 2015). Basal media play important role as culture medium but different species of orchid react differently to each basal media. For instance, *Dimorphorchis lowii* prefer VW basal medium (Birhalawati *et al.,* 2004) while *Vanda coerulea* germinate better in MS basal medium (Hrahsel and Thangjam, 2015). Capsule maturity also affect germination frequencies where Vasudevan and Van Staden (2010) and Zhao *et al.,* (2013) reported that seeds orchid *Ansellia Africana* and *Dendrobium wangliangii* germinate the highest when seeds harvested on 129 and 240 days after pollination. Besides that, incorporation of complex additives and carbon source further improve germination rate for orchid *Trichoglottis tenera* when culture in medium with banana powder (Bastin and Jeyachandran, 2015) and orchid *Vanda dearei* in presence of 1% (w/v) sucrose (Jualang *et al.,* 2010). Photoperiod also significantly promoted seed germination and protocorm development for orchid *D. wangliangii* which require 16 hour light to germinate (Zhao *et al.,* 2013). Protocorm multiplication and development was also influenced