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IN VITRO PROPAGATION AND MOLECULAR CHARACTERIZATION OF SOMACLONAL VARIATION IN PHALAENOPSIS GIGANTEA

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IN VITRO PROPAGATION AND MOLECULAR CHARACTERIZATION OF SOMACLONAL VARIATION IN *PHALAENOPSIS GIGANTEA*

By

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IN VITRO PROPAGATION AND MOLECULAR CHARACTERIZATION OF SOMACLONAL VARIATION IN *PHALAENOPSIS GIGANTEA*

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Phalaenopsis gigantea (Elephant's Ear orchid) is the largest species of *Phalaenopsis* genus originating from the lowland forests of Malaysia and Indonesia. Deforestation and over-collection have resulted in the extinction of this orchid. *P. gigantea* has the potential of producing beautiful hybrids and currently research on micropropagation using plant growth regulators of this orchid is ongoing. Chitosan is an environmentally friendly carbohydrate polymer and has been reported to stimulate growth of some plant species, including orchids. Multiplication was undertaken through *in vitro* inoculation of PLBs in liquid New Dogashima medium (NDM) and Vacin and Went (VW) medium supplemented with different concentrations of chitosan (0, 5, 10, 15, 20 and 25 mg/L) during 8 weeks of culture. The best response was established at 10 mg/L of chitosan supplementation in both media with the mean number of 177 and 147 PLBs formed on VW and NDM, respectively. After 6 weeks of culture in liquid media, some PLBs differentiated producing juvenile leaves and

the best response was obtained on NDM at 20 mg/L chitosan with mean number of 66 leaves. To establish an efficient treatment combination in semi solid culture for enhancing PLBs multiplication and subsequent shoot regeneration, solid NDM and VW medium supplemented with various concentrations of chitosan (0, 5, 10, 15, 20 and 25 mg/L) and thidiazuron (TDZ) (0, 0.1, 0.5 mg/L) were used. The optimum treatment for PLB multiplication in solid medium was NDM at 10 mg/L chitosan in combination with 0.1 mg/L TDZ with the mean number of 353 PLBs after 20 weeks of cultivation. NDM containing 10 mg/L chitosan and 0.1 mg/L TDZshowed a 19-fold increase in fresh weight. Whilst, the efficiency of shoot regeneration in semi solid VW was higher than NDM and the best response was observed on VW in addition with 10 mg/L chitosan and 0.5 mg/L TDZ (16), VW at 20 mg/L chitosan (15) and VW including 15 mg/L chitosan and 0.5 mg/L TDZ (13). In order to assess the genetic fidelity among initial PLBs and proliferated PLBs obtained at the end of each two week's sub-culture from the optimum treatment (10 mg/L chitosan). Eight inter-simple sequence repeat (ISSR) primers were finally selected from 10 used for initial screening. The ISSR primers generated 55 clear band classes with 0% polymorphism. The somaclonal variations among mother plant (MP) and PLBs from the sub-cultures of optimum treatment in PLB multiplication (solid NDM supplemented with 10 mg/L chitosan and 0.1 mg/L TDZ) have been estimated. The primers selected produced 67 bands with 11 of it being polymorphic. The highest number of polymorphic bands (3) was obtained using primers I65 and I2 with 27.3% polymorphism. It was found that no genetic changes occurred among mother plant and PLBs after 4, 8 and 12 weeks of culture. After 16 and 20 weeks of culture, PLBs were 95% and 80% similar to MP, respectively. In summary, the present report expressed that the addition of 10 mg/L chitosan in liquid medium could provide a

promising *in vitro* culture system to stimulate PLBs proliferation withoutany somaclonal variation up to 16 weeks of culture. The incorporation of 10 mg/L chitosan and 0.1 mg/L TDZ in solid NDM was also efficient for PLB proliferation. However, it resulted in 20% dissimilarity with the mother plant after 20 weeks of culture.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMBIAKAN *IN VITRO* DAN PENCIRIAN MOLEKUL VARIASI SOMAKLONAL *PHALAENOPSIS GIGANTEA*

Oleh

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Phalaenopsis gigantea (orkid telinga gajah) adalah spesies terbesar dalam genus *Phalaenopsis* yang berasal dari hutan tanah pamah di Malaysia dan Indonesia. Penebangan hutan dan pengumpulan orkid yang berleluasa telah menyebabkan spesies ini. *P. gigantea* pupus. Orkid ini berpotensi untuk menghasilkan hibrid yang cantik dan kajian pembiakan mikro keatas orkid ini sedang dijalankan dengan giat. Chitosan merupakan polimer karbohidrat mesra alam dan telah dilaporkan untuk merangsang pertumbuhan beberapa spesies tumbuhan, termasuk orkid. Peningkatan multiplikasi PLBs telah dijalankan secara *in vitro* melalui inokulasi PLBs dalam medium cecair New Dogashima (NDM) dan medium Vacin dan Went (VW) yang ditambah dengan chitosan (0, 5, 10, 15, 20 dan 25 mg/L) pada kepekatan yang berlainan sepanjang 8 minggu dikultur. Respons terbaik telah dicapai dengan penambahan chitosan sebanyak 10 mg/L dalam kedua-dua media dengan purata PLBs masing-masing sebanyak 177 dan 147 dalam VW dan NDM. Selepas 6 minggu pengkulturan dalam media cecair, beberapa PLBs telah membentuk daun juvana dan

respons terbaik telah diperolehi dalam NDM pada 20 mg/L chitosan dengan purata sebanyak 66 daun. Medium pepejal NDM dan VW yang ditambah dengan pelbagai kepekatan chitosan (0, 5, 10, 15, 20 dan 25 mg/L) dan thidiazuron (TDZ) (0, 0.1, 0.5 mg/L) telah digunakan untuk mendapatkan satu rawatan kombinasi yang cekap dalam kultur pepejal yang berupaya meningkatkan multiplikasi PLBs dan penjanaan semula pucuk. Rawatan optimum bagi multiplikasi PLBs dalam medium pepejal adalah NDM dengan kombinasi 10 mg/L chitosan dan 0.1 mg/L TDZ dengan purata 353 PLBs selepas 20 minggupengkulturan. Medium NDM pejal yang mengandungi 10 mg/L chitosan dan 0.1 mg/L TDZ telah menghasilkan peningkatan 19 kali ganda dalam berat segar PLB. Sementara itu, keberkesanan penjanaan semula pucuk dalam VW pepejal adalah lebih tinggi daripada NDM dan respons terbaik telah diperhatikan pada VW dengan penambahan 10 mg/L chitosan dan 0.5 mg/L TDZ (16), VW dengan penambahan 20 mg/L chitosan (15) dan VW dengan penambahan 15 mg/L chitosan dan 0.5 mg/L TDZ (13). Lapan inter-simple sequence repeat' (ISSR) telah dipilih daripada sepuluh yang digunakan dalam penyaringan awaldan penentuan ketulenan genetik diantara PLBs induk berbanding dengan PLBs yang diperoleh pada akhir setiap dua minggu sub-kultur daripada rawatan optimum (10 mg/L chitosan) sepanjang pengkulturan. Primer ISSR telah menjana 55 jalur yang jelas dengan polimorfisma sebanyak 0%. Variasi somaklonal dalam kalangan tumbuhan induk (MP) dan PLBs sekunder daripada sub-kultur rawatan optimum multiplikasi PLB yang berbeza (NDM pepejal yang ditambah dengan 10 mg/L chitosan dan 0.1 mg/L TDZ) telah dianggarkan. Lapan primer ISSR menghasilkan 67 jalur dengan 11 jalur polimorfik. Bilangan jalur polimorfik terbanyak (3) telah diperoleh menggunakan primers I65 dan I2 dengan polimorfisma sebanyak 27.3%. Perubahan genetik tidak berlaku dalam kalangan tumbuhan induk dan PLBs sekunder selepas 4,

8 dan 12 minggu dikultur. Terdapat sebanyak 95% dan 80%, persamaan di antara PLBs yang dimultiplikasi dengan PLB induk selepas 16 dan 20 minggu dikultur, masing-masing. Secara kesimpulan, laporan ini menyatakan bahawa penambahan 10 mg/L chitosan dalam medium cecair boleh merangsang proliferasi PLBs kultur *in vitro* tanpa menyebabkan variasi somaklonal selama 16 minggu. Penggabungan 10 mg/L chitosan dan 0.1 mg/L TDZ dalam NDM pepejal juga berkesan untuk percambahan PLBs. Walau bagaimanapun, ia menyebabkan perbezaan sebanyak 20% dengan tumbuhan induk selepas 20 minggu dikultur.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

SAMIRA SAMARFARD

Date: 10 May 2013

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ABA	Abscissic Acid
ANOVA	Analysis of Variance
BAP	6-Benzylaminopurine
Bp	Base pair
CTAB	Cetylmethylammonium bromide
CW	Coconut Water
DNA	Deoxyribonucleic Acid
DNMRT	Duncan New Multiple Range Test
EDTA	Ethylenediaminetetra-acetic acid
ISSR	Inter Simple Sequence Repeat
M	Molar
μ*M*	Micromolar
Mm	mili Molar
NAA	Naphthalene Acetic Acid
NaOH	Sodium Hydroxide
NDM	New Dogashima Medium
NTSYS	Numerical Taxonomy and Multivariate Analysis system
PAL	Phenylalonine Ammenio-Lyase
PCR	Polymerase Chain Reaction
PLBs	Protocorom-like-bodies
RAPD	Random Amplified Polymorphic DNA
RCBD	Randomized Complete Block Design

RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RNase	Ribonuclease
SAMPL	Selective Amplified Microsatellite Polymorphism Length
SSR	Simple Sequence Repeat
TDZ	Thidiazuron
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
VW	Vacin and Went (tissue culture medium)

CHAPTER 1

INTRODUCTION

The family *Orchidaceae* includes about 7% of all angiosperms and is regarded as one of the largest families of this group. It constitutes more than 25,000 recognized species, propagated around the world exhibiting highest genetic range in tropical areas (Thammasiri, 2002). Most orchid species are dispersed mainly in the Neotropical zone and the majority are epiphytes with many being rupiculous, terrestrial and propagated in marshy regions (Pansarin and Pansarin, 2011). Orchids are esteemed as ornamentals and cut flowers because of their marvelous beauty and long lasting flowers. Currently their production stand as multi-million dollar floral industries in several countries like Singapore, Australia, Thailand, Malaysia and several others (Chugh *et al.*, 2009).

The genus *Phalaenopsis* is one of the most important epiphytic monopodial orchid, known as the moth orchid due to similarity of the flowers to night active moth butterflies (Nash, 2003), valued for its attractive cut flowers and potted plants (Chai *et al.*, 2002), a long shelf-life and large diversities (Zheng *et al.*, 2008). Commercial production of potted *Phalaenopsis* exists in Taiwan, China, Netherlands, Germany, United States, and Japan (Griesbach, 2002). About 85–90% of orchid sales among all commercial orchids in the USA are *Phalaenopsis* because of their easiness of arrangement to meet specific market dates, high wholesale value, and long shelf life (Nash, 2003).

Phalaenopsis gigantea (Elephant's Ear orchid) is the largest species in the genus, occurring in the lowland forests of Sabah in Malaysia; but deforestation and over-collection have resulted in near extinction of this species (Rodrigues and Kumar, 2009). The species has the capability of producing attractive hybrids (Niknejad *et al.*, 2011). The species name comes from its vast leaf size and propagation is typically performed through the configuration of new buds induced at the bases of mature plants. However, the procedure is much unfrequented due to low number of new buds initiated by a mature plant (Shu-guo, 2008).

Tissue culture techniques have been used not only for rapid propagation on a large scale for orchids, but also for conservation purposes (Murdad *et al.*, 2006). Various explants and combination and concentrations of plant growth regulators have been significant factors for *in vitro* propagation of orchids. However, enhancement of multiplication, total yield and successive maturity into plantlets without mutation rates are most preferred (Pornpienpakdee *et al.*, 2010). Application of some growth stimulators enhances the rate of growth during *in vitro* multiplication. Chitosan is a cationic polymer and N-deacetylated product derivative of chitin which is present in shells of crustaceans and cell wall of fungi. This component is an environmentally friendly carbohydrate polymer and has been reported to stimulate the growth of some plant species, including orchids (Nge *et al.*, 2006). Similar to some other orchids, *Phalaenopsis gigantea* is inherently difficult to propagate and the supplementation of growth stimulants like chitosan and thidiazuron (TDZ) in tissue culture medium can provide an alternative means for multiplication of protocorm-like bodies (PLBs). Propagation through PLB formation is prefered by commercial growers of orchids due to the large number of PLBs that can be obtained within a relatively short period

of time. There are two rate-limiting steps in mass production of *Phalaenopsis* PLBs: growth and multiplication. These steps require precise cultural conditions including medium composition and growth regulation, and it is necessary to reduce the time for growth and multiplication of PLBs (Park *et al.*, 1996).

The process of sub-culturing is an important stage in order to produce more PLBs and subsequent plantlet regeneration. However, excessive sub-culturing can result in unpredictable mutations. Somaclonal variations can occur due to several reasons such as types of tissue culture media, plant growth regulators and their concentrations, explant types and number of sub-culturing cycles (Reuveni *et al.*, 1986). The use of some synthetic PGRs at sub and supra-optimal concentrations was reported to be effective in inducing somaclonal variations in some crops (Martins *et al.*, 2004). The occurance of somaclonal variation is an important issue in plant tissue culture especially in ornamerntal plants because it can result in the production of undesirable off-types. The assessment of somaclonal variation by using molecular markers like Inter Simple Sequence Repeat (ISSR) will give indication on the limit to the number of sub cultures and duration of cultures.

The present study was conducted to optimize PLB multiplication of *Phalaenopsis gigantea* with chitosan and TDZ supplementation in different growth media. The study was also conducted to detect the genetic stability between mother plant and PLBs obtained from different sub-cultures of the optimal treatments. Inter Simple Sequence Repeats (ISSR) molecular technique has been applied in order to verify the genetic fidelity of PLBs produced during the process of multiplication in *Phalaenopsis gigantea*. Therefore, the study focuses on the following objectives:

(1) To determine the optimum chitosan concentration for promoting PLB proliferation in two types of liquid medium.

(2) To determine the optimum chitosan and TDZ combination for enhancing PLBs proliferation on two types of solid medium.

(3) To assess genetic stability of PLBs produced from different subcultures of optimum treatment by the Inter Simple Sequence Repeats (ISSR).

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