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DETERMINATION OF GENETIC RELATIONSHIPS AMONG *PHALAENOPSIS* Spp. USING RANDOM AMPLIFIED POLYMORPHIC DNA AND *IN VITRO* PROPAGATION OF *PHALAENOPSIS GIGANTEA*

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By

AZADEH NIKNEJAD

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

July 2009

In The Name of Allah, the Most Gracious, the Most Merciful

Specially Dedicated

To

The soul of my Father who wished to see me succeed

My dear Mother and Brother who always have faith in me

Abstract of the thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

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Chairman: Associate Professor Mihdzar Abdul Kadir, PhD

Faculty: Agriculture

Phalaenopsis, with long arching sprays of flowers, is among the most beautiful flowers in the world. *Phalaenopsis* is an important genus and one of the most popular epiphytic monopodial orchids, grown commercially for production of cut flowers and potted plants. Most of them have different and interesting morphological characteristics which have different value to the breeders.

Study was carried out using molecular characterization through Random Amplified Polymorphic DNA (RAPD) analyses for 20 species of *Phalaenopsis* was conducted to determine their genetic variation and relationships. Among the 20 primers used for these analyses, 10 showed polymorphism, with 26 to 54 DNA fragments amplified per primer. A total of 414 polymorphic fragments were generated by these 10 primers and then they were used for correlation grouping analysis. The highest value of Similarity

index was 0.28 between *Phalaenopsis violacea malaysia* and *Phalaenopsis violacea witte*. The dendrogram resulting from UPGMA hierarchical cluster analysis separated the species into three groups: the first group consisted of five species of *Ph. violacea blue*, *Ph. belina*, *Ph. violacea malaysia*, *Ph. violacea witte*, and *Ph. gigantea*; the second group included *Ph. lamelligera*, *Ph. amabilis*, *Ph. parishii*, *Ph. labbi nepal*, *Ph. speciosa*, *Ph. lobbi yellow*, *Ph. venosa*, *Ph. hieroglyphica*, and *Ph. maculata*; the third group consist of *Ph.* Minho Princess, *Ph.* Leopard prince, *Ph. mannii*, *Ph. modesta*, *Ph. cornucervi* and *Ph. pantherina*. RAPD markers can thus be successfully applied to this economically important group of orchids to determine relationship between species of these orchids.

Phalaenopsis gigantea one of the most difficult to grow and has the potential of producing beautiful hybrids was selected for further study in developing a rapid and efficient *in vitro* propagation technique, this technique can also be tested for other species in the future.

Ripe capsules of *Phalaenopsis gigantea* were collected and sterilized. Seed germination was conducted using Vacin and Went (VW) medium supplemented with coconut water (CW) and 6-benzylaminopurine (BAP) and kinetin (KIN). Protocorm-like bodies (PLBs) were successfully induced and plantlets were produced after 60 days. BAP at 1 mgL^{-1} in combination with 2 mgL^{-1} KIN produced the highest number of plantlets followed by treatment 1 mgL^{-1} BAP and 1 mgL^{-1} KIN.

Protocorm-like bodies (PLBs) were successfully induced from leaf segments using New Dogashima medium (NDM) with BAP, Thidiazuron (TDZ), and KIN, each at 0.01, 0.1, 0.5 and 1.0 mg/L alone and in combination with naphthalene acetic acid (NAA), at 0.01, 0.1, 0.5 and 1.0 mgL^{-1}, within 6-8 weeks of culture. The highest percentage of callus formation (100%) was obtained from treatment containing 1 mgL^{-1} NAA in combination with 0.1 mgL^{-1} TDZ followed by treatment supplemented with 1 mgL^{-1} NAA and 0.5 mgL^{-1} TDZ (76.56%).Plant regeneration from PLBs was achieved in PGR-free NDM basal medium.

There was no PLB observed on full and half-strength MS media supplemented with different concentrations of 2, 4-D, BAP and TDZ. Similarly, no PLB was observed on VW and ½ MS media supplemented with different concentrations and combinations with 2, 4.-D, BAP, TDZ and CW or NAA. No PLB was also observed on NDM medium with different concentrations and combinations with BAP and NAA. However multiplication of PLB was observed in liquid media of basal MS, VW, and NDM with or without sucrose. The highest fresh weight of PLBs was obtained from VW medium supplemented with CW.

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

PENENTUAN HUBUNGAN GENETIK DI ANTARA *PHALAENOPSIS* Spp. MENGGUNAKAN POLIMORFIK DNA TERAMPLIFIKASI RAWAK DAN PEMBIAKAN *IN VITRO* UNTUK *PHALAENOPSIS GIGANTEA*

Oleh

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Pengerusi: **Prof. Madya Mihdzar Abdul Kadir, PhD**

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Phalaenopsis, dengan tangkai bunga yang panjang melengkung, adalah antara bunga yang tercantik di dunia. *Phalaenopsis* adalah orkid epifit monopodial yang penting dan paling popular ditanam untuk pengeluaran bunga keratan dan tanaman pasuan secara komersil. Kebanyakkannya mempunyai sifat morphologi yang mempunyai nilai berbeza kepada pembiakbaka.

Satu kajian penyifatan molekul melalui Polimorfik DNA Teramplifikasi Rawak (RAPD) analisis bagi 20 spesis *Phalaenopsis* telah dilakukan untuk menentukan jarak genetik dan perkaitannya. Antara 20 primer yang digunakan bagi analisis RAPD, 10 primer primer ini telah menunjukkan polimorfik, dan mengikut jenis primer, 26 hingga 54 serpihan telah diperluaskan. Sejumlah 414 serpihan polimorfik telah dihasilkan oleh 10 primer dan digunakan untuk analisis korelasi kumpulan. Nilai Indeks Kesamaan

yang tertinggi adalah 0.28 diantara *Phalaenops*is *violaceae malaysia* dan *Phalaenopsis violaceae witte*. Dendrogram yang terhasil daripada UPGMA analisis kelompok hierarki mengasingkan spesis asal kepada tiga kumpulan : Kumpulan pertama mempunyai lima spesis *Ph. violaceae blue, Ph. belina, Ph. violaceae malaysia, Ph. violaceae witte* and *Ph. gigantea;* Kumpulan kedua termasuklah *Ph. lamelligera, Ph. amabilis, Ph parishii, Ph. labbi nepal, Ph. speciosa, Ph. lobii yellow, Ph. venosa, Ph. hieroglyphica* dan *Ph. maculata;* Kumpulan Ketiga termasuklah *Ph.*Minho Princess *Ph.* Leopard Prince, *Ph.* mannii, *Ph. modesta, Ph. cornu cervi* dan *Ph. pantherina.* Kajian ini menunjukkan bahawa penunjuk RAPD boleh digunakan dalam mengenalpasti pertalian bagi kumpulan orkid yang penting dari segi ekonomi.

Phalaenopsis gigantea adalah satu antara orkid yang sukar dibiak dan mempunyai potensi untuk menghasilkan hibrid yang menarik telah dipilih untok kajian selanjutnya dalam membentuk teknik pembiakan *in vitro* yang pantas dan efficien. Teknik ini juga akan diuji untuk species yang lain pada masa hadapan.

Lengai *Phalaenopsis gigantea* yang matang telah diambil dan disteril. Percambahan biji benih telah dilakukan dengan menggunakan medium Vacin dan Went (VW) yang ditambah dengan air kelapa (CW) dan 6-benzil aminopurina (BAP) dan kinetin (KIN). Protokom (PLB) dan anak benih telah terhasil selepas 60 hari kultur. BAP pada kadar 1 mgL^{-1} dengan kombinasi 2 mgL^{-1} KIN menghasilkan bilangan anak benih yang tertinggi diikuti oleh rawatan dengan 1 mgL^{-1} BAP dan 1 mgL^{-1} KIN. PLB berjaya diaruhkan

daripada segmen daun menggunakan medium New Dogashima (NDM) dengan rawatan BAP, Thidiazuron (TDZ) dan KIN masing-masing pada kadar 0.01, 0.1, 0.5 dan 1.0 mgL^{-1} sendirian dan dengan kombinasi naftelena asid asetik (NAA) pada kadar 0.01, 0.1, 0.5 dan 1.0 mgL^{-1} dalam jangkamasa kultur selama 6-8 minggu. Pembentukan kalus yang tertinggi (100%) telah didapati daripada rawatan yang mengandungi 1 mgL^{-1} NAA dengan kombinasi 0.1 mgL^{-1} TDZ diikuti dengan rawatan yang ditambah dengan 1 mgL^{-1} NAA dan 0.5 mgL^{-1} TDZ (76.56%). Regenerasi PLBs tercapai dalam medium asas NDM tanpa hormon.

Tiada PLB terbentuk pada media MS berkekuatan penuh dan separa yang ditambah dengan beberapa kepekatan 2,4-D, BAP dan TDZ. PLB juga tidak terbentuk pada media VW dan MS separa kekuatan yang ditambah dengan kekuatan dan kombinasi berbeza 2,4-D, BAP, TDZ dan CW atau NAA. PLB juga tidak terbentuk pada media NDM dengan kepekatan dan berkombinasi antara BAP dan NAA. Walau bagaimanapun, PLB didapati terbentuk pada media asas cair MS, VW dan NDM samada dengan atau tanpa sukrosa. Berat segar PLB yang tertinggi telah didapati daripada media VW yang ditambah dengan CW.

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also to Mr Esmaeil Shahsavari, and Mr Karim Sorkheh for their help and support and special thanks to my dear friends Mr Hossein Kamaladini and Fatemeh Haddadi and Naghmeh Nejat for sharing their knowledge and moral support. Finally, special thanks to my friend and housemate, Motahareh Nobakht for consoling me and listening to me, throughout my study.

Finally, I pray that I shall be a good steward of this honour.

I certify that a Thesis Examination Committee has met on 27 July 2009 to conduct the final examination of Azadeh Niknejad on her thesis entitled "Determination of Genetic Relationships Among *Phalaenopsis* Spp. Using Random Amplified Polymorphic DNA and *In Vitro* Propagation of *Phalaenopsis gigantea*" in accordance with Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Master of Science.

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory committee were as follows:

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Date: 16 November 2009

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 any other institution.

_____

Azadeh Niknejad

Date: 26 November 2009

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
BAP	6-Benzylaminopurine
Bp	base pair
CTAB	Cetylmethylammonium bromide
CV	Coefficient of Variations
CW	Coconut Water
DNA	Deoxyribonucleic acid
DNMRT	Duncan New Multiple Range Test
dNTP	deoxynucleotide triphosphate
EDTA	Ethylenediaminetetra-acetic acid
FAA	Formalin Acetic Acid
Kin	Kinetin
M	Molar
MANOVA	Multivariate Analysis of Variance
MgCl2	Magnesium chloride
µM	micromolar
mM	mili Molar
MS	Murashige and Skoog (tissue culture medium)

NAA	Naphthalene Acetic Acid
NaCL	sodium Chloride
NDM	New Dogashima Medium
NTSYS	Numerical Taxonomy and Multivariate Analysis System
PCR	Polymerase Chain Reaction
PIC	Polymorphic Information Content
PLBs	Protocorom-like-bodies
PVP	Polyvinyl pyrrolidone
RAPD	Random Amplified Polymorphic DNA
RCBD	Randomized Complete Block Design
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolution per minute
SSR	Simple Sequences Repeat
TAE	40 mM Tris-Cl, 20mM sodium acetate, 1 mM EDTA
Taq	Thermus aquaticus
TDZ	Thidiazuran
TE	Tris-EDTA buffer
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
VW	Vacin and Went (tissue culture medium)

CHAPTER 1

INTRODUCTION

Orchid constitutes an order of royalty in the world of ornamental plants. They are of immense horticultural importance and also play a very useful role to balance the forest ecosystems (Kaushik, 1983). They are one of the most pampered plants and occupy top position among all flowering plants valued for cut flower production and as potted plants, which fetch a very high price in the international market (Kalimuthu *et al*, 2007).

Discoveries have established that orchids appeared on earth long before humans. The oldest orchid fossil was found dating back 15 million years ago (Jacquet, 1994). During the BC period, records revealed that consumable orchid bulbs were used as food, to ward off evil spirits, to stimulate sexual powers and also for medical purposes such as oral contraceptives (Hunt, 1987).

Phalaenopsis species have been used in numerous breeding programs by both amateur and professional horticulturists (Huang *et al*., 2004). However, conventional breeding is slow and difficult as it requires two to three years to complete a life cycle. *Phalaenopsis* has large genome size of 38 chromosomes with different lengths for different species, making cultivar improvement among commercial varieties difficult due to sexual

incompatibility. Several sexual hybridizations are often required to improve even just only for one trait (Portia *et al*., 2005).

Phalaenopsis gigantea is the largest species in the genus *Phalaenopsis*. It is known as the Elephant Ear orchid because of its extremely large leaves, it is mainly found in Sabah and Kalimantan (the Malaysian and Indonesian parts of Borneo respectively) and has been declared as an endangered species in Appendix II of the Convention of International Trade of Endangered Species (C.I.T.E.S).

Phalaenopsis gigantea has produced outstanding novelty hybrids and has become a much sought after species that results in over-collection from its natural habitat leading to eventual extinction. The species has been reported as difficult to propagate and grow. In nature, multiplication of the species is usually by seeds formed at the end of a flowering season. The rate of germination has been known to be extremely low and infrequent. It has also been known to propagate itself through the formation of new buds initiated at the bases of mature plants. Wide phenotypic variations occur within the species and this has made genetic relationships within and between species difficult to establish.

The study attempted at to determine genetic relationships between and within the species and hybrids of *Phalaenopsis* using PCR-based molecular marker of Random Amplified Polymorphic DNA (RAPD) and determining the relationships of the species

under study within the genus using cluster analysis, the present study attempts to establish an optimiz protocols for the propagation of *Phalaenopsis gigantea* through *in vitro* culture by use of seeds and tissue (leaf).

The study focuses on the following objectives: (1) Determination of genetic relationship of *Phalaenopsis* species using Random Amplified Polymorphic DNA (2) To evaluate the phases of seed germination in selected species (*Phalaenopsis gigantea),* (3) To determine the optimum culture medium for the callus culture and Protocorms-Like Bodies (PLBs) from leaf explants, (4) To establish the optimum liquid culture medium for Propagation of PLB

The study attempts to complement existing knowledge on *Phalaenopsis gigantean*, the molecular studies on genetic distances evaluate the genetic relationship of the genus *Phalaenopsis* as well as resolving relationships among the species under study and particularly on its propagation with potential application in large-scale production of the species; thus reducing the pressure of over-exploitation from the wild.

CHAPTER 2

LITERATURE REVIEW

2.1 Orchidaceae

The Orchidaceae family is one of the largest families of flowering plants on our planet which has been estimated that there are over 70,000 species in the world, and that only half of them have been discovered. Orchids are most plentiful (both in numbers and varieties) in the equatorial regions, but have also been discovered in temperate climates and some even in places close to the tundra regions. Orchids have become the most popularly grown flowers in the world. It does not take much to realize why orchids have such a huge range of sizes, shapes, colours, flowering and growing habits (Simon, 2008).

A typical orchid flower is unique in that it has both male and female parts in one flower. It comprises of two petals, three sepals, a lip, a column, side lobes, an ovary and an anther, where pollen is found. This combination is not found in any other flower. In the wild, orchid is pollinated by insects, which are attracted to its fragrance or bright colours.