UNIVERSITI PUTRA MALAYSIA

INDUCINDUCTION OF PROTOCORM-LIKE BODIES, SYNTHETIC SEED PRODUCTION AND CRYOPRESERVATION IN Phalaenopsis bellina (Rchb.f.) Christenson

AMIR ALI KHODDAMZADEH

FP 2011 2

INDUCINDUCTION OF PROTOCORM-LIKE BODIES, SYNTHETIC SEED PRODUCTION AND CRYOPRESERVATION IN *Phalaenopsis bellina* (Rchb.f.) Christenson

By

AMIR ALI KHODDAMZADEH

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

January 2011

DEDICATION

To

My lovely Wife Maryam

and

My dear parents

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

INDUCINDUCTION OF PROTOCORM-LIKE BODIES, SYNTHETIC SEED PRODUCTION AND CRYOPRESERVATION IN *Phalaenopsis bellina* (Rchb.f.) Christenson

By

AMIR ALI KHODDAMZADEH

January 2011

Chairman: Associate Professor Uma Rani A/P Sinniah, PhD

Faculty : Agriculture

Phaleanopsis bellina (Rchb.f.) Christenson is one of the important orchid species originating from Malaysia. It is an orchid which is known to be difficult to propagate even using *in-vitro* techniques. This study was carried out to establish an *in-vitro* system for induction and proliferation of *Phaleanopsis bellina*. Furthermore attempt was made to convert the PLBs into synthetic seed as well as to establish a method to store the synthetic seeds both for short and long-term. An *in-vitro* culture procedure was established to induce protocorm-like bodies (PLBs) from leaf segments of *Phalaenopsis bellina* directly from epidermal cells without intervening callus on half-strength modified Murashige and Skoog (MS) medium supplemented with naphthaleneacetic acid (NAA; 0, 0.1, 1.0 mg/L) and thidiazuron (TDZ; 0, 0.1, 1.0, 3.0 mg/L). The best response was established at 3 mg/l TDZ which induced 78% of leaf segments to form a mean number of 15.5 PLBs per explant after 16 weeks of

culture. No PLBs were found when leaf segments were cultured on half-strength modified MS medium supplemented with 0.1 and 1 mg/l NAA. The best induction percentage for auxin: cytokinin combination was using 1.0 mg/l NAA and 3.0 mg/l TDZ which gave 72% induction with 11 PLBs per explant. Once successfully induced, it is important to maximize the proliferation and utilization of the PLBs. In this regard, semi-solid half-strength MS and liquid Vacin and Went (VW) media with and without sucrose were used in order to find the highest survival and number of PLBs proliferation after three months in culture. Half-strength MS medium showed an average of 9 PLBs with 60% survival and mean fresh weight of 0.5g in comparison with VW medium with and without sucrose which showed an average of 4.8 and 5.3 PLBs per explant followed by 55 and 57.5% of survival respectively. Histological observations revealed that the adaxial surfaces near wounded regions had the highest number of PLBs compared to other regions of explants. Also, SEM micrographs showed that leaf derived PLB (LDP) were formed from leaf segment after 16 weeks of culture.

Twelve decamer RAPD primers were used to estimate the somaclonal variation among the mother plant, the initially induced PLBs and proliferated PLBs after 3 and 6 months in culture. Eight out of twelve primers produced 172 bands with 18 polymorphic bands in all the treatments. The amplified products varied between 125 to 8000 bp. Among the primers used, P 16 produced the highest number of bands (29) while primer OPU 10 produced the lowest number (15). The range of similarity coefficient was from 0.83 to 1.0 among the different sub-cultures and mother plant. It was found that minimal or no changes occurred between the mother plant and the

PLBs produced after 3 months of induction. The induced PLBs were then subcultured for six months for proliferation and this resulted in about 17% dissimilarity with mother plant. Micropropagation of *Phalaenopsis bellina* can be carried out successfully using ½ strength MS media for 6 months but further proliferation may result in somaclonal variation which might change the prolific characteristic of this orchid.

In-vitro PLBs of *Phalaenopsis bellina* obtained from PLBs explants on modified semi-solid half-strength MS medium, 4-5 mm in diameter, were selected for encapsulation with different concentration of sodium alginate (3, 4 and 5%) and calcium chloride (25, 50, 75 and 100 mM) and none encapsulated PLBs as a control. PLBs encapsulated with 4% sodium alginate in 75 mM calcium chloride showed the best encapsulation combination on survival of PLBs after two weeks of incubation at 5°C giving the survival of 70 and 65%. Subsequently, PLBs encapsulated with 4% sodium alginate + 75 mM calcium chloride were used for evaluating the storage durations (15, 30, 45 and 60 days) and temperatures (5, 15, and 25°C). The highest PLBs survival of 70% was observed after 15 days storage at 5°C followed by 30 days of storage at 5°C with 50% of survival frequency. The best survival percentage in case of storage temperature for the synthetic seeds were 5°C >15°C > 25°C. The highest PLB fresh weight was 0.36g which belong to the encapsulated PLBs stored for 15 days at 5°C. In addition the lowest fresh weight of 0.19g belonged to encapsulated PLBs stored for 60 days at 25°C. The moisture content (MC) of the

PLBs decreased sharply with increasing storage temperature and duration to 10.2% after 60 days at 25°C.

As synthetic seeds should technically function as normal seeds, it would be useful to establish a method to store them both for short and long time. This study indicated that *Phalaenopsis bellina* PLBs were successfully cryopreserved by encapsulation-dehydration method. The highest re-growth of cryopreserved explants was observed when PLBs were pretreated in half-MS medium supplemented with 30 g/l sucrose for 3 days followed by preculturing on 0.75 M sucrose for 3 days. In addition, the highest sucrose concentration at 0.78 g/l was measured using HPLC in 100g of PLBs precultured on 0.75 M for 3 days. Encapsulated PLBs were dehydrated with silica gel for 6 h prior to immersion in liquid nitrogen for 1 h. Protocorm viability was tested by the 2, 3, 5-triphenyltetrazoliumchloride (TTC) assay and re-growth ability was assessed by determining the survival percentage after 2 weeks recovery. The survival rate of cryopreserved PLBs was 30% while highest viability percentage by TTC assay was 46.6%. Finally, dehydration time after freezing and thawing affected positively electrolyte leakage (EL) of the PLBs. Non-dehydrated PLBs showed the highest EL (85.3%) while the lowest amount (53%) was achieved after 6 h dehydration with silica gel.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN JASAD BERBENTUK PROTOKOM, PENGHASILAN BIJIBENIH SINTETIK DAN KRIOAWETAN DALAM *Phalaenopsis bellina* (Rchb.f.) Christenson

Oleh

AMIR ALI KHODDAMZADEH

Januari 2011

Pengerusi: Associate Professor Uma Rani A/P Sinniah, PhD

Fakulti : Agriculture

Phaleanopsis bellina (Rchb.f.) Christenson merupakan salah satu daripada spesies orkid yang berasal daripada Malaysia. Ia merupakan jenis orkid yang agar sukar untuk di biakkan walaupun dengan menggunakan teknik *in vitro*. Kajian ini dijalankan untuk memperolehi teknik *in-vitro* yang sesuai bagi menghasilkan *Phaleanopsis bellina*. Tambahan, teknik untuk menukarkan PLBs kepada biji benih sintetik dan teknik penyimpanan biji benih sintetik untuk jangka pendek dan panjang juga telah di jalankan. Satu kajian telah dijalankan untuk mengaruh jasad berbentuk protokom (PLBs) daripada cebisan daun *Phalaenopsis bellina* secara langsung dari sel epidermal tanpa melibatkan pembentukan kalus pada media Murashige and Skoog yang diubahsuai (MS) berkepekatan separuh yang telah ditambahkan dengan asid naphtaleneasetik (NAA; 0, 0.1, 1.0 mg/L) dan thidiazuron (TDZ; 0, 0.1, 1.0, 3.0 mg/L).

Tindak balas yang terbaik diperolehi pada media 3 mg/l TDZ dimana 78% daripada bahagian daun teraruh membentuk purata 14 PLBs bagi setiap eksplan selepas enam belas minggu dikultur. Tiada pembentukan PLBs kedapatan apabila cebisan daun dikultur pada media MS terubahsuai berkepekatan separuh yang ditambah dengan 0.1 dan 1 mg/l NAA. Peratus aruhan yang terbaik bagi kombinasi auksin : sitokinin adalah pada kombinasi NAA dan TDZ pada 1.0 dan 3.0 mg/l yang menghasilkan aruhan sebanyak 72% dengan sembilan PLBs untuk setiap eksplan. Setelah berjaya diaruh, adalah penting untuk memaksimumkan proses proliferasi dan penggunaan PLBs. Untuk mengkaji kesan proliferasi, media MS separa pejal berkepekatan separuh dan media Vacin dan Went (VW) cecair dengan dan tanpa sukrosa telah digunakan untuk mendapatkan bilangan PLBs dan kemandirian yang tertinggi selepas tiga bulan pengkulturan media MS berkepekatan separuh menghasilkan purata 9 PLBs dengan 60% kemandirian dan purata berat segar ialah 0.5g berbanding media VW dengan dan tanpa sukrosa yang menghasilkan purata 4.8 and 5.3 PLBs untuk setiap eksplan diikuti dengan kemandirian sebanyak 55 and 57.5%. Pemerhatian histologi membuktikan bahawa permukaan atas daun berhampiran dengan luka mempunyai bilangan PLBs yang paling tinggi berbanding dengan kawasan lain pada eksplan. Mikrograf SEM juga menunjukkan bahawa PLB yang terhasil daripada daun (LDP) terbentuk dari keratin daun selepas 16 minggu tempoh kultur.

Dua belas primer “decamer RAPD” telah digunakan untuk mengkaji variasi somaklonal diantara pokok induk, PLBs yang teraruh pada peringkat awal, dan PLBs selepas 3 dan 6 bulan di dalam kultur. Lapan daripada dua belas primer telah

menghasilkan 172 jalur dengan 18 jalur polimorfik dalam semua rawatan. Produk yang telah diamplifikasikan berbeda antara 125 kepada 8000 bp. Di antara primer yang digunakan, P 16 menghasilkan bilangan jalur yang paling tinggi (29) manakala primer OPU 10 menghasilkan bilangan yang paling rendah (15). Julat pekali kesamaan adalah dari 0.83 kepada 1.0 antara subkultur yang berbeza dan pokok induk. Didapati bahawa perubahan minima atau tiada perubahan berlaku antara pokok induk dan PLBs yang terhasil selepas 3 bulan aruhan. PLBs yang teraruh seterusnya disubkultur untuk proliferasi selama enam bulan dan ini menyebabkan lebih kurang 17% perbezaan dengan pokok induk. Mikropropagasi *Phalaenopsis bellina* boleh dilakukan dengan menggunakan MS media berkepekatan separuh untuk tempoh 6 bulan tetapi, proliferasi seterusnya mungkin menyebabkan terjadinya variasi somaklonal yang boleh mengubah ciri-ciri prolifik orkid ini.

PLBs *in vitro Phalaenopsis bellina* berukuran 4-5 mm telah dipilih untuk dikapsulkan menggunakan kepekatan berbeza iaitu natrium alginat (3, 4 dan 5%) dan kalsium klorida (25, 50, 75 dan 100 mM). PLBs yang tidak dikapsulkan dijadikan sebagai kawalan. PLBs yang dikapsulkan dengan 4% natrium alginat di dalam 75 mM kalsium klorida menunjukkan kombinasi yang terbaik sebagai agen pembentukan gel ke atas kemandirian PLBs selepas penyimpanan selama dua minggu pada 5°C yang memberikan peratus kemandirian sebanyak 70. Selanjutnya PLBs yang dikapsulkan dengan 4% natrium alginat + 75% kalsium klorida telah digunakan untuk menilai kebolehan penyimpanan untuk jangka masa simpanan yang berbeza (15, 30, 45 dan 60 hari) pada suhu yang berlainanan (5, 15, dan 25°C). Peratus kemandirian yang tertinggi dengan 70% didapati selepas 15 hari pada 5°C

diikuti dengan 45 hari penyimpanan pada 5 ° C dengan 50% frekuensi kemandirian. Dalam kajian terkini peratus kemandirian tertinggi mengikut urutan suhu penyimpanan untuk biji benih sintetik adalah 5 ° C > 15 ° C > 25 ° C. Berat segar PLB tertinggi adalah 0.36g dimiliki oleh PLBs yang dikapsulkan dan disimpan selama 15 hari pada suhu 5 °C. Tambahan, selepas 60 hari pada suhu 25°C berat segar menurun kepada 0.19g dan PLB tidak menunjukkan kebernasan. Kandungan air (MC) pada PLBs berkurangan dengan cepat kepada 10.2% pada suhu 25°C setelah disimpan selama 60 hari.

Secara teknikal biji benih sintetik seharusnya boleh berfungsi seperti biji benih normal, adalah bermanfaat sekiranya dapat mengwujudkan kaedah penyimpanan untuk jangka pendek dan panjang. Kajian ini menunjukkan bahawa PLB *Phalaenopsis bellina* berjaya dikrioawetkan dengan menggunakan kaedah pengkapsulan-dehidrasi. Pertumbuhan semula tertinggi eksplan yang telah dikrioawetkan diperolehi ketika PLBs dipra-rawat selama 3 hari menggunakan MS media berkepekatan separuh dengan penambahan 30 g/l sukrosa dan diikuti dengan pra-kultur pada 0.75 M sukrosa selama 3 hari. Tambahan, kandungan sukrosa tertinggi iaitu sebanyak 0.78 g/l telah direkod dalam 100 gram PLBs dengan menggunakan HPLC. PLBs yang dikapsulkan telah didehidrasi menggunakan gel silika selama 6 jam sebelum direndam ke dalam cecair nitrogen selama 1 jam. Kebernasan protokom telah diuji menggunakan assay 2, 3, 5-trifeniltetrazoliumklorida (TTC) dan kebolehan untuk tumbuh semula dinilai dengan mendapatkan peratus kemandirian setelah 2 minggu pemulihan. Kadar kemandirian PLBs yang telah dikrioawet adalah 30%, manakala peratus asai TTC adalah 46.6%.

Akhirnya, tempoh dehidrasi selepas pembekuan dan pencairan menunjukkan perbezaan kebocoran elektrolit (EL) dimana PLBs yang tidak dihidrasi memberikan nilai bacaan EL tertinggi (85.3%) berbanding dengan nilai terendah (53%) yang diperolehi selepas dehidrasi selama 6 jam menggunakan gel silika.

ACKNOWLEDGEMENTS

My first gratitude goes to Allah Almighty who let me finish this journey. I thank God for HIS gifts of patience, wisdom and the perseverance to pursue my dream of attaining academic excellence.

I would like to thank my academic supervisor, Associate Professor Dr. Uma Rani for her patience and selfless help towards improving my academic performance. I appreciate the advice you have given me over these years and without you it would have been impossible to successfully complete this project. I would also like to thank my committee members; Associate Professor Dr. Mihdzar Abdul Kadir, Associate Professor Dr. Saleh Kadzimin and Professor Dr. Maziah Mahmood for their professional contributions and suggestions towards my academic success.

My deepest gratitude goes to my family. I thank my lovely wife, Maryam Ghafoori, for her love, calmness, contributions and sharing the ups and downs. I will never forget UPM not only as our university, but for the place where we met each other and got married. To my mother, Mojgan, and my father, Mahmoud, for unflagging love and support throughout my life and being my haven. I have no suitable word that can fully describe their everlasting love to me. Thanks are also extended to my dear sister, Asal, for her support. Also, I would like to thank my father and mother in law for their encouragements. Finally, I appreciate all my friends who have made UPM a home away from home.

I certify that a Theses Examination Committee has met on 31 January 2011 to conduct the final examination of Amir Ali Khoddamzadeh on his thesis entitled entitled "INDUCTION OF PROTOCORM-LIKE BODIES (PLBs), SYNTHETIC SEED PRODUCTION AND CRYOPRESERVATION in *Phalaenopsis bellina* (Rchb.f.) Christenson " in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Pertanian Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the relevant degree of Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Mohd Ridzwan Abdul Halim, PhD
Associated Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Maheran Abdul Aziz, PhD
Associated Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Nur Ashikin Psyquay Abdullah, PhD
Assistant Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Paul Thomas Lynch, PhD
Professor
Faculty of Education, Health and Sciences
University of Derby
(External Examiner)

BUJANG KIM HUAT, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of requirement for degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Uma Rani A/P Sinniah, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Mihdzar B Abdul Kadir, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Saleh B Kadzimin, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Maziah Mahmood, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

HASANAH MOHD GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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 other institutions.

_____

AMIR ALI KHODDAMZADEH

Date: 31 January 2011

TABLE OF CONTENTS