UNIVERSITI PUTRA MALAYSIA

ESTABLISHMENT OF TISSUE CULTURE PROTOCOLS FOR Curculigo latifolia DRYAND AND DETERMINATION OF UNIFORMITY AMONG PLANTLETS USING SSR MARKERS

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FP 2012 51

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By

NAHID BABAEI

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

June 2012

DEDICATION

Dedicated to

My parents for their endless and boundless love, support, encouragement

My beloved husband Yousof Gheisari for his sacrifices, understanding and tremendous support throughout my study, and

My sweet heart Farnaz for making my life more meaningful

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

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Chairman: Nur Ashikin Psyquay Abdullah, PhD
Faculty: Agriculture

Curculin found in Curculigo latifolia fruit has great potential for the pharmaceutical industry. Studies were conducted with the objectives of establishing a tissue culture protocol for C. latifolia and to determine uniformity among plantlets and their mother plants using SSR markers. Although the plants are found abundantly in Malaysia, commercial fruit production from superior varieties is at the early stage. Propagation through seeds and rhizomes are poor and planting using materials harvested directly from the forest is precarious as the genetics of this plant is understudied. Therefore tissue culture provides the means of producing true to type and uniform seedlings in mass numbers. Plants were collected from Jelebu, Negeri Sembilan and maintained under 70% shade with regular watering for at least six months. Petioles and shoot tips were used as explants but this resulted in very high in vitro contamination. Therefore, a sterilization protocol suitable for C. latifolia was developed. Sterilization and

browning elimination were performed by pre-treatment with different antioxidants (citric acid, ascorbic acid and PVP (0.1%)), fungicide (bavistin 0.1%), and antibiotic (chloramphinicol 0.1%) on an orbital shaker for 9 hours followed by disinfecting with mercuric chloride (0.1%) for 5 minutes with 62.5% of shoot tips and 100% survival of explants after 45 days of culture.

Petiole explants resulted in a higher percentage of explant (83.3%); however, there was more necrosis than with shoot tips, and there was a lower percentage of explant survival (49.2%). Thus, shoot tips were preferred for use as explants for regeneration. The direct regeneration of shoots from explants were optimized using Murashige and Skoog (1962) medium supplemented with different concentrations of single thidiazuron (TDZ) treatment (0, 0.5, 1, 1.5, 2 mgl^{-1}) or in combination with IBA (0, 0.25, 0.5 mgl^{-1}). The best results in terms of percentage of regeneration (77.8% and 83.3%), number of shoots (3.08 and 7.52) and shoot length (1.43 and 2.71 cm) were from treatment consisting of 0.5 mgl^{-1} TDZ and 0.25 mgl^{-1} after 10 and 14 weeks of culture, respectively. Meristems were elongated and roots were induced to generate a single plant in the control treatment. In this study it was also observed that a single concentration of 2 mgl^{-1} TDZ produced the highest percentage of scalp induction (77.8%).

Scalp induction from shoot tip explants on MS medium with different concentrations of TDZ (0, 1, 2, 3, 4 mgl^{-1}) showed that TDZ at a concentration of 3 mgl^{-1} was the best treatment for percentage of scalp induction (83.3%). In determining the growth pattern and best time for sub-culturing, the scalp weighing 1.1 $\pm$ 0.02 g was transferred onto MS medium containing 3 mgl^{-1} TDZ.

This confirmed that four weeks interval was the best time for scalp sub-culturing. Scalp maintenance studies were performed on MS medium supplemented with different concentration of TDZ (0, 1, 2, 3, 4 mgl^{-1}) with sub-culturing at four week intervals for three subcultures. The results indicated that 3 mgl^{-1} was the best concentration of TDZ for scalp maintenance (5.91 g) and the highest growth index (4.91) was obtained after the third subculture.

Root induction and elongation studies were performed by separating regenerated shoots longer than 2 cm and culturing onto MS medium supplemented with different concentrations of IBA (0, 0.25, 0.5, 0.75 mgl^{-1}). Shoots sub-cultured every four weeks showed that MS medium devoid of plant growth regulators was the best treatment for percentage root induction (86.7%) and root length (4.87 cm), but IBA at a concentration of 0.25 mgl^{-1} induced more roots (7.64) after eight weeks of culture.

SSR markers were developed using 3′ and 5′ anchored ISSR markers. Amplified PCR products were ligated into vector and transformed into *E.coli* after purification. The positive clones were sequenced and SSR primers were designed using the PRIMER3 software (http:// www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). These primers were tested on 12 accessions of wild *C. latifolia* collected from all states of Peninsular Malaysia. From the 33 designed SSR primers, 22 primers were able to amplify *C. latifolia* target DNA and 11 primer pairs were polymorphic. The number of observed alleles (Na) per locus ranged from three to six. The allele size was between 141 and 306 bp. The observed heterozygosity ranged from 0.00 to 0.37, whereas the expected

heterozygosity ranged from 0.52 to 0.81. The polymorphic information content (PIC) value of each locus was between 0.59 and 0.81, with an average of 0.67 showing that the primers were highly polymorphic. Genetic variations observed in the mother plants when they were analyzed using the 11 primers. Plantlets from *in vitro* cultures showed the same allele sizes when compared to their mother plants and generic uniformity was observed among the plantlets. Plantlets reached 2.5 cm in height before separation from explants and sub-culturing thus avoiding somaclonal variations. The plantlets were then prepared for acclimatization and transfered to the nursery. This study demonstrated a new breakthrough for producing uniform plantlets from *in vitro* cultures for *C. latifolia*. It established a sterilization technique, optimal conditions for direct and indirect regeneration of uniform plantlets and finally provided a means of producing seedlings from superior hybrids with desirable fruits.

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

PENWUJUDAN PROTOKOL KULTUR TISU BAGI *Curculigo latifolia* DRYAND DAN PERKEMBANGAN KESERAGAMAN DI KALANGAN ANAK POKOK MENGGUNAKAN PENANDA SSR

Oleh

NAHID BABAEI

Jun 2012

Pengerusi: Nur Ashikin Psyquay Abdullah, PhD

Fakulti: Pertanian

Curculin yang dijumpai didalam buah *Curculigo latifolia* mempunyai potensi yang besar dalam industri farmaseutikal. Kajian telah dijalankan dengan objektif, mewujudkan protokol kultur tisu untuk *C. latifolia* dan menentukan keseragaman dikalangan anak pokok dan pokok induk dengan menggunakan penanda SSR. Walaupun pokok ini boleh didapati dengan banyak di Malaysia, namun pengeluaran buah komersial daripada varieti yang lebih baik tidak pernah dijalankan. Pembiakan melalui biji benih dan rizom adalah tidak elok dan sesuai, oleh itu kultur tisu menyediakan cara penghasilan benih yang tulen dan seragam dalam kuantiti yang banyak. Pokok-pokok diambil dari Jelebu, Negeri Sembilan dan diselenggarakan dibawah 70% teduhan dengan penyiraman tetap selama sekurang-kurangnya enam bulan. Petiol dan tunas pucuk digunakan sebagai eksplan tetapi ianya menyebabkan kontaminasi *in vitro* yang tinggi. Oleh itu, protokol pensterilan yang khusus untuk *C. latifolia* telah dibangunkan.

Pensterilan dan pembuangan perang dilakukan dengan rawatan awal menggunakan antioksidan yang berbeza (asid sitrik, asid askorbik dan PVP (0.1%)), racun kulat (bavistin 0.1%), dan antibiotik (chloramphinicol 0.1%) di atas penggoncang orbit selama 9 jam diikuti dengan menyahjangkit menggunakan mercuric chloride (0.1%) untuk 5 minit dengan 62.5% tunas pucuk yang steril dan 100% eksplan hidup selepas 45 hari kultur.

Eksplan petiol menunjukkan peratusan yang lebih tinggi untuk eksplan steril (83.3%); walaubagaimanapun, terdapat lebih banyak nekrosis berbanding dengan tunas pucuk, dan terdapat peratusan yang lebih rendah untuk eksplan hidup (49.2%). Oleh itu, tunas pucuk dipilih untuk digunakan sebagai eksplan bagi pertumbuhan baru. Pertumbuhan secara langsung untuk tunas daripada eksplan dioptimakan dengan menggunakan Murashige dan Skoog (1962) sederhana ditambah dengan kepekatan rawatan thidiazuron (TDZ) tunggal (0, 0.5, 1, 1.5, 2 mgl^{-1}) atau dalam kombinasi dengan IBA (0, 0.25, 0.5 mgl^{-1}). Keputusan terbaik dari segi peratusan pertumbuhan (77.8% dan 83.3%), bilangan tunas (3.08 dan 7.52) dan panjang tunas (1.43 dan 2.71 cm) daripada rawatan yang mengandungi 0.5 mgl^{-1} TDZ and 0.25 mgl^{-1} selepas 10 dan 14 minggu kultur. Meristem memanjang dan akar diaruh untuk menjana pokok tunggal di dalam rawatan kawalan. Dalam kajian ini juga diperhatikan bahawa kepekatan tunggal 2 mgl^{-1} TDZ menghasilkan peratusan tertinggi untuk mengaruh skalp.

Aruhan skalp daripada eksplan tunas di atas media MS dengan kepekatan TDZ yang berbeza (0, 1, 2, 3, 4 mgl^{-1}) menunjukkan TDZ pada kepekatan 3 mgl^{-1} adalah rawatan yang terbaik untuk peratusan aruhan skalp (83.3%). Dalam

menentukan corak pertumbuhan dan masa terbaik untuk sub kultur, skalp seberat 1.1 ± 0.02 g dipindahkan ke dalam media MS yang mengandungi 3 mgl^{-1} TDZ, empat minggu adalah masa terbaik untuk sub kultur skalp. Kajian penyelenggaraan skalp dilaksanakan di dalam media MS dengan kepekatan TDZ yang berbeza (0, 1, 2, 3, 4 mgl^{-1}) yang telah di sub kultur selama empat minggu berselang tiga sub kultur. Keputusan menunjukkan 3 mgl^{-1} adalah kepekatan TDZ yang terbaik untuk penyelenggaraan skalp (5.91 g) dan indek pertumbuhan yang tertinggi (4.91) telah diperolehi selepas tiga sub kultur.

Kajian aruhan dan pemanjangan akar telah dilaksanakan dengan mengasingkan pertumbuhan tunas yang lebih panjang daripada 2 cm dan dikultur di dalam media MS dengan kepekatan IBA yang berbeza (0, 0.25, 0.5, 0.75 mgl^{-1}). Pucuk di subkultur setiap empat minggu menunjukkan media tanpa MS adalah rawatan yang terbaik untuk peratusan aruhan akar (86.7%) dan panjang akar (4.87 cm), tetapi IBA pada kepekatan 0.25 mgl^{-1} mengaruh lebih akar (7.64) selepas dikultur lapan minggu.

Penanda SSR telah diperkembangkan denyan penggunaan 3’ dan 5’ penanda ISSR bersauh. Produk PCR terhurai telah digabungkan ke dalam vektor dan berubah kepada *E.coli* selepas penulenan. Klon positif dijujuk dan primer SSR dibentuk menggunakan perisian PRIMER3 (http:// www.genome.wi.mit.edu/cgi-bin/primer/ptimer3.cgi). Primer yang dibangunkan, diuji pada 12 jenis *C. latifolia* liar yang dikumpul daripada semua negeri di Semenanjung Malaysia. Daripada 33 primer SSR yang dibentuk, 22 primer mampu untuk menghurai DNA target *C. latifolia* dan 11 pasang primer menunjukkan polimorfik. Bilangan allel (Na) per

lokus berjulat diperhatikan dari tiga ke enam. Saiz allel adalah diantara 141 dan 306 bp. Heterozigositi yang diperhati berjulat daripada 0.00 hingga 0.37, manakala heterozigositi dijangka berjulat daripada 0.52 hingga 0.81. Nilai kandungan informasi polimorfik (KIP) untuk setiap lokus adalah diantara 0.59 dan 0.81, dengan purata 0.67 menunjukkan primer adalah sangat polimorfik. Variasi genetik yang diperhatikan pada tumbuhan induk apabila dianalisis dengan menggunakan 11 primer seperti dijangka. Anak pokok daripada kultur *in vitro* menunjukkan saiz allel yang sama berbanding pokok induknya dan keseragaman generik diperhatikan di kalangan anak pokok. Anak pokok yang seragam telah mencapai ketinggian 2.5 cm sebelum pengasingan daripada eksplan dan sub kultur untuk mengelakkan variasi soma-klon. Anak pokok kemudian disediakan untuk penyesuaian dan dipindah ke nurseri. Kajian ini menghastikan penyelesaian baharu dalam penghasilan anak pokok yang seragam daripada kultur *in vitro* untuk *C. latifolia*. Ianya meliputi teknik pensterilan, pengoptimaan keadaan untuk pertumbuhan langsung dan tidak langsung bagi anak pokok dan akhirnya menyedikan kaedah penghasilan benih daripada hibrid yang terbaik dengan buah yang diinginkan.

ACKNOWLEDGEMENTS

All praises and thanks due to Allah Almighty for His Mercy and Grace.

I would like to express my sincere thanks to Dr. Nur Ashikin Psyquay Abdullah, chairman of my supervisory committee, for her dedicated efforts, support, invaluable advice and intellectual guidance during the accomplishment of this research work. I would also like to thank my supervisory committee members, Prof. Dr. Ghizan Saleh and Assoc. Prof. Dr. Thohirah Lee Abdullah for their guidance, assistance and encouragement throughout the period of this study. I greatly appreciate all the help they availed to me while pursuing my studies.

I would like to thank the Ministry of Agriculture, Malaysia, from which Dr. Nur Ashikin Psyquay Abdullah obtained this research grant under the e-Science Fund (05-01-04-SF1051). I would also like to appreciate and thank Universiti Putra Malaysia, for providing me the two semesters Graduate Research Fellowship (GRF).

I am indebted to the staff members of Field 2, Universiti Putra Malaysia for the technical assistance while conducting my field experiments. I am also grateful to the laboratory technicians of the Department of Crop Science and University Agriculture Park, Universiti Putra Malaysia. The help and assistance provided by Mr. Rosdi Abd Ghani, Mr. Mohd Helmi Hamisan, Mr. Daud Mustam, Mr. Suhaimi Aman and Pn. Maininah Tahir are highly appreciated. My sincere appreciations also goes to my colleagues and fellow students in the Plant Breeding

laboratory, Crop Science Department, Universiti Putra Malaysia for their support, encouragements, co-operation and involvements in discussions during the study.

My deepest gratitude goes to my parents, sisters, brothers for their love, support and encouragement. Finally, a very heartfelt gratitude and appreciation goes to my husband, Yousof Gheisari and my beloved daughter Farnaz Gheisari for their constant encouragement, patience, great companion and moral support throughout my graduate program and for being there for me.

I certify that a Thesis Examination Committee has met on 12 June 2012 to conduct the final examination of Nahid Babaei on her thesis entitled " Establishment of tissue culture protocol for Curculigo litofolia dryand and determination of uniformity among plantlets using SSR markers " in accordance with the Universities and Univresiti 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 Doctor of Philosophy

Members of the Thesis Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement of the degree of PhD of Agricultural Science. The members of the Supervisory Committee were as follows:

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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 other institutions.

NAHID BABAEI

Date: 12 June 2012

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