UNIVERSITI PUTRA MALAYSIA

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IN VITRO PROPAGATION OF *CITRUS HYSTRIX* AND ASSESSMENT OF GENETIC UNIFORMITY USING RAPD MARKERS

By

ENG WEE HIANG

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

July 2013

Dedicated to:

My Parents
George Ngu

Abstract of thesis presented to the Senate of Universiti Putra Malaysia
in fulfilment of the requirement for the degree of Master of Science

IN VITRO PROPAGATION OF *CITRUS HYSTRIX* AND ASSESSMENT OF GENETIC UNIFORMITY USING RAPD MARKERS

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July 2013

Chairperson: Associate Professor Maheran Abdul Aziz, PhD

Faculty: Agriculture

Recent studies in *Citrus hystrix* largely focused on its usage in citriculture, pharmaceutical and nutritional values. This newly emerged plant urgently needs biotechnological studies especially on the establishment of its micropropagation protocol. The objectives of this study are (a) to develop an efficient micropropagation system using various juvenile explants of *C. hystrix* seedlings, (b) to elucidate genetic uniformity of plantlets derived from the juvenile explants using RAPD markers and (c) to develop an effective sterilization technique and induction of multiple shoot formation from nodal segments of mature field grown *C. hystrix*.

In vitro seedling and field-grown mature *C. hystrix* was used as source of explants to assess regeneration capability. In this study, various concentrations of BAP were assessed to determine the optimum concentration for regeneration. Leaves abscission occured during regeneration stage. To overcome the problem, Ca-gluconate and

silver nitrate were amended into medium containing optimized concentration of BAP. In rooting stage, continuous auxin treatment and auxin pulse treatment were tested to determine efficient rooting method. Types of potting mixture were assessed to determine the best potting mixture for survival of deflasked *in vitro* plantlets. For sterilization study using field-grown mature plant, different types and combination of antibiotics were tested to reduce latent bacterial contamination. Medium with combination of BAP, GA_3 and $AgNO_3$ were tested to determine the best medium for shoot regeneration for field-grown mature plant. RAPD markers were used for screening uniformity of regenerants from *in vitro* seedling.

The optimum regeneration medium for shoot tip was MS medium + 2.22 µM BAP + 4 mM Ca-glu + 20 µM $AgNO_3$ + 30 g/L sucrose, inducing four shoots per explant. For epicotyls, hypocotyls, primary roots and cotyledons, the optimum regeneration medium was MS medium + 2.22 µM BAP + 10 µM $AgNO_3$ + 30 g/L sucrose which induced five, four, three and two shoots, respectively. For *in vitro* rooting, auxin pulse treatment with 9840 µM IBA in MS medium for 16 h prior to transfer to MS medium + 20 µM $AgNO_3$ produced significantly the highest number of roots (2 roots) and highest rooting percentage (87.50%). In the acclimatization study, medium consisting of soil : Peatgro : sand (1 : 1 : 1) was the best attaining a survival percentage of plantlets at 83.33%. Genetic stability of the plantlets was assessed using RAPD markers. Most of the plantlets were identical to the mother plant based on RAPD banding pattern. Eighteen out of twenty samples had Jacaard's similarity coefficient of 1.0000 indicating 90% of the regenerants were likely to be clones.

For effective sterilization of nodal segments derived from mature field grown *C. hystrix* the use of 500 mg/L cefotaxime as part of the surface sterilization procedure successfully reduced percentage of latent bacterial contamination and resulted in significantly the highest survival percentage (61.25%) after eight weeks of culture. The optimised regeneration medium for nodal segments of mature plants of *C. hystrix* was MS medium + 2.22 µM BAP + 20 µM $AgNO_3$ + 30 g/L sucrose inducing three shoots per explant. Through this study, an efficient micropropagation system has been developed that could provide a mean for mass propagation, and as a tool in genetic modification and *in vitro* germplasm conservation of *C. hystrix*.

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

PEMBIAKAN *IN VITRO CITRUS HYSTRIX* DAN PENILAIAN KESAMAAN GENETIK MELALUI RAPD

Oleh

ENG WEE HIANG

Julai 2013

Pengerusi: Profesor Madya Maheran Abdul Aziz, PhD

Fakulti: Pertanian

Kajian terkini dalam *Citrus hystrix* lebih tertumpu kepada kegunaannya dalam penanaman sitrus, perubatan dan nilai pemakanan. Tanaman ini amat memerlukan kajian bioteknologi terutamanya dalam pembangunan protokol pembiakan mikro. Objektif kajian ini adalah untuk (a) membangunkan sistem pembiakan mikro yang cekap dengan menggunakan pelbagai eksplan juvenil daripada anak benih *C. hystrix*, (b) menilai kesamaan genetik anak pokok daripada eksplan juvenil dengan menggunakan penanda RAPD dan (c) membangunkan teknik sterilisasi yang efektif dan mengaruh pembetukan pucuk berganda pada segmen nod *C. hystrix* yang diperoleh dari pokok matang di ladang.

Anak benih *in vitro* dan pokok matang dari ladang bagi *C. hystrix* telah digunakan sebagai bahan tanaman untuk menilai keupayaan pertumbuhan semulanya. Dalam

kajian ini, pelbagai kepekatan BAP telah dinilai untuk menentukan kepekatan BAP yang paling sesuai untuk pertumbuhan semula. Keguguran daun berlaku ketika peringkat pertumbuhan semula. Bagi mengatasi masalah ini, Ca-gluconate dan argentum nitrat telah ditambahkan ke dalam medium yang mengandungi kepekatan BAP yang terbaik. Di peringkat pengakaran, rawatan auksin berterusan dan rawatan auksin sementara dikaji bagi mengenalpasti kaedah merangsang pengakaran terbaik. Pelbagai jenis campuran medium memasu dinilai bagi menentukan campuran terbaik untuk anak pokok yang dikeluarkan dari berkas kultur. Bagi kajian pembasmian pencemar-pencemar pokok matang dari ladang, pelbagai jenis antibiotik dan campuran antibiotik-antibiotik telah digunakan untuk mengurangkan pencemaran bakteria lanjutan. Medium yang terdiri daripada kombinasi BAP, GA_3 dan $AgNO_3$ dinilai bagi menentukan medium yang paling sesuai untuk pertumbuhan semula pokok matang. Penanda-penanda RAPD digunakan untuk menilai keseragaman genetik anak-anak pokok hasil pertumbuhan semula *in vitro*.

Medium pertumbuhan semula yang optimum untuk pucuk adalah medium MS + 2.22 µM BAP + 4 mM Ca-glu + 20 µM $AgNO_3$ + 30 g/L sukrosa yang menghasilkan empat pucuk per eksplan. Bagi epikotil, hipokotil, akar utama dan kotiledon, medium regenerasi optimum adalah medium MS + 2.22 µM BAP + 10 µM $AgNO_3$ + 30 g/L sukrosa masing-masing menghasilkan empat, empat, tiga dan dua pucuk. Untuk pengakaran, rawatan auksin sementara dengan 9840 µM IBA dalam medium MS selama 16 jam sebelum dipindahkan ke medium MS + 20 µM $AgNO_3$ menghasilkan jumlah akar (2 akar) dan peratusan pengakaran (87.50%) tertinggi. Dalam kajian penyesuaian, medium yang terdiri daripada tanah : Peatgro : pasir (1 : 1 : 1)

merupakan campuran yang terbaik dengan mencapai peratusan hidup tertinggi (83.33%) untuk anak pokok berakar. Kesamaan genetik anak pokok dinilai dengan menggunakan penanda RAPD. Berdasarkan corak jalur RAPD, didapati kebanyakan anak pokok adalah seiras dengan pokok induk. Lapan belas daripada dua puluh sampel menpunyai nilai kesamaan koefficient Jacaard 1.0000 menunjukkan 90% anak pokok berkemungkinan adalah klon.

Untuk pensterilan segmen nod *C. hystrix* dari pokok matang di ladang penggunaan 500 mg/L cefotaxime sebagai sebahagian daripada prosidur pensterilan telah berjaya menurunkan peratusan pencemaran bakteria dan menghasilkan peratusan hidup tertinggi (61.25%) yang ketara selepas lapan minggu. Medium regenerasi optimum untuk keratan nod dari pokok matang adalah medium MS + 2.22 µM BAP + 20 µM $AgNO_3$ + 30 g/L sukrosa menghasilkan tiga pucuk per eksplan. Melalui kajian ini, sistem pembiakan mikro yang cekap telah dibangunkan sesuai untuk kegunaan pembiakan bahan tanaman secara besar-besaran, dan sebagai kaedah pengubahsuaian genetik dan pengekalan germaplasma *C. hystrix* secara *in vitro*.

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I certify that a Thesis Examination Committee has met on 15 July 2013 to conduct the final examination of Eng Wee Hiang on his thesis entitled "*In Vitro* Propagation of *Citrus hystrix* and Assessment of Genetic Uniformity Using RAPD Markers" in accordance with the 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 was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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