UNIVERSITI PUTRA MALAYSIA

SOMATIC EMBRYOGENESIS FROM IMMATURE MALE FLOWERS OF BANANA (*MUSA* SPP. CV. RASTALI)

AINI MOHD ZAINOL AZLIN

FP 2008 6

SOMATIC EMBRYOGENESIS FROM IMMATURE MALE FLOWERS OF BANANA (*MUSA* SPP. CV. RASTALI)

By

AINI MOHD ZAINOL AZLIN

MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA

2008

SOMATIC EMBRYOGENESIS FROM IMMATURE MALE FLOWERS OF BANANA (*MUSA* SPP. CV. RASTALI)

By

AINI MOHD ZAINOL AZLIN

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

May 2008

musa

Fruits Of Paradise

is

Dedicated To

My dearest parents

Mohamad Zainol Azlin Ariffin
Hasiah Hashim

My thoughtful siblings and brother in-law

Aida and Jafar
Mohd Fikril Hadi

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

SOMATIC EMBRYOGENESIS FROM IMMATURE MALE FLOWERS OF BANANA (*MUSA* SPP. CV. RASTALI)

By

AINI MOHD ZAINOL AZLIN

May 2008

Chairman : Associate Professor Maheran Abdul Aziz, PhD

Faculty : Agriculture

This study was carried out with the main objective of establishing a plant regeneration system through somatic embryogenesis in *Musa* spp *cv.* Rastali. The study included induction of embryogenic callus and somatic embryos from immature male flowers, initiation of cell suspension cultures from the embryogenic callus and somatic embryos, proliferation of somatic embryos from the embryogenic cell suspension followed by embryo maturation and germination.

In the study on embryogenic callus induction from immature male flowers of banana *cv.* Rastali, the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) and different flower cluster positions on the embryogenic callus formation was determined. Various concentrations of 2,4-D (0, 1, 2 and 4 mg/L) were incorporated into M1 medium consisting of MS medium, 1 mg/L IAA, 1mg/L NAA, 30 g/L sucrose and 7 g/L agarose.

The levels of 2,4-D affected the embryogenic callus initiation and somatic embryo formation in *cv.* Rastali. The highest percentage of embryogenic callus formation (53.9 %) was obtained on treatment with 2 mg/L 2,4-D in *cv.* Rastali for all flower cluster positions assessed.

The effect of flower cluster positions on percentage of embryogenic callus formation showed the highest percentage (48.4 %) was from flower cluster position 8 in *cv.* Rastali. Interaction between the different flower cluster positions and 2,4-D concentrations produced the highest percentage of embryogenic callus formation (83.8 %) from flower cluster position 8 on treatment with 2 mg/L 2,4-D in *cv.* Rastali.

After six months on the callus induction medium, calli varying from yellowish, creamy to white were formed. A positive embryogenic response was shown by the appearance of individual somatic embryos amongst the callus produced on 2 and 4 mg/L 2,4-D and this indicated an ideal callus.

The initial phase of cell suspension establishment comprised of a mixture of cell aggregates and heterogenous cells that varied from embryogenic cells, non-embryogenic cells and elongated cells. The suspension cultures were refreshed monthly to improve the suspension quality and to obtain homogeneous embryogenic cell cultures.

After a duration of one month, with agitation of the suspension culture, attached embryogenic cells broke free from the cell aggregates. Cells stained with fluorescein diacetate (FDA) fluoresced bright green when observed under a microscope with UV attachment indicating the cells were viable. It offers a quick visual assessment on percentage of cell viability. Meanwhile, Evan's blue staining was also used to check the cell viability to complement the FDA assesment. When cells were treated with dilute (0.025 %) solution of Evan's blue, intact and viable cells remained unstained whilst damaged cells took up the stain.

The growth of suspended cells in S1 and S2 media over five subcultures showed a sporadic pattern. S1 medium consisted of half strength MS macronutrients, MS micronutrients, Dhed'a Vitamins, 10 mg/L ascorbic acid, 1.1 mg/L 2,4-D, 0.25 mg/L zeatin and 20 g/L sucrose, while S2 medium consisted of MS medium, 1 mg/L biotin, 1 mg/L 2,4-D, 99 mg/L glutamine, 100 mg/L malt extract (Sigma) and 44 mg/L sucrose. Cell growth was determined by counting cells using a haemocytometer. Highest cell count of 77 per ml was attained at subculture 2 in S2 medium while in S1 medium cells reached their highest growth rate of 75 per ml at subculture 5. Microscopic observation of the cell suspension in liquid S1 medium at the third subculture showed cells with small, distinct and voluminous nucleus as well as dense cytoplasm. In the meantime, closely attached cells with compact cytoplasm; mainly composed of embryogenic cells were observed in liquid S2 medium before sieving at the second subculture. Sieving out the elongated and vacuolated cells generated uniform and single meristematic cells in the medium, producing embryogenic cell

suspensions that were less heterogeneous. After three months, with sieving and subculture, *Musa* sp. *cv*. Rastali produced a very fine, light yellow embryogenic suspension culture most suitable for embryo maturation and germination study. In the final stage after the fifth subculture, the embryogenic cell suspensions were transferred into MS liquid medium without hormone and showed formation of globular somatic embryos.

Within one month after placing 1 mL of embryogenic cell suspension (ECS) onto a filter paper placed on M3 medium containing SH salts, MS vitamins, 1 mg/L biotin, 0.05 mg/L zeatin, 0.1 mg/L kinetin, 45 g/L sucrose, 10 g/L lactose, 100 mg/L glutamine, 230 mg/L proline, 0.2 mg/L NAA, 0.14 mg/L 2iP, 100 mg/L malt extract and 3 g/L phytagel, clusters of smooth globular to polar shaped somatic embryos creamy white in colour appeared. Such clusters of globular somatic embryos with hyaline protuberances formed many cell lines that allowed the selection of high quality lines. The creamy-like globular structures were transferred in clumps onto M4 germination medium consisting of MS macronutrients, MS micronutrients, MS Fe EDTA, Morel and Wetmore modified vitamin, 0.2 mg/L IAA, 30 g/L sucrose, 2 g/L phytagel and supplemented with different concentrations of BAP (0, 0.1, 0.5, 1.5 and 2.0 mg/L). After a duration of two months on the M4 germination medium, radicles and hairy roots emerged from the somatic embryos. A month later, development of the whitish radicles from the globular somatic embryos became prominent followed by the formation of greenish plumules. The radicles elongated into roots and the greenish plumules developed into shoots. Treatment with 0.5 mg/L

BAP gave the highest regeneration percentage of 8.9 % compared to the control at 2.3 %. BAP concentrations of more than 0.5 mg/L resulted in stunted growth of plantlets and a decrease in the regeneration percentage.

Plantlets obtained were transferred to hormone-free MS medium and placed in light condition for formation of chloroplasts and further growth of the shoots and roots. Histological study clearly showed the shoot and root development from somatic embryos of *Musa* sp. *cv.* Rastali. The shoot arised from in between the leaf primordia that were attached to the mother tissue. The shoot apex arrangement could be clearly seen through lateral section of the same specimen. The root portion was easily detached during sectioning nevertheless it was clearly evident in a separate histological section. The presence of shoot and root poles in the histological observation confirmed the bipolar nature of the somatic embryos.

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

EMBRIOGENESIS SOMA DARIPADA BUNGA JANTAN BELUM MATANG *MUSA* SPP. KULTIVAR RASTALI

Oleh

AINI MOHD ZAINOL AZLIN

May 2008

Pengerusi : Profesor Madya Maheran Abdul Aziz, PhD

Fakulti : Pertanian

Penyelidikan ini dilaksanakan dengan objektif utama untuk mewujudkan sistem regenerasi melalui embriogenesis soma bagi *Musa* spp. kultivar Rastali. Kajian ini merangkumi induksi kalus embriogenik dan embrio soma daripada bunga jantan belum matang, inisiasi kultur sel ampaian daripada kalus embriogenik dan embrio soma, proliferasi embrio soma daripada sel ampaian embriogenik diikuti dengan pematangan serta percambahan embrio.

Bagi kajian induksi kalus embriogenik daripada bunga jantan belum matang pisang kultivar Rastali, kesan asid diklorofenoksi asetik (2,4-D) dan posisi kluster bunga jantan yang berbeza terhadap pembentukan kalus embriogenik ditentukan. Pelbagai kepekatan 2,4-D (0, 1, 2 and 4 mg/L) ditambah ke dalam medium M1 yang mengandungi medium MS dengan 1 mg/L IAA, 1mg/L NAA, 30 g/L sukrosa dan 7 g/L agarose.

Kepekatan 2,4-D didapati memberi kesan terhadap inisiasi kalus embriogenik dan pembentukan embrio soma bagi kultivar ini. Peratus pembentukan kalus embriogenik yang tertinggi (53.9 %) diperoleh pada rawatan 2 mg/L 2, 4-D bagi kultivar Rastali untuk kesemua posisi kluster yang diuji.

Kesan posisi kluster bunga jantan terhadap peratus pembentukan kalus embriogenik menunjukkan peratus tertinggi (48.4 %) adalah pada posisi kluster ke 8 bagi bunga jantan kultivar Rastali. Interaksi di antara posisi kluster bunga jantan dan kepekatan 2,4-D memberikan peratus tertinggi pembentukan kalus embriogenik (83.8 %) pada kluster bunga posisi ke 8 pada rawatan 2 mg/L 2, 4-D bagi kultivar Rastali.

Selepas enam bulan di atas medium induksi kalus, pelbagai variasi kalus daripada warna kekuningan, krim dan keputihan terbentuk. Respon embriogenik yang positif ditunjukkan menerusi kehadiran embrio soma individu dicelah kalus yang terbentuk pada kepekatan 2 dan 4 mg/L 2, 4-D, dan ini adalah kalus yang ideal.

Fasa awal pembentukan sel ampaian didapati mengandungi campuran sel agregat dan sel heterogenus yang terdiri daripada sel embriogenik, sel tidak embriogenik dan sel berbentuk panjang dan bervakuol. Larutan kultur sel ampaian diperbaharui pada setiap bulan bagi memperbaiki kualiti sel yang diampai dan untuk mendapatkan kultur sel ampaian embriogenik yang sekata.

Selepas tempoh sebulan dikocak di dalam sel ampaian, sel embriogenik terlerai daripada sel agregat. Sel yang diwarnai dengan fluoresin diasetat (FDA) didapati berwarna hijau terang apabila dilihat di bawah mikroskop dengan kelengkapan ultra-ungu menandakan sel adalah hidup. Ianya memberikan kaedah visual yang pantas dalam penelitian peratus sel hidup. Sementara itu, pewarna Evan's blue boleh digunakan menggantikan FDA untuk menentukan peratus sel hidup. Sel hidup yang diuji dengan cairan (0.025 %) Evan's blue tidak diwarnai kerana hanya sel tidak hidup akan meresap pewarna tersebut.

Lengkungan kultur sel ampaian diantara S1 dan S2 bagi lima kali subkultur menunjukkan corak yang tidak menentu. Medium S1 mengandungi separuh kepekatan MS makronutrien, MS mikronutrien, Dhed'a Vitamin, 10 mg/L asid askorbik, 1.1 mg/L 2,4-D, 0.25 mg/L zeatin dan 20 g/L sukrosa manakala medium S2 mengandungi medium MS, 1 mg/L biotin, 1 mg/L 2,4-D, 99 mg/L glutamin, 100 mg/L ekstrak maltosa (Sigma) dan 44 mg/L sukrosa. Penambahan sel dihitung dengan menggunakan hemositometer. Bilangan tertinggi 77 sel per ml diperoleh pada subkultur 2 bagi medium S2, manakala bagi medium S1 kadar pertumbuhan sel tertinggi pada 75 per ml dicapai pada subkultur ke-5. Pengamatan mikroskopik sel ampaian di dalam medium S1 pada subkultur ke-3 menunjukkan nukleus sel adalah kecil, ketara dan mempunyai ruang yang besar serta sitoplasma yang tumpat. Sementara itu, sel yang melekat diantara satu sama lain dengan sitoplasma yang padat terdiri daripada komposisi sel embriogenik yang didapati di dalam medium S2 sebelum disaring pada subkultur ke-2.

Penyaringan sel panjang dan sel bervakuol menghasilkan sel meristematik yang sekata dan tunggal di dalam medium, membentuk kultur sel ampaian embriogenik yang kurang heterogenus. Fasa terakhir selepas subkultur ke-5, sel ampaian embriogenik dipindahkan ke dalam media cecair tanpa hormon menunjukkan pembentukkan embrio soma globul.

Dalam tempoh sebulan selepas meletakkan 1 mL kultur sel ampaian embriogenik di atas kertas turas pada medium M3 yang mengandungi medium SH, MS vitamin, 1 mg/L biotin, 0.05 mg/L zeatin, 0.1 mg/L kinetin, 45 g/L sukrosa, 10 g/L laktosa, 100 mg/L glutamin, 230 mg/L prolin, 0.2 mg/L NAA, 0.14 mg/L 2iP, 100 mg/L ekstrak maltosa dan 3 g/L phytagel, kluster embrio soma berbentuk globul licin sehingga berbentuk polar dengan warna krim keputihan muncul. Kluster embrio soma globul dengan unjuran hailin membentuk warisan sel yang membenarkan pemilihan warisan yang berkualiti tinggi.

Struktur globular berwarna krim dipindahkan ke medium percambahan M4 yang mengandungi MS makronutrien, MS mikronutrien, MS Fe EDTA, modifikasi vitamin Morel dan Wetmore, 0.2 mg/L IAA, 30 g/L sukrosa and 2 g/L phytagel dan dibekalkan dengan kepekatan BAP berbeza (0, 0.1, 0.5, 1.5 and 2.0 mg/L). Selepas dua bulan di atas medium percambahan M4, radikel dan akar rerambut muncul daripada embrio soma. Sebulan kemudian, pembentukan radikel yang keputihan bertambah jelas diikuti dengan pembentukan pumul kehijauan daripada embrio soma globular. Radikel memanjang membentuk akar dan plumul kehijauan membentuk

pucuk. Rawatan dengan 0.5 mg/L BAP memberikan peratus regenerasi tertinggi pada 8.9 % berbanding kawalan pada 2.3 %. Kepekatan BAP yang melebihi 0.5 mg/L merencatkan pertumbuhan plantlet dan menyebabkan penurunan peratus regenerasi.

Plantlet yang terhasil dipindahkan pada medium MS tanpa hormon dan diletakkan di bawah cahaya bagi pembentukan kloroplas dan seterusnya tumbesaran pucuk dan akar. Kajian histologi menunjukkan pertumbuhan pucuk dan akar daripada embrio soma *Musa* sp. *cv.* Rastali. Pucuk muncul daripada tapak diantara tunas daun yang bersambungan dengan tisu induk. Susunan tunas aksil boleh dilihat melalui keratan rentas spesimen yang sama. Bahagian akar mudah tertanggal sewaktu `*sectioning*', walau bagaimanapun ia jelas dilihat pada keratan histologi akar yang berasingan. Kehadiran pucuk dan akar di dalam pengamatan histologi ini membuktikan sifat bipolar embrio soma.

ACKNOWLEDGEMENT

In the name of Allah, the Most Almighty and Gracious for giving me the blessing and strength to complete this study. First and foremost, I would like to convey my deepest gratitude to the chairman of the supervisory committee, Assoc. Prof. Dr. Maheran Abdul Aziz, also my mentor, for her endless assistance and guidance throughout my research. Thank you for being patience with me. I am also indebted to my co-supervisory committee members, En. Azmi Abd Rashid and Assoc. Prof. Dr. Norihan Salleh for their continuous advice and suggestion during my study.

Not forgetting Prof. Rahman Abd Razak and Assoc. Prof. Dr. Abd Ghani Yunus for giving me the opportunity to use the Plant Microstructure and Anatomy and Scanning Electron Microscophy Laboratory. I would also like to express my thanks to Mrs. Asiah Othman and Mr. Daud Mustam, for their technical assistance.

I wish to acknowledge all members of Makmal *In Vitro* particularly Dr. Humayum Kabir, for his help during the early days of the research and the person who introduced me to the grandeur of banana. Not forgetting Beverlien Christine Daiman, Dalila Bahrun, Agus Sutanto, Budi Winarto, Lim Swee Ping, Norazwa Salim, Roshailila, Norwaty Bita, Suleiman Elhorry, Syaiful Bahri Panjaitan, Tengku Laila

Kamaliah, Kaynoosh Kashefi, Arash Rafat, Tajul Abdullah and Elixon Sunian for their upmost encouragement while making the environment pleasurable and comfortable to me. Lots of thanks also goes specially to Teguh Prasetyo, Norizah Jaafar, Fatimah Abdol Latif, Ooi Mei Kim, Yohannes, Rozaimah, Tuhc, Mei An, Norliza, Fadhilah, Ummi, Rina Ariffin, Chin, Maiyastri and Puspita Deswina.

This dissertation is especially dedicated to my parents Mohd Zainol Azlin Ariffin and Hasiah Hashim, sister and brother in-law Aida Mohd Zainol Azlin and Jafar, brother Mohd Fikril Hadi, for their prayers, affection, tolerance and encouragement for the achievement of my study.

Last but not least, loads of thanks to all my friends whom I did not list above, who made it possible for me to complete this study. This study was supported by PASCA.

I certify that an Examination Committee met on 20 Mei 2008 to conduct the final examination of Aini Mohd Zainol Azlin on her Master of Science thesis entitled "Somatic Embryogenesis From Immature Male Flowers of Banana (*Musa* spp. *cv.* Rastali)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the Master of Science.

Members of the Examination Committee are as follows:

Halimi Mohd. Saud, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Saleh Kadzimin, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

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

Normah Mohd Noor, PhD
Professor
Faculty of Science and Technology,
National Universiti of Malaysia
(External Examiner)

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

Date: 26 August 2008

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirements for the degree of Master of Science. The members of the Supervisory Committee are as follows :

Maheran Abdul Aziz, PhD
Associate Professor,
Faculty of Agriculture,
Universiti Putra Malaysia,
(Chairman)

Azmi Abdul Rashid, M. Phil
Faculty of Agriculture,
Universiti Putra Malaysia,
(Member)

Norihan Saleh, PhD
Associate Professor,
Faculty of Biotechnology and Biomolecular Science,
Universiti Putra Malaysia,
(Member)

_____
AINI IDERIS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date : 11 September 2008

DECLARATION

I hereby declare that the thesis is based on my original work except for equations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

_____

Aini Mohd Zainol Azlin

Date:

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES