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

AINI MOHD ZAINOL AZLIN

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By

AINI MOHD ZAINOL AZLIN

MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA

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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
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)

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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 assessment. 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 diklorofenoksii 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 terbentu. 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 heterogensus. Fasa terakhir selepas subkultur ke-5, sel ampaian embriogenik dipindahkan ke dalam media ceair tanpa hormon menunjukkan pembentukan 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 xi
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.

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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

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATIONS</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>viii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xiii</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>xv</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>xvii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xxi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xxi</td>
</tr>
<tr>
<td>LIST OF PLATES</td>
<td>xxiii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xxvi</td>
</tr>
</tbody>
</table>

## CHAPTER

1. **GENERAL INTRODUCTION**
   
   Objectives
   
   3

2. **LITERATURE REVIEW**
   
   Botany of Banana
   
   5
   
   Nutrition of banana
   
   6
   
   Tissue Culture of Banana
   
   8
   
   Plant Growth Regulators
   
   11
   
   Auxins
   
   11
   
   Cytokinins
   
   13
   
   Somatic Embryogenesis
   
   15
   
   Embryogenic Callus Induction
   
   19
   
   Cell Suspension Cultures
   
   21
   
   Cell number and Cell viability
   
   22
   
   Histological Studies
   
   24
3 INDUCTION OF EMBRYOGENIC CALLUS AND SOMATIC EMBRYO FROM IMMATURE MALE FLOWERS OF MUSA SPP.

- Introduction 26
- Materials and Methods 27
- Research Location 27
- Plant Materials 27
- Preparation of Explants and Sterilisation 27
- Preparation of Stock Solutions 31
- Preparation of Culture Media 31
- Incubation of Cultures 32
- Experimental Design and Data Analysis 32
- Effect of 2,4-D and Flower Cluster Position on Embryogenic Callus in Banana in cv. Rastali (AAB) 33
- Morphological of Embryogenic Callus in Banana cv. Rastali (AAB) 33

Results 34

- Effect of 2,4-D and Flower Cluster Position on Embryogenic Callus Formation in Banana Cultivar Rastali 34
- Morphological of Embryogenic Callus in Banana cv. Rastali (AAB) 38

Discussion 46

4 ESTABLISHMENT AND MAINTENANCE OF EMBRYOGENIC CELL SUSPENSION CULTURES 49

- Introduction 49
- Materials and Methods 50
- Results 52
- Discussion 65
## 5 EMBRYO MATURATION AND GERMINATION

### Introduction

Materials and Methods

Histological Study

Fixation

Vacuum

Dehydration

Infiltration

Embedding

Microtoming

Staining, Mounting and De-Waxing

Experimental Design and Data Analysis

### Results

### Discussions

## 6 GENERAL DISCUSSION AND CONCLUSION

### REFERENCES

### APPENDICES

### BIODATA OF STUDENT

### LIST OF PUBLICATIONS
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Effect of different concentrations of 2,4-D in M1 medium on embryogenic callus formation of banana cv. Rastali (observed after 6 months of culture)</td>
</tr>
<tr>
<td>3.2</td>
<td>Effect of different flower cluster positions on embryogenic callus formation of banana cv. Rastali on M1 medium (observed after 6 months of culture)</td>
</tr>
<tr>
<td>3.3</td>
<td>Interaction between 2,4-D concentrations and flower cluster positions on percentage of embryogenic callus formation of banana cv. Rastali on M1 medium (observed after 6 months of culture)</td>
</tr>
<tr>
<td>3.4</td>
<td>Effect of different concentrations of 2,4-D on callus characteristics of cv. Rastali by the end of six months of culture</td>
</tr>
<tr>
<td>4.1</td>
<td>Liquid media assessed for the establishment of embryogenic cell suspension cultures</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Growth of suspended cells in liquid S1 and S2 media over five subculture cycles</td>
<td>58</td>
</tr>
<tr>
<td>5.1</td>
<td>Percentage of plantlet germination of banana cv. Rastali on M4 medium containing different concentrations of BAP (mg/L)</td>
<td>87</td>
</tr>
<tr>
<td>6.1</td>
<td>A flowchart on methodology of somatic embryogenesis from immature male flowers of Musa spp. cv. Rastali</td>
<td>93</td>
</tr>
</tbody>
</table>
LIST OF PLATES

<table>
<thead>
<tr>
<th>Plate</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Banana plant sample collected from Malaysian Agriculture Research and Development Institute (MARDI). (A) Male flower inflorescence of banana cv. Rastali with rose purplish-coloured ovoid bracts; (B) Banana plants at the institute</td>
</tr>
<tr>
<td>3.2</td>
<td>Explants obtained from reproductive organs of banana plant. A) Bracts were removed exposing the inflorescence tip; B) Male flower clusters from position 1 to 10</td>
</tr>
<tr>
<td>3.3</td>
<td>Morphology of male flower cluster of cv. Rastali on M1 medium with 2,4-D. (A) Swelling and curling of male flower clusters after one month culture; (B) Enlargement of male flower clusters one to two months later</td>
</tr>
<tr>
<td>3.4</td>
<td>Response of calli on different concentrations of 2,4-D from immature male flowers of banana cv. Rastali after six months of culture. (A) Flower hand which swelled with no callus formation at 0 mg/L of 2,4-D; (B) nodular callus with phenolic secretion at the base of the explant causing browning of the callus on 1 mg/L of 2,4-D; (C) mucilagenous and yellowish nodular callus with embryogenic capacity on 2 mg/L of 2,4-D; and (D) compact, friable and nodular creamy callus with embryogenic capacity on 4 mg/L 2,4-D</td>
</tr>
<tr>
<td>3.5</td>
<td>Types of callus produced from male flowers of cv. Rastali, mostly from positions 9 and 10. (A) White hardened, stunted cauliflower-like callus; (B) callus with roots emerging directly from it; (C) vitreous and glassy-like callus, non-embryogenic; (D) non-embryogenic callus bearing greenish bodies; All callus were initiated after six months of culture</td>
</tr>
<tr>
<td>3.6</td>
<td>Stages of development of somatic embryos from embryogenic callus of cv. Rastali during one month on Dhed’a solid medium. (A) Globular and mature individual somatic embryos (arrowed) emerging from an embryogenic complex; (B) A globular-stage somatic embryo (arrowed); (C) Close up view of a globular-stage</td>
</tr>
</tbody>
</table>