

**THE DEVELOPMENT OF PLANT REGENERATION SYSTEM
FROM CALLUS OF PINEAPPLE (*Ananas comosus* L.)**

By

ANGELA EE DE SILVA

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

February 2005

*To all
whose chief end
is to
glorify God
and to
enjoy Him forever.*

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

**THE DEVELOPMENT OF PLANT REGENERATION SYSTEM
FROM CALLUS OF PINEAPPLE (*Ananas comosus* L.)**

By

ANGELA EE DE SILVA

February 2005

Chairman: Mihdzar Abdul Kadir, PhD

Faculty: Agriculture

Malaysia's production of canned pineapples has been decreasing since 1992. Two important factors that have been a hindrance to the progress of this industry are competition from other producers and the increasing demand for fresh pineapples. Current varieties need to undergo qualitative improvements. Genetic modification, breeding and selection are some crop improvement techniques that are not successful at the moment in developing varieties that can replace current world varieties. Somaclonal variation is another technique for obtaining desirable variants, which have been achieved in crops such as sugarcane, wheat and sorghum. Highly stable variants that can be transmitted to progenies, and a more controlled change of their characteristics than those of induced mutations were achieved. However, this technique requires a plant regeneration system from callus cells. These cells have a tendency to mutate, and more cells are mutated under prolonged culture and rapid proliferation, and so generate more variants for selection. Therefore, the objectives of this project are to induce calli, proliferate old calli and regenerate shoot from calli.

For calli induction, meristemic globular bodies (MGB) of Moris and Josapine, were cultured in various levels of auxin naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic (2,4-

D). The highest percentage of MGB forming calli was observed in treatment NAA 14 and 10 mg/L for Moris and Josapine respectively, at the end of 16 weeks.

For calli proliferation, 18 month-old calli were cultured in various levels of NAA, 2,4-D, b-naphthoxyacetic acid (BNOA) and p-chlorophenoxyacetic acid (4-CPA) auxins for 12 weeks, and then in various levels of casein hydrolysate (CH) and coconut water (CW) in the presence of NAA 6 mg/L for 12 weeks. Among the various levels of the four auxins, NAA 6 mg/L proliferated healthy and high mean calli fresh weight. However, NAA 6mg/l supplemented with CW and CH also gave healthy and generally higher mean calli fresh weight than NAA 6mg/L alone. NAA 6 mg/L alone was considered the most economical treatment for calli proliferation, while NAA 6mg/L supplemented with 15%v/v CW and 300mg/L CH gave significantly highest mean calli fresh weight and was considered the best treatment for rapid calli proliferation.

For shoot regeneration, 18 month-old calli were cultured in various levels of NAA, 2,4-D, BNOA and 4-CPA auxins for 12 weeks, and then in combinations of various levels of auxins (BNOA and 2,4-D) and cytokinins (Benzylaminopurine [BAP], Kinetin and Adenine) for 12 weeks. Among the various levels of the four auxins, 2,4-D at 1mg/L regenerated high number of shoots, and was considered the best treatment for high shoot regeneration from calli that were considered as high competency calli. However, regeneration response from these calli were gradually decreasing, such that treatment BNOA 6mg/L combined with BAP 1mg/L (with subculture) (that statistically gave highest number of regenerated shoots) and an extended culture period of 12 weeks (without subculture), generated mean number of shoots was considered as not satisfactory. Subsequently, the 18 month old-calli (now 27 months) were cultured in various combination levels of CH and CW for 12 weeks, but these failed to

show any regenerated shoots from calli that were now considered low competency calli. However, calli obtained from treatment NAA 6 mg/L + 300mg/l CH + 15%v/v CW (rapid calli proliferation treatment, and now 27 months old), preserved calli competency and regenerated highest mean number of shoots in treatment 10%v/v CW and 200mg/l CH, and was considered the best treatment for regeneration of shoots from low competency calli.

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

**PEMBENTUKKAN KALUS DAN REGENERASI PUCUK DARI KALUS UNTUK
NANAS (*Ananas comosus* L.)**

Oleh

ANGELA EE DE SILVA

February 2005

Pengerusi: Mihdzar Abdul Kadir, PhD

Fakulti: Pertanian

Sejak tahun 1992, prestasi pengeluaran nanas tin Malaysia semakin merosot. Dua faktor penting dan cabaran utama perkembangan industri ini adalah persaingan dari pengeluar nanas dunia dan permintaan terhadap nanas segar yang semakin meningkat. Baka yang sedia ada perlu dipertingkatkan nilai kualitatifnya. Setakat ini, modifikasi genetik, pembiakbakaan dan pemilihan adalah teknik pemajuan tanaman yang kurang berjaya dalam menghasilkan baka yang lebih baik atau setanding dengan baka dunia. Variasi somaklonal, adalah satu lagi teknik yang berupaya menghasilkan baka baru yang lebih baik, dan teknik ini telah berjaya diaplikasikan pada beberapa tanaman seperti tebu, gandum dan sorghum. Teknik ini membolehkan modifikasi genetik yang lebih terkawal berbanding dengan teknik modifikasi genetik secara langsung, serta menghasilkan baka yang stabil dan boleh mewariskan kualiti barunya kepada generasi seterusnya. Namun begitu, teknik ini memerlukan sistem regenerasi pokok melalui sel kalus. Sistem ini merangkumi tiga bahagian iaitu; pembentukan kalus, pertumbuhan kalus, dan regenerasi pucuk dari kalus. Kalus adalah fasa sel yang cenderung mengalami mutasi, dan lebih banyak kalus mengalami mutasi apabila dikultur untuk jangkamasa yang panjang dan dipercepatkan pertumbuhannya, dan seterusnya menghasilkan

variasi untuk pemilihan baka yang lebih baik. Maka, objektif projek ini adalah, pembentukkan kalus, pertumbuhan kalus dan regenerasi pucuk dari kalus.

Dalam kajian pembentukkan kalus, MGB baka Moris dan Josapine telah dikultur dalam pelbagai kepekatan auksin NAA dan 2,4-D selama 12 minggu. Peratus pembentukkan kalus yang tertinggi dari MGB telah dikenalpasti dalam rawatan NAA 14 dan 10 mg/L untuk baka Moris dan Josapine masing-masing, pada akhir minggu ke-16.

Dalam kajian pertumbuhan kalus, kalus berumur 18 bulan telah dikultur dalam pelbagai kepekatan auksin NAA, 2,4-D, BNOA dan 4-CPA selama 12 minggu, dan kemudian dikultur dalam kombinasi pelbagai kepekatan CH dan CW berserta NAA 6 mg/L untuk 12 minggu. Rawatan NAA 6mg/L telah menghasilkan pertumbuhan kalus yang sihat dan purata berat basah kalus yang tinggi berbanding di antara rawatan keempat auksin tersebut. Tetapi rawatan NAA 6mg/L berserta CW dan CH, telah menghasilkan purata berat basah kalus yang lebih tinggi. Rawatan dengan hanya NAA 6mg/L telah diterima sebagai rawatan yang berekonomi untuk penghasilan kalus, manakala rawatan NAA 6 mg/L + 15%v/v CW + 300mg/L CH telah menghasilkan purata berat basah kalus yang tertinggi dan menunjukkan perbezaan yang bererti.

Dalam kajian regenerasi pucuk dari kalus, kalus berumur 18 bulan telah dikultur dalam pelbagai kepekatan auksin NAA, 2,4-D, BNOA dan 4-CPA selama 12 minggu, dan kemudian dikultur dalam gabungan pelbagai kepekatan auksin (BNOA dan 2,4-D) dan sitokinin (BAP, Kinetin dan Adenine) selama 12 minggu. Di antara rawatan keempat-empat auksin tersebut, rawatan 2,4-D 1mg/L telah menghasilkan bilangan regenerasi pucuk yang tertinggi dari kalus, maka rawatan ini diterima sebagai rawatan yang terbaik untuk regenerasi bilangan pucuk yang tinggi dari kalus, dan kalus ini secara relatif dianggap sebagai kalus cergas.

Tetapi bilangan regenerasi pucuk dari kalus semakin berkurang dengan bertambahnya jangkamasa pengkulturannya, sehinggakan gabungan rawatan BNOA 6mg/L dengan BAP 1mg/L (walaupun bilangan regenerasi pucuknya adalah tertinggi mengikut statistik, melalui amalan subkultur), serta tambahan pengkulturan selama 12 minggu lagi (amalan tanpa subkultur), telah menghasilkan bilangan pucuk yang tidak memuaskan. Seterusnya, kalus 18 bulan tersebut (sekarang genap 27 bulan), dikultur dalam gabungan pelbagai kepekatan CH dan CW selama 12 minggu, tetapi kalus ini telah langsung hilang kecergasannya untuk menghasilkan pucuk, dan kalus ini dianggap sebagai kalus kurang cergas. Tetapi, kalus yang diperolehi dari rawatan NAA 6mg/L + 15%v/v CW + 300mg/L CH (rawatan yang telah diterima sebagai terbaik untuk penghasilan kalus yang cepat, sekarang turut genap 27 bulan), telah berjaya memelihara kecergasan kalus dan menghasilkan bilangan pucuk yang tertinggi dalam rawatan 10%v/v CW + 200mg/L CH.

ACKNOWLEDGEMENTS

The author relays her utmost gratitude to the Almighty God who has preserved her until the completion of her degree and for giving her guidance through her family, her supervisors and trusted friend.

The author expresses gratitude to her family, father (Peter De Silva), mother (Tan Ee Hva) and brothers (Joel Ee De Silva and Joash Ee De Silva), who not for them, she would not have considered her achievements to come thus far.

The author expresses gratitude to her supervisors, Dr. Mihdzar Abdul Kadir (chairman), Dr Maheran Abdul Aziz and Dr. Saleh Kadzimin (co-supervisors), for their academic guidance, advice and cooperation throughout the course of this study.

Last but not least, her gratitude to her trusted academic mentor and friend, who was determined that she finish what she started.

I certify that an Examination Committee met on 14th February 2005 to conduct the final examination of Angela Ee De Silva on her Master of Science thesis entitled “The Development of Plant Regeneration System from Callus of Pineapple (*Ananas comosus* L.)” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Halimi Mohd. Saud, PhD

Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Siti Nor Akmar Abdullah, PhD

Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Nor Aini Ab Shukor, PhD

Faculty of Forestry
Universiti Putra Malaysia
(Internal Examiner)

Ahmad Tarmizi Hashim, PhD

Principal Research Officer
Malaysian Palm Oil Board
Selangor
(External Examiner)

GULAM RUSUL RAHMAT ALI, PhD

Professor/Deputy Dean,
School of Graduate Studies
Universiti Putra Malaysia

Date: 22 APR 2005

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

Mihdzar Abdul Kadir, PhD

Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Maheran Abdul Aziz, PhD

Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Saleh Kadzimin, PhD

Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD

Professor/ Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations 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.

ANGELA EE DE SILVA

Date:

TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vi
AKNOWLEDGEMENTS	ix
APPROVAL	x
DECLARATION	xii
LIST OF TABLES	xv
LIST OF FIGURES	xvii
LIST OF PLATES	xviii
LIST OF ABBREVIATIONS	xvi
CHAPTER	
I INTRODUCTION	1
General Objectives	5
II LITERATURE REVIEW	6
Malaysian Pineapple Industry and World Scenario	6
Situation and Matters Arising	13
Low Cost Production	13
Consumer Preferences	19
Strategies and Achievements in Pineapple Crop Improvement Programmes	21
Local Varieties	22
Genetic Transfer	25
Selection	26
Breeding and Selection	27
Somaclonal Variation	30
The Callus Development and Shoot Regeneration System from 18-month Old Calli	36
Callus Cells (Calli)	37
Indirect Organogenesis	38
Plant Growth Regulators and Supplements	41
The Three Main Objectives in this Project	44
III CALLUS INDUCTION	46
Introduction	46
Materials and Methods	48
Results and Discussions	50
Conclusions	65

IV	CALLUS PROLIFERATION	66
	Introduction	66
	Materials and Methods	70
	Results and Discussions	73
	Conclusions	102
V	SHOOT REGENERATION FROM CALLUS	103
	Introduction	103
	Materials and Methods	107
	Results and Discussions	111
	Conclusions	146
VI	GENERAL CONCLUSIONS AND DISCUSSIONS	148
	Possible Future Research on Pineapple from Established Callus Development and Shoot Regeneration System	152
	BIBLIOGRAPHY	158
	APPENDICES	168
	BIODATA OF AUTHOR	171

LIST OF TABLES

Table	Page
2.1 Price of fresh fruit for canning (cent/kg)	9
3.1 Auxin types and levels for callus induction from MGB	49
3.2 Effects of auxin levels on calli induction from MGB of the Moris cultivar with time without subculturing for 16 weeks	53
3.3 Effects of auxin levels on calli induction from MGB of the Josapine cultivar with time without subculturing for 16 weeks	53
4.1 Auxin types and levels for callus proliferation in Study 1	71
4.2 CH and CW treatment combinations for callus proliferation in Study 2	72
4.3 Effects of different auxins on calli fresh weight, after 12 weeks of culture	74
4.4 Ratio of calli yield per unit cost of auxin	84
4.5 Effects of CW and CH on calli fresh weight, after 12 weeks of culture	86
4.6 Ratio of calli yield per unit cost of auxin	93
4.7 Effects of five concentration levels of CH on mean calli fresh weight, after 12 weeks of culture	94
4.8 Effects of four concentration levels of CW on mean calli fresh weight, after 12 weeks of culture	95
4.9a Effects of various CW levels without CH on mean calli fresh weight, after 12 weeks of culture	96
4.9b Effects of CH at 100 mg/L with various CW levels on mean calli fresh weight, after 12 weeks of culture	97
4.9c Effects of CH at 200 mg/L with various CW levels on mean calli fresh weight, after 12 weeks of culture	97
4.9d Effects of CH at 300 mg/L with various CW levels on mean calli fresh weight, after 12 weeks of culture	98
4.9e Effects of CH at 400 mg/L with various CW levels on mean calli fresh weight, after 12 weeks of culture	99
5.1 Auxin types and levels for shoot regeneration from calli in Study 1 of Chapter 4	108
5.2 Combined levels of auxin and cytokinin for shoot regeneration from calli in Study 3	109

5.3	CH and CW treatment combinations for shoot regeneration from calli in Study 4	110
5.4	Effects of different auxins on mean number of shoots regenerated from calli, after 12 weeks of culture	112
5.5	Effects of auxin and cytokinin combinations on mean number of shoots regenerated from calli, after 12 weeks of culture	117
5.6	Effects of auxin and cytokinin combinations on mean number of shoots regenerated from calli without subculture, after 12 weeks of culture	130
5.7	Effects of CH and CW on mean number of shoots regenerated from calli, after 12 weeks of culture	134

LIST OF FIGURES

Figure		Page
2.1	Malaysian production and export of canned pineapple products, 1990-2002 (Malaysian Agricultural Information and Index, 2003/2004)	8
2.2	World canned pineapple export by major countries, 1990-2000 (Malaysian Agricultural Information and Index, 2003/2004)	11
2.3	Major world market for Malaysian canned pineapple, 1990-2001 (Malaysian Agricultural Information and Index, 2003/2004)	12
2.4	Indirect organogenesis developmental sequence (Hicks, 1980)	40
2.5	Model system for organogenesis (Christianson and Warwick, 1985)	40
4.1	Effects of various auxins on calli fresh weight, after 12 weeks of culture	78
4.2	Effects of CH and CW on calli fresh weight	100
5.1	Effects of different auxins and their levels on shoot regeneration from calli, after 12 weeks of culture	113
6.1	Static conceptual model for plant regeneration system from callus of pineapple (<i>Ananas comosus</i> L.) cv. Moris	151

LIST OF PLATES

Plate		Page
3.1	Calli induction from Moris MGB under different NAA levels, at the 8 th week without subculture	54
3.2	Calli induction from Moris MGB under different NAA levels, at the 12 th week without subculture	54
3.3	Calli induction from Moris MGB under different NAA levels, at the 16 th week without subculture	56
3.4	Calli induction from Moris MGB under different NAA levels, after the 16 th week without subculture	56
3.5	Control for Moris MGB, at the 16 th week without subculture	57
3.6	Calli induction from Josapine MGB under different NAA levels, at the 8 th week without subculture	59
3.7	Calli induction from Josapine MGB under different NAA levels, at the 12 th week without subculture	59
3.8	Calli induction from Josapine MGB under different NAA levels, at the 16 th week without subculture	61
3.9	Calli induction from Josapine MGB under different NAA levels, after the 16 th week without subculture	61
3.10	Control for Josapine MGB, at the 16 th week without subculture	62
4.11	Calli proliferation under different NAA levels in Study 1, after 12 weeks of culture	76
4.12	Calli proliferation under different 2,4-D levels in Study 1, after 12 weeks of culture	79
4.13	Calli proliferation under different BNOA levels in Study 1, after 12 weeks of culture	80
4.14	Calli proliferation under different 2,4-D levels in Study 1, after 12 weeks of culture	82
4.15	Control in Study 1, after 12 weeks of culture	83
4.21	Calli proliferation under CH at 0 mg/L combined with different CW levels in Study 2, after 12 weeks of culture	87

4.22	Calli proliferation under CH at 100 mg/L combined with different CW levels in Study 2, after 12 weeks of culture	88
4.23	Calli proliferation under CH at 200 mg/L combined with different CW levels in Study 2, after 12 weeks of culture	88
4.24	Calli proliferation under CH at 300 mg/L combined with different CW levels in Study 2, after 12 weeks of culture	90
4.25	Calli proliferation under CH at 400 mg/L combined with different CW levels in Study 2, after 12 weeks of culture	90
4.26	Control in Study 2, after 12 weeks of culture	91
5.11	Shoot regeneration from calli under BNOA at 2 mg/L combined with three different cytokinins at three levels each in Study 3, after 12 weeks with subculture (first period)	119
5.12	Shoot regeneration from calli under BNOA at 6 mg/L combined with three different cytokinins at three levels each in Study 3, after 12 weeks with subculture (first period)	120
5.13	Shoot regeneration from calli under BNOA at 8 mg/L combined with three different cytokinins at three levels each in Study 3, after 12 weeks with subculture (first period)	121
5.14	Shoot regeneration from calli under 2,4-D at 1 mg/L combined with three different cytokinins at three levels each in Study 3, after 12 weeks with subculture (first period)	123
5.15	Shoot regeneration from calli under 2,4-D at 4 mg/L combined with three different cytokinins at three levels each in Study 3, after 12 weeks with subculture (first period)	124
5.16	Shoot regeneration from calli under 2,4-D at 6 mg/L combined with three different cytokinins at three levels each in Study 3, after 12 weeks with subculture (first period)	125
5.17	Shoot regeneration from calli under BNOA and 2,4-D controls and general control in Study 3, after 12 weeks with subculture (first period)	127
5.18	Shoot regeneration from calli under BNOA and 2,4-D controls and general control in Study 3, after 12 weeks without subculture (second period)	128
5.21	Shoot regeneration from calli under CH at 0 mg/L combined with different levels in Study 4, after 12 weeks of culture (second part)	136
5.22	Shoot regeneration from calli under CH at 100 mg/L combined with different levels in Study 4, after 12 weeks of culture (second part)	136

5.23	Shoot regeneration from calli under CH at 200 mg/L combined with different levels in Study 4, after 12 weeks of culture (second part)	138
5.24	Shoot regeneration from calli under CH at 300 mg/L combined with different levels in Study 4, after 12 weeks of culture (second part)	138
5.25	Shoot regeneration from calli under CH at 400 mg/L combined with different levels in Study 4, after 12 weeks of culture (second part)	140
5.26	Shoot regeneration from calli under C2, C2, C3 and C5 in Study 4, after 12 weeks of culture (second part)	140
5.27	Control in Study 4, after 12 weeks of culture (second part)	141

LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
4-CPA	p-chlorophenoxyacetic acid
ADE	Adenine
BAP	6-benzylaminopurine
BNOA	b-naphtoxyacetic acid
CH	casein hydrolysate
CW	coconut water
MS	Murashige and Skoog basic culture media
NAA	naphthaleneacetic acid
MGB	Meristemic globular bodies