IN VITRO PROPAGATION AND MUTATION INDUCTION OF *DENDRANTHEMA GRANDIFLORA* TZVELEV

By

MI CHAN MON

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

April 2006

Dedicated to:
My father U Kyan Yit
My mother Daw Khin Sein

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

IN VITRO PROPAGATION AND MUTATION INDUCTION OF _DENDRANTHEMA GRANDIFLORA_ TZVELEV

By

MI CHAN MON

April 2006

Chairrman: Associate Professor Saleh Bin Kadzimin, PhD

Faculty: Agriculture

The genus *Dendranthema* (Family: Asteraceae) is a popular cut flower or pot plant species of high economic value cultivated around the world. The genus has more than 100 species of annuals and perennial herbs and shrubs used as floral crops as well as tea and source of other products such as pyrethrum. *In vitro* propagation using meristems, shoot tips etc. have been successfully applied as a means of large-scale production system for disease-free plants.

The present study is carried out in two parts: *in vitro* propagation and mutation induction with the objectives of developing a protocol for an *in vitro* method of propagation using ray florets and creation of variations in *Dendranthema grandiflora* Tzvelev by combining the techniques of *in vitro* culture and radiation-induced mutagenesis.

In developing protocol for rapid propagation, *in vitro* culture was established by using ray florets on two types of basal media, such as Murashige and Skoog (MS) and Gamborg (B_5) media, containing various levels of 6-benzylaminopurine (BAP) (0, 0.5, 1.0 and 2.0 mg/L) and α–naphthaleneacetic acid (NAA) (0, 0.2, 0.5, 1.0 and 2.0 mg/L). In this study the highest percentage of callus formation was observed after 8 weeks on MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA. However, the highest number of shoot multiplication was obtained from MS basal medium containing 2.0 mg/L BAP and 1.0 mg/L NAA. No growth responses were observed on MS and B_5 basal media with BAP alone, whereas some roots developed on NAA alone in both types of basal media. After 10 weeks of culture many explants turned brown and died in the B_5 basal medium.

The highest callus proliferation in terms of fresh and dry weight as established on MS basal media containing 2.0 mg/L BAP and 1.0 mg/L NAA followed by treatment with 2.0 mg/L BAP with 1.0 and 2.0 mg/L NAA after 20 weeks of culture. The highest number of adventitious shoot formation (6.0 shoots per explant) was observed in MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA. There were significant interactions between both growth regulators on the number of shoot per explant and height of shoots produced.

In the radiation-induced mutagenesis study, irradiation treatments was performed on callus derived from ray florets, and on fresh ray florets using gamma rays from

^{60}Co source at levels of 0 Gy, 10 Gy, 20 Gy, 30 Gy, 40 Gy and 50 Gy at a dose rate of 1.858 Gy/sec. Radiosensitivity test recorded a 100 % survival rate of callus on control treatment and subsequent decrease on treatments at higher doses. The highest growth of callus was observed in control treatment and subsequently growth rate decreased correspond to increasing doses. Similarly, irradiated ray florets recorded a 100% survival rate in the control treatment. Treatments at 10, 20, 30, 40 and 50 Gy gave survival rates of 83.3%, 73.3%, 26.6%, 23.3% and 6.6% respectively.

The study concluded that the optimum dose for ray floret on growth rate of callus and survival were between 18.8 - 28.4 Gy. The optimum dose for irradiated callus based on growth rate and survival were between 26.9 - 36.2 Gy. The results from the experiments indicated that mutation induction can be performed on both ray florets and callus at LD_{50} 18.8 - 28.4 Gy and 26.9 - 36.2 Gy respectively. The formation of shoots was observed on control (0 Gy) and 10 Gy treatments. The mean number of adventitious shoots from non-irradiated samples (control) was 2.72 ± 0.09 compared to 2.5 ± 0.18 for treatment at 10 Gy. No growth responses were observed from ray florets culture at all levels of treatment. Therefore, the development of new variety through mutation induction of *Dendranthema*, it is recommended to irradiate the explants at 10 Gy or lower.

Amplified Fragment Length Polymorphism (AFLP) technique was carried out to detect the variation on genomic DNA of callus samples irradiated at different doses. The highest numbers of polymorphic bands were observed with primer combinations E-AGC + M-CAG at 20 Gy and E-AGG + M-CAT at 10 Gy. The smallest number of polymorphic bands was found with primer combination E-AAG + M-CTC at 30 and 40 Gy. Primer combination E-ACG + M-CAA produced the most number of polymorphic bands (80) when compared to other primer combinations which were used in the experiment. There were no definite correlation between the different levels of irradiation to the number of polymorphic bands and unique polymorphic bands detected. Higher numbers of polymorphic bands and unique polymorphic bands were detected by the various primer combinations at doses 10 and 20 Gy.

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

PEMBIAKAN *IN VITRO* DAN ARUHAN MUTASI *DENDRANTHEMA GRANDIFLORA* TZVELEV

Oleh

MI CHAN MON

April 2006

Pengerusi: Profesor Madya Saleh Bin Kadzimin, PhD

Fakulti: Pertanian

Chrysanthemum (Keluarga : Asteraceae) adalah satu genus bunga keratan dan pasuan. Genus ini mempunyai lebih daripada 100 spesis tumbuhan tahunan dan herba saka yang digunakan sebagai tanaman hiasan dan teh, dan sebagai sumber pelbagai produk seperti pyrethrum. Pembiakan *in vitro* dengan menggunakan meristem, mercu pucuk dan lain-lain telah berjaya digunakan sebagai satu sistem pengeluaran anak benih bebas penyakit secara besar-besaran.

Kajian ini telah dijalankan dalam dua bahagian : pembiakan *in vitro* dan aruhan mutasi dengan tujuan untuk menjana satu protokol bagi pembiakan tanaman yang cepat dan untuk penciptaan variti yang baru dan lebih baik melalui gabungan teknik kultur *in vitro* dan mutagenesis aruhan radiasi.

Dalam menjana protokol bagi pembiakan cepat, kultur *in vitro* telah dilakukan dengan menggunakan *ray floret* di atas dua jenis medium asas, Murashige dan Skoog (MS) dan Gamborg (B_5) yang mengandungi berbagai paras benzil amino purina (BAP) (0, 0.5, 1.0, dan 2.0 mg/L) dan asid naptelina asetik (NAA) (0, 0.2, 0.5, 1.0, dan 2.0 mg/L). Dalam kajian ini, peratusan yang tertinggi dalam pembentukan kalus yang diperhatikan selepas 8 minggu ialah pada rawatan MS yang diperkaya dengan 2.0 mg/L BAP dan 1.0 mg/L NAA. Walau bagaimanapon, penggandaan bilangan pucuk yang bercambah didapati pada medium asas MS yang mengandungi 2.0 mg/L BAP dan 1.0 mg/L NAA. Tiada respon yang direkodkan pada rawatan MS dan B_5 yang diperkaya dengan BAP sahaja, manakala sedikit akar bercambah pada kedua-dua medium asas dengan tambahan NAA sahaja. Selepas 10 minggu dalam kultur, eksplan dalam medium asas B_5 menjadi coklat dan mati.

Proliferasi kalus yang tertinggi dari segi berat segar dan kering telah diperolehi pada medium asas MS yang mengandungi 2.0 mg/L BAP dan 1.0 mg/L NAA diikuti dengan rawatan 2.0 mg/L BAP dengan 1.0 dan 2.0 mg/L NAA selepas 20 minggu dikultur. Bilangan pucuk adventitus yang tertinggi (6.0 pucuk bagi satu eksplan) telah diperolehi dari rawatan MS yang ditambah dengan 2.0 mg/L BAP dan 1.0 mg/L NAA. Interaksi yang ketara berlaku diantara kedua-dua hormon dari aspek bilangan pucuk bagi setiap eksplan dan ketinggian pucuk yang dikeluarkan.

Dalam kajian mutagenesis melalui radiasi, aruhan telah dilakukan keatas kalus yang diperolehi daripada kajian di atas dan juga keatas *ray floret* segar dengan menggunakan sinaran gamma dari sumber ^{60}Co pada paras 0 Gy, 10 Gy, 20 Gy, 30 Gy, 40 Gy dan 50 Gy pada kadar dos 1.858 Gy/sec. Ujian radiosensitiviti memberikan 100% hidup pada kalus kawalan dan peratusan menurun dengan peningkatan dos radiasi. Pertumbuhan kalus yang tertinggi didapati pada rawatan kawalan dan kadar pertumbuhan didapati menurun dengan peningkatan dos. *Ray floret* dalam rawatan kawalan yang diradiasi memberi peratusan hidup 100%. Rawatan pada 10, 20, 30, 40 dan 50 Gy masing-masing memberi peratusan hidup 83.3, 73.3, 26.6, 23.3 dan 6.6.

Kajian merumuskan bahawa dos optimum bagi kadar pertumbuhan kalus dari ray floret dan peratusan hidup adaah diantara 18.8-28.4 Gy. Dos optimum bagi kaus yang diradiasi berdasarkan kadar pertumbuhan dan hidup adaah diantara 26.9-36.2 Gy. Pembentukan pucuk berlaku keatas rawatan kawalan (0 Gy) dan 10 Gy. Bilangan purata pucuk adventitus dari rawatan kawalan ialah 2.72 ± 0.09 berbanding 2.5 ± 0.18 dari rawatan 10 Gy. Tiada pertumbuhan berlaku pada kultur *ray floret* di semua paras rawatan.

Teknik Amplified Fragment Length Polymorphism (AFLP) telah dilakukan bagi menentukan variasi keatas genom DNA kalus yang diradiasi pada berbagai paras. Bilangan jalur polimorfik tertinggi didapati pada kombinasi primer E-AGC + M-CAG pada rawatan 20 Gy dan E-AGG + M-CAT pada rawatan 10 Gy.

Bilangan jalur polimorfik terendah didapati pada kombinasi E-AAG + M-CTC pada rawatan 30 dan 40 Gy. Kombinasi primer yang terbaik dalam kajian ini ialah E-ACG + M-CAA yang menghasiklkan jalur polimorfik yang tertinggi (80) diantara dos yang dikaji.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and thanks to Assoc. Prof. Dr. Saleh Kadzimin, Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia (UPM) for his valuable guidance and supervision.

I would also like to express my gratitude to members of my Supervisory Committee, Assoc. Prof. Dr. Maheran Abdul Aziz, Faculty of Agriculture, Universiti Putra Malaysia, and Dr. Mohd. Nazir Basiran, Research Officer, Agrotechnology and Bioscience Division, Malaysian Institute for Nuclear Technology Research (MINT) for their encouragement and guidance in carrying out this research.

I am grateful to Managing Director, Myanma Perennial Crops Enterprise, Ministry of Agriculture and Irrigation, Union of Myanmar for official leave given to me and to SEAMEO SEARCA for the scholarships granted to undertake a postgraduate program at the Universiti Putra Malaysia.

I would also like to thank Mr. Abdul Rahman Sidam and Miss Suzieyani Abd. Latiff of the Tissue Culture Laboratory, Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia for their kind assistance in tissue culture work during the course of the study.

I also would like to extend my thanks to Miss Affrida Abu Hassan, Mr. Shuhaimi Shamsudin, Miss Putri Noor Faizah bt. Megat Mohd. Tahir and staff in Agrotechnology and Bioscience Division, Malaysian Institute for Nuclear Technology Research (MINT) for their kindness and help in molecular work.

I certify that an Examination Committee has met on 21 April 2006 to conduct the final examination of Mi Chan Mon on her Master of Agricultural Science thesis entitled "*In Vitro* Propagation and Mutation Induction of *Dendranthema grandiflora* Tzvelev" 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:

Mihdzar Bin Abdul Kadir, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Faridah Quamaruz Zaman, PhD
Institute of Bioscience
Universiti Putra Malaysia
(Intrernal Examiner)

Rusea Go, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Intrernal Examiner)

Vilasini Pillai, PhD
Biotechnology Research Centre
Malaysian Agriculture Research and Development Institute (MARDI)
(External Examiner)

_____

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

Date:

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 Agricultural Science. The members of the Supervisory Committee are as follows:

Saleh Bin Kadzimin, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Maheran Abd. Aziz, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Mohd. Nazir Bin Basiran, PhD
Research Officer
Agrothechnology and Bioscience Division
Malaysian Institute for Nuclear Technology Research (MINT)
(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.

_____

MI CHAN MON

Date:

TABLE OF CONTENTS

		Page
DEDICATION		ii
ABSTRACT		iii
ABSTRAK		vii
ACKNOWLEDGEMENTS		xi
APPROVAL		xiii
DECLARATION		xv
LIST OF TABLES		xix
LIST OF FIGURES		xxi
LIST OF PLATES		xxii
LIST OF ABBREVIATIONS		xxiv
CHAPTER		
I	**INTRODUCTION**	1
II	**LITERATURE REVIEW**	5
	Part I: *In vitro* propagation	5
	Dendranthema	5
	Dendranthema flower	6
	Dendranthema cultivars	6
	Propagation of *Dendranthema*	8
	Dendranthema tissue culture	9
	Establishment of aseptic cultures and surface sterilization	11
	Basal media	12
	Plant growth regulators	12
	Culture environment	14
	Part II: *In vitro* mutation induction	15
	Mutation	15
	Mutation breeding	16
	Induction of mutation	17
	Physical mutagens	17
	Chemical mutagens	19
	Irradiation dose	21
	Mutation induction in *Dendranthema*	24
	Molecular markers	25
	Amplified fragment length polymorphism	26

AFLP adapters and primers 27

III **MATERIALS AND METHODS** 29
Part I: *In vitro* propagation 29
Study location 29
Plant materials 29
Surface sterilization techniques 32
Culture media 33
Placement of explants 33
Incubation condition 34
Rooting and acclimatization 35
Subculture and data collection 37
Experimental design and data analysis 38

Part II: *In vitro* mutation induction 39
Irradiation 39
Genomic DNA isolation 42
Quantification of DNA 42
Quality determination of isolated DNA 43
AFLP procedure 43
Restriction endonuclease digestion of the DNA and ligation of the adapters 44
Pre-selective amplification of target sequences 44
Selective amplification 45
Gel electrophoresis 46

IV **RESULTS AND DISCUSSION** 49
Part I: *In vitro* propagation 49
Effects of NAA and BAP concentrations in MS medium on growth of callus 49
Effects of NAA and BAP concentrations in MS medium on adventitious shoot formation 55
Effects of NAA and BAP concentrations in B5 medium on the mean % of callus formation 61

Part II: *In vitro* mutation induction 64
Determination of LD_{50} and radiosensitivity test of *Dendranthema* 64
Effect of gamma radiation on callus formation 68
Effect of gamma radiation on adventitious shoot formation 74
DNA extraction 77
Quantification of DNA 79
Effects of gamma irradiation on genetic variability of *Dendranthema* 81

V **OVERALL CONCLUSION** 90

BIBLIOGRAPHY 94
APPENDICES 106
BIODATA OF THE AUTHOR 122