UNIVERSITI PUTRA MALAYSIA

MICROPROPAGATION OF Cyclanthus bipartitus POITEAU EX A. RICHARD AND ASSESSMENT OF ITS GENETIC VARIABILITY

NUR FAUZANA MOHD KASIM

FP 2015 20

MICROPROPAGATION OF *Cyclanthus bipartitus* POITEAU EX A. RICHARD AND ASSESSMENT OF ITS GENETIC VARIABILITY

© COPYRIGHT UPM

By

NUR FAUZANA MOHD KASIM

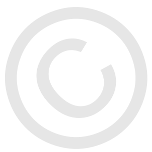
Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

June 2015

COPYRIGHT

All material contained within the thesis, including without limitation text, logos, icons, photographs and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright © Universiti Putra Malaysia



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

MICROPROPAGATION OF *Cyclanthus bipartitus* POITEAU EX A. RICHARD AND ASSESSMENT OF ITS GENETIC VARIABILITY

By

NUR FAUZANA MOHD KASIM

June 2015

Chair: Associate Professor Yahya Awang, PhD

Faculty: Agriculture

Cyclanthus bipartitus is characterized as a rhizomatous, and terrestrial shrub with divided leaves and the plant can grow up to 460 cm in height. The plant can be propagated using seeds but seed set is very low, as the pollination process for this plant requires a specific pollinator. Even though this plant can be propagated by cutting and division, micropropagation seems to be the best method for commercial purposes as mass multiplication can be done at a faster rate compared to the conventional method. Thus, this study was carried out to develop an efficient protocol for micropropagation of *C. bipartitus*. More specifically, the objectives of the study were to determine suitable source of explants and to evaluate the effects of varying concentration of 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA) (as plant growth regulators), and concentration of sucrose (as a carbon source) for micropropagation of *C. bipatitus*. The study also aimed at determining the genetic variability of regenerated plants following micropropagation protocols adopted.

Type of explants used in this study was first determined by excising 1 cm of explants from petiole and basal stem and 1 cm^2 explants from distal and basal lamina, and culturing them in sterilized MS medium for containing BAP and NAA 10 weeks. Compared to other explants, distal lamina and basal lamina generated equally high number of shoots (with a mean of 49 shoots/explant). The shoot was also found to be significantly longer than those generated by other explant. Due to its superiority, explants from lamina part of the plant were used in the following experiments.

An experiment to determine a suitable level of sucrose added to MS medium supplemented with BAP and NAA concentration was also performed. Explants were cultured in sterilized MS medium containing 1.0 mg/L of BAP and 0.5 mg/L NAA with five different concentrations of sucrose: 20 g/L, 25 g/L, 30 g/L, 35 g/L and 40 g/L. The highest number of shoots/explant (54.88), tallest shoots (3.80 cm), highest number of roots (3.12) and longest root (0.78 cm) were

© COPYRIGHT UPM

obtained from explants cultured in MS media containing a combination of 1.0 mg/L of BAP, 0.5 mg/L of NAA and 30 g/L of sucrose after 10 weeks of culture. Genetic variability of regenerated plants at the DNA level was also analyzed by using random amplified polymorphic DNA (RAPD) molecular markers. Ten arbitrary primers were screened for RAPD use. Primers that produced score-able bands were chosen to analyse polymorphism in regenerated plant DNA. By PCR amplification, 26 score-able bands were amplified from 5 primers out of 10 arbitrary primers screened, where 18 of them were polymorphic and 8 were monomorphic, which gave 69.2% of polymorphism frequency. In conclusion, explants from lamina part of the plant were used for propagating *Cyclanthus bipartitus in vitro* in MS medium supplemented with BAP and NAA concentrations of 1.0 mg/L and 0.5 mg/L, respectively. Regenerated plants from the micropropagation were shown to have 69.2% of polymorphism frequency, which indicates the occurrence of genetic variation subcultured plants

© COPYRIGHT UPM

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

PEMBIAKAN MIKRO DAN PENILAIAN VARIASI GENETIK BAGI POKOK *Cyclanthus bipartitus* POITEAU EX A. RICHARD

Oleh

NUR FAUZANA MOHD KASIM

Jun 2015

Pengerusi: Professor Madya Yahya Awang, PhD

Fakulti: Pertanian

Cyclanthus bipartitus dicirikan sebagai rhizomatous dan renek dengan daun yang terbelah di bahagian tengah. Tumbuhan ini juga boleh mencapai ketinggian sehingga 490 cm. Walaupun tumbuhan ini boleh dibiakkan melalui kaedah keratan, kaedah pembiakan *in vitro* adalah kaedah terbaik untuk tujuan komersial kerana ia mampu menghasilkan hasil yang banyak tetapi pada kadar tempoh yang singkat berbanding kaedah konvensional. Selain itu, proses pendebungaan bagi tumbuhan ini memerlukan pendebunga tertentu. Oleh itu, kajian ini dijalankan untuk membangunkan protokol berkesan untuk kaedah pembiakan *in vitro* bagi pokok *C. bipartitus.* Lebih khusus, objektif kajian ini adalah untuk menentukan sumber eksplan yang sesuai daripada tumbuhan ini untuk menilai kesan yang berbeza-beza menggunakan kepekatan 6-benzylaminopurine (BAP) dan 1-naphthaleneacetic acid (NAA) (sebagai hormon pertumbuhan tumbuhan), dan kepekatan sukrosa (sebagai sumber karbon) untuk pembiakan *C. bipatitus in vitro*. Kajian ini juga bertujuan untuk menentukan variasi genetik tumbuhan yang terhasil susulan daripada pembiakan *in vitro* yang dilakukan.

Jenis eksplan yang digunakan dalam kajian ini pertama kalinya ditentukan dengan memotong eksplan berukuran 1 cm untuk eksplan dari bahagian tangkai daun dan dasar pokok 1 cm^2 daripada bahagian daun. Pengkulturan kesemua eksplan dilakukan di dalam media yang telah disterilkan selama 10 minggu. Eksplan dari bahagian hujung dan pangkal daun menghasilkan jumlah pucuk yang sama (dengan min 49 pucuk / eksplan). Eksplan bahagian hujung daun juga didapati menhasilkan daun yang lebih panjang berbanding eksplan yang lain. Justeru itu, eksplan daripada bahagian daun telah digunakan dalam eksperimen berikutnya.

Penentuan kadar sukrosa yang sesuai untuk ditambah kepada media MS yang dibekalkan dengan BAP dan NAA juga telah dilakukan. Eksplan dikulturkan dalam MS media yang mengandungi 1.0 mg/L daripada BAP dan 0.5 mg/L NAA dengan lima kepekatan sukrosa yang berbeza, iaitu 20 g/L, 25 g/L, 30 g/L, 35 g/L dan 40 g/L. Bilangan tertinggi pucuk per eksplan (54.88), pucuk

© COPYRIGHT UPM

tertinggi (3.80 cm), jumlah tertinggi akar (3.12) dan akar terpanjang (0.78 cm) telah diperolehi daripada eksplan yang dikulturkan di dalam MS media yang mengandungi gabungan 1.0 mg/L BAP, 0.5 mg/L NAA dan 30 g/L sukrosa selepas 10 minggu.

Variasi somaklonal di dalam tumbuhan yang terhasil juga dianalisis menggunakan penanda molekul RAPD (Random Amplified Polymorphic DNA) dalam menentukan variability genetic susulan daripada pembiakan *in vitro* yang dilakukan. Sepuluh primer telah dipilih secara rawak dan telah disaring untuk penggunaan analisis RAPD. Primer yang menghasilkan ban yang boleh diskor telah dipilih untuk menganalisis tahap polemik DNA. Dengan menggunakan PCR (polymerized chain reaction), 26 ban yang boleh diskor telah diamplifikasikan dari 5 primer yang terpilih, yang mana 18 daripada mereka adalah polimorfik dan 8 pula adalah monomorfik, justeru memberikan 69.2% kekerapan polymorfisma. Kesimpulannya, eksplan daripada bahagian daun tumbuhan yang digunakan untuk pembiakan *Cyclanthus bipartitus* secara *in vitro* dalam media MS yang dibekalkan dengan BAP dan NAA masing-masing berkepekatan 1.0 mg/L dan 0.5 mg/L. Tumbuhan dijana dari mikropropagasi yang telah ditunjukkan mempunyai 69.2% daripada frekuensi polymorfisma, di mana nilai ini menunjukkan bahawa terdapatnya variasi di dalam genetic kesemua pokok yang telah digunakan.

© COPYRIGHT UPM

ACKNOWLEDGEMENTS

I would like to express my gratitude to the following people who have helped me a lot in order for me to complete my master study. My Supervisor, Associate Professor Dr. Yahya Awang for his continuous support and kindness, critisism and encouragement, patience and guidance during my study and Associate Professor Dr. Saleh Kadzimin for his support and fatherly guidance and advices with my project.

I also would like to thank all staff in the Department of Crop Science, UPM, especially to Mr. Abdul Aziz Ismail, Mr. Mazlan Bangi, Mr. Mohd Helmy, Mrs. Mazlina Alias, and Mrs. Salmah Kassim. I am also indebted to my dear friends who were always there for me in times of hardship during my study, Miss Nur Izzah, Mrs. Nazihah, Mrs. Noor Shahida, Mrs. Nurul Hawa, Miss Asmaa Nafeesa, Mrs. Nur Adilah, Miss Sakina, Miss Aishah and Miss Minny. Million thanks are also due to the Malaysian government for the scholarship given to me throughout my study period provided to Universiti Putra Malaysia.

Finally, I'm fully indebted to my family, especially my parents and brothers for their prayers and support; also my aunties and uncles who have been showering me with tender, love and care. Above all, ALLAH SWT the Most Gracious and Most Merciful for He has given me the will and strength to continue doing work, complete my project and made things go well.

© COPYRIGHT UPM

© COPYRIGHT UPM

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:

Yahya Awang, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

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

BUJANG KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

© COPYRIGHT UPM

Declaration by graduate student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: ________________________ Date: __________________

Name and Matric No.: NUR FAUZANA MOHD KASIM (GS29145)

© COPYRIGHT UPM

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: ______________________________
Name of Chairman of Supervisory Committee: Assoc. Prof. Yahya Awang

Signature: ______________________________
Name of Member of Supervisory Committee: Assoc. Prof. Saleh Kadzimin

© COPYRIGHT UPM

TABLE OF CONTENTS

		Page
ABSTRACT		i
ABSTRAK		iii
ACKNOWLEDGEMENTS		v
APPROVAL		vi
DECLARATION		vii
LIST OF TABLES		xi
LIST OF FIGURES		xiv
LIST OF ABBREVIATIONS		xvi

CHAPTER

1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	4
	2.1 *Cyclanthus bipartitus*	4
	2.2 Propagation of *Cyclanthus bipartitus*	6
	2.3 Tissue culture	6
	2.4 Type of explants used in tissue culture	7
	2.5 Culture medium	9
	2.6 Plant growth regulators	9
	2.6.1 Cytokinin	9
	2.6.2 Auxin	10
	2.7 Carbon source in tissue culture	12
	2.8 Somaclonal variations	12
	2.9 Random amplified polymorphic DNA (RAPD)	14
3	**EVALUATION OF EXPLANT TYPES FOR MICROPROPAGATION OF *CYCLANTHUS BIPARTITUS***	16
	3.1 Introduction	16
	3.2 Materials and methods	16
	3.2.1 Plant materials	16
	3.2.2 Preparation of MS medium	17
	3.2.3 Preparation of PGR	17
	3.2.4 Treatment	17
	3.2.5 Data collection	19
	3.2.5.1 Number of shoots	19
	3.2.5.2 Number of roots	19
	3.2.5.3 Shoot height	19
	3.2.5.4 Root length	19
	3.2.6 Experimental design and statistical analysis	19
	3.3 Results and discussion	19
	3.4 Conclusion	23

© COPYRIGHT UPM

4	**EFFECTS OF DIFFERENT CONCENTRATIONS OF BAP AND NAA ON SHOOT AND ROOT FORMATION**	24
	4.1 Introduction	24
	4.2 Materials and methods	24
	4.3 Experiment I	24
	4.3.1 Materials and methods	24
	4.3.2 Results and discussion	25
	4.4 Experiment II	27
	4.4.1 Materials and methods	27
	4.4.2 Results and discussion	28
	4.5 Conclusion	30
5	**EFFECTS OF DIFFERENT CONCENTRATIONS OF SUCROSE ON SHOOT AND ROOT FORMATION**	31
	5.1 Introduction	31
	5.2 Experiment I	31
	5.2.1 Materials and methods	31
	5.2.2 Results and discussion	32
	5.3 Experiment II	33
	5.3.1 Materials and methods	33
	5.3.2 Results and discussion	34
	5.4 Conclusion	36
6	**EVALUATION OF GENETIC VARIABILITY OF REGENERATED PLANTS**	37
	6.1 Introduction	37
	6.2 Materials and methods	37
	6.2.1 Plant materials	37
	6.2.2 Plant materials for DNA extraction	38
	6.2.3 Preparation of template DNA	38
	6.2.4 Quantification of extracted DNA	39
	6.2.5 Screening of RAPD primers	39
	6.2.6 DNA amplification	40
	6.2.7 DNA electrophoresis	40
	6.2.8 Data analysis	41
	6.3 Results and discussion	41
	6.4 Conclusion	53
7	**SUMMARY AND GENERAL CONCLUSION**	54
REFERENCES		56
APPENDICES		64
BIODATA OF STUDENT		70

© COPYRIGHT UPM

LIST OF TABLES

Table		Page
4.1	Treatments used in Experiment I	25
4.2	Observation of explants grown on medium with different combinations of concentrations of BAP and NAA	26
4.3	Number of shoots, shoot height, number of roots and root length obtained when explants were cultured in MS medium supplemented with different combinations of BAP and NAA concentrations	27
4.4	Treatment combinations used in Experiment II	28
4.5	Number of shoots, shoot height, number of roots and root length produced by explants after 10 weeks of culture in MS medium supplemented with different combinations of BAP and NAA concentrations	29
5.1	Number of shoots, shoot length, number of roots and root length	32
5.2	Combinations of BAP, NAA and sucrose added to culture medium	34
5.3	Numbers of shoots, shoot length, number of shoots and root length affected by different combinations of concentrations of BAP, NAA and sucrose	35
6.1	List of 10 oligo primers used for RAPD primer screening	40
6.2	The purity DNA ratio and DNA concentration obtained from extractions of DNA from mother plant and offspring.	42
6.3	Primers used in RAPD analysis of genetic stability in *Cyclanthus bipartitus* regenerated plants and number of score able bands produced by each primer	43
6.4	Summary of amplification products produced by primer OPE-01	44
6.5	Summary of amplification products produced by primer OPE-05	45
6.6	Summary of amplification products produced by primer OPE-08	46
6.7	Summary of amplification products produced by primer OPE-13	47
6.8	Summary of amplification products produced by primer OPE-15	48
6.9	Summary of amplification products produced by primer OPE-19	49
6.10	Summary of similarity matrix using Jaccard's coefficient	50

© COPYRIGHT UPM

6.11	Similarity percentage between parental plant and somaclones	51

© COPYRIGHT UPM

LIST OF FIGURES

Figures		Page
2.1	Accurate illustration of *Cyclanthus bipartitus* plant showing divided adult leaves (AL) and undivided young leaf (George *et al.*, 1982)	5
2.2	Structural formula of plant growth regulators	11
3.1	Illustrated image of a stock plants and each number indicates part of plant that was used for culturing. Part 1 is the distal lamina; part 2 is the basal lamina; part 3 is the petiole and part 4 is the basal stem	18
3.2	Effects of source of explants on number of shoots of micropropagated *C. bipartitus*	20
3.3	Effects of source of explants on length of shoots of micropropagated *C. bipartitus*	21
3.4	Effects of source of explants on number of roots of micropropagated *C. bipartitus*	21
3.5	Effects of source of explants on length of roots of micropropagated *C. bipartitus*	22
6.1	Gel electrophoresis image of OPE-01 primer. The first column was loaded with DNA ladder, which can be broken down into bands of sizes 10000 base pairs (bp), 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1000 bp, 750 bp, 500 bp and 250 bp, respectively, reading from up to bottom.	44
6.2	Gel electrophoresis image of OPE-05 primer. The first column was loaded with DNA ladder, which can be broken down into bands of sizes 10000 base pairs (bp), 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1000 bp, 750 bp, 500 bp and 250 bp, respectively, reading from up to bottom.	45
6.3	Gel electrophoresis image of OPE-08 primer. The first column was loaded with DNA ladder, which can be broken down into bands of sizes 10000 base pairs (bp), 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1000 bp, 750 bp, 500 bp and 250 bp, respectively, reading from up to bottom.	46
6.4	Gel electrophoresis image of OPE-13 primer. The first column was loaded with DNA ladder, which can be broken down into bands of sizes 10000 base pairs (bp), 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1000 bp, 750 bp, 500 bp and 250 bp, respectively, reading from up to bottom.	47

© COPYRIGHT UPM

6.5	Gel electrophoresis image of OPE-15 primer. The first column was loaded with DNA ladder, which can be broken down into bands of sizes 10000 base pairs (bp), 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1000 bp, 750 bp, 500 bp and 250 bp, respectively, reading from up to bottom.	48
6.6	Gel electrophoresis image of OPE-19 primer. The first column was loaded with DNA ladder, which can be broken down into bands of sizes 10000 base pairs (bp), 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1000 bp, 750 bp, 500 bp and 250 bp, respectively, reading from up to bottom.	49
6.7	Cluster analysis from Table 6.10 produced dendogram constructed using UPGMA (unweighted pair-group method with arithmetic averages, which represents relationships of similarity among parental plant and somaclones	52

© COPYRIGHT UPM

LIST OF ABBREVIATIONS

^{o}C	degree celcius
ANOVA	analysis of variance
BA/BAP	6-benzylaminopurine
Bp	base pair
cm	centimeter
DMRT	Duncan's Multiple Range Test
EDTA	ethylenediaminetetraacetic acid
Et al	et alia
g	Gram
HCl	hydrogen chloride
IBA	indole-3-butyric acid
Kn	kinetin
L	litre
mg	milligram
mg/L	milligram per litre
µM	micromolar
μ mol m^{-2} s^{-1}	micromole per meter square per second
MS	Murashige and Skoog
NAA	naphthalene acetic acid
NaCl	sodium chloride
PGRs	plant growth regulators
pH	hydrogen ion concentration/-log(H^{+})
RCBD	randomized complete block design
TDZ	thidiazuron
%	percent

© COPYRIGHT UPM

CHAPTER 1

INTRODUCTION

Cyclanthus bipartitus comes from the Cyclanthaceae family, which consists of 222 species in 12 genera. Cyclanthaceae family can be found exclusively in neotropical areas, which includes herbs, vines, and epiphytes; which most species prefer humid habitat at low and medium high altitudes (Erikson, 1994). The family can be divided into two subfamilies, Cyclanthoideae and Carludovicoideae; in which the former contains only the genus *Cyclanthus* and the latter the remaining 11 genera (Beach, 1982).

Cyclanthus bipartitus makes a beautiful landscape plant as it can grow up to 460 cm in height. Its leaves are shaped in extreme V-shaped formation, and can grow up to about 122 cm wide. This plant develops inflorescence that is an erect spadix, which bears both staminate and pitillate flowers arranged in alternating cycles along its length (Beach, 1982),

Not only that this plant makes a beautiful landscape plant, there also have been reports on its medicinal values used by the indigenous people for ethnomedicine, such as a cure for ant's bite fever (Valadeau, 2010), to prevent hair loss (Luziatelli *et al.*, 2010), and a cure for snakebite (Odonne *et al.*, 2013). Apart from ethnomedicine use, *Cyclanthus bipartitus* can also be used for canine ethnoveterinary medicine for hunting dogs in order to cure them from an ant's or a wasp's sting on the eyes.

Propagation of this plant can be done by cutting and division. However, for rapid mass propagation, micropropagation may serve as a good technique for commercial production compared to the conventional method. Hence, the right method or protocol to perform tissue culture should be employed in order to optimize production in the shortest time frame.

Micropropagation follows several detailed stages. The first stage is the initiation stage, where a portion of a plant, called explant, is taken from an "*in vivo*" mother plant and brought into the laboratory for sterilization process. Explants are disinfected using sterile water, alcohol and bleach; all these steps are being performed in a laminar flow hood to prevent explants being comtaminated. The second stage of tissue culture is multiplication, the sterilized explants are placed in sterilized flasks that contain culture media with desired ingredients needed for the explants to develop into new plants. At this stage, the desired outcome would be to have the explant to produce shoots from a callus. All cultures needed to be sub-cultured into fresh media in order to lengthen its life after they have used up what was supplemented in the media.

The third stage of tissue culture is the elongation stage, where all regenerated plants are transferred onto a medium that helps the shoots to elongate. All

© COPYRIGHT UPM

steps are carried out in a laminar flow hood to prevent any contamination by bacteria or fungi. At this stage, stems would grow longer and begins to look like a little plant, which often referred as regenerated plantlets. The last stage of tissue culture, which is the fourth stage, is the acclimatization stage. Regenerated plantlets are transferred into a sterilized soil for hardening process under greenhouse environment. Over time, regenerated plants will acclimate to the greenhouse condition.

The correct choice of explant material would lead to the success of tissue culture (George *et al.*, 2008). This is to ensure the effectiveness of tissue culture and achievement of the highest rate of multiplication, as seen in micropropagation of *Anthurium andraeanum* (Atak and Celik, 2009).

To date, no proper research had been published on the topic of micropropagation of *Cyclanthus bipartitus*. However, a number of research has been done on its relatives that can be used as references to this research. The most important thing in tissue culture procedure is to prepare the appropriate medium for explants to maximize its ability to fully utilize elements supplemented, and initiate the process of organ regeneration. It is crucial to supply the suitable concentration of plant growth regulators (especially cytokinin and auxin) into the medium in order to enhance and help the growth of the explants. Culture medium supplemented with auxins and cytokinins have been used to propagate many commercial ornamental plants by *in vitro* techniques (Preil, 2003; Rout and Jain, 2004).

Generally, cytokinin helps in shoot induction while auxin helps in root induction both working by stimulating cell division and differentiation (Trigiano and Gray, 2010). Two common cytokinins used in tissue culture are kinetin (Kn) and 6-benzylaminopurine (BAP). BAP was found to be more effective compared to other cytokinins (Varshney, 2012). This is due to the fact that BAP induces production of endogenous hormones, such as zeatin, or it is readily metabolized by plant tissues, compared to the other synthetic plant growth regulators (Zaerr and Mapes, 1982).

On the other hand, common auxins used for tissue culture procedure are the indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA), which are widely used in combination with cytokinin (Trigiano and Gray, 2010). When cytokinin was combined with optimal auxin concentrations, the synergic influence was evident in both shoots and roots induction. In a study done by Varshney (2012), it showed that the addition of NAA to BAP distinctly enhanced the percentage of regeneration and number of shoots per explant.

Plant cells, tissues and organs are grown *in vitro* on media supplemented with artificial and exogenous nutrients needed for growth and development of the plantlets. The success of an *in vitro* culture as a mean of plant propagation is influenced by culture media composition. Micronutrients, macronutrients, plant growth regulators, amino acids, vitamins, nitrogen supplements and carbon source (sugars) are the elements required for rapid growth of plantlets in *in vitro* condition. Sugars are required in the culture media to replace the carbon,

© COPYRIGHT UPM

which plants normally obtain from atmosphere and fixed by in vivo photosynthesis for growth and development (Yaseen *et al*., 2013).

Sucrose is known to be the most widely used as a major transport sugar in the phloem sap of many plants. It is also often assumed to be the sugar of choice in cell and tissue culture media as it is the most common carbohydrate in the phloem sap of many plants apart from being cheap and easily available (Thompson and Thorpe, 1987; Ahmad *et al*., 2007; Fuentes *et al.,* 2000).

Tissue culture has been accepted as a common way to propagate crop plants for commercial purposes. Originally, all plants regenerated from cell or tissue culture were expected to have genetic materials identical to that of the parent plant. In spite of this, phenotypic variation was observed to be abundant amongst regenerated plants (Rasheed *et al*., 2005). This variation was later termed as somaclonal variation and defined as phenotypic and genetic variation among clonally propagated plants of a mother plant.

The presence of somaclonal variation has been related to growth regulators, variability of cultivar, the age of cultivars in culture, level of ploidy, explants sources and other endogenous culture conditions (Skirvin *et al*., 1994). As chemicals present in culture medium such as 6-benzyladenine (BA), indole-3-acetic acid (IAA) and 2,4-Dichlorophenoxyacetic acid may enhance the rate of this variation. Plantlets produced via *in vitro* propagation may have different genetic materials compared to the parents and this possibility is examined in this study.

Objectives of study

The first objective of this study was to determine suitable explants type to be used for commercial micropropagation of *Cyclanthus bipartitus*. Secondly, this study was conducted to determine the combination of BAP, NAA and sucrose used to supplement the MS medium used to culture the explants in order to obtain optimal growth of explants to plantlet. In the end of this study, DNAs of regenerated plantlets were tested to determine its somaclonal variation level when compared to mother plant.

© COPYRIGHT UPM

REFERENCES

Abbasi, B., Khan, M., Guo, B., Bokhari, S. & Khan, M. (2010). Efficient regeneration and antioxidative enzyme activities in *Brassica rapa* var. turnip. *Plant Cell, Tissue and Organ Culture,* 105(3), 337-344.

Ahmad, T., Abbasi, N. A., Hafiz, I. A. & Ali, A. (2007). Comparison of sucrose and sorbitol as main carbon energy source in morphogenesis of peach rootstock GF-677. *Pakistan Journal of Botany*, 39(4), 1269-1275.

Al-Bahrany, A. (2002). Effect of phytohormones on *in vitro* shoot multiplication and rooting of lime *Citrus aurantifolia* (Christm.) Swing. *Scientia Horticulturae,* 95(4), 285-295.

Atak, C. & Celik, O. (2009). Micropropagation of *Anthurium andraeanum* from leaf explants. *Pakistan Journal of Botany*, 41(3), 1155-1161.

Beach, J. (1982). Beetle Pollination of *Cyclanthus bipartitus* (Cyclanthaceae). *American Journal of Botany,* 69(7), 1074-1081.

Bellamine, J., Penel, C., Greppin, H. & Gasper, T. (1998). Confirmation of the role of auxin and calcium in the late phases of adventitious root formation. *Plant Growth Regulators*, 26, 191-194.

Bhojwani, S. & Razdan, M. (1983). *Plant tissue culture: Theory and practice* (pp. 94). Amsterdam: Elsevier.

Cloutier, S. & Landry, B. (1994). Molecular markers applied to plant tissue culture. *In Vitro Cell Development and Biology of Plant*, 30, 32–39.

Cuenca, B. & Vieitez, A. M. (2000). Influence of carbon source on shoot multiplication and adventitious bud regeneration in *in vitro* beech culture. *Plant Growth Regulators*, 32(1), 1-12.

Cunha, A. & Fernandes-Ferreira, M. (1999). Influence of medium parameters on somatic embryogenesis from hypocotyl explants of flax (*Linum usitatissimum* L.). *Journal of Plant Physiology,* 155(4-5), 591-597.

Damasco, O. P., Godwin, I. D., Smith, M. K. & Adkins, S. W. (1996). Gibberellic acid detection of dwarf off-types in micropropagated Cavendish bananas. *Australian Journal of Experimental Agriculture*, 36(2), 237–341.

Dan, Y. H. & Stephens, C. T. (1991). Studies of protoplast culture types and plant regeneration from callus-derived protoplasts of *Asparagus oficinalis* L. cv. Lucullus 234. *Plant Cell Tissue and Organ Culture,* 27(3), 321-331.

© COPYRIGHT UPM

Daub, M. E. (1986). Tissue culture and the selection of resistance to pathogens. *Annual Review of Phytopathology*, 24, 159–186

Debnath, S. (2003). Improved shoot organogenesis from hypocotyl segments of lingonberry (*Vaccinium vitis-idaea* L.). *In Vitro Cellular and Developmental Biology - Plant,* 39(5), 490-495.

DendroUPGMA: Dendrogram construction using the UPGMA algorithm. (2002). Retrieved August 8, 2015.

Dimitrov, B., Tashera, K., Zagorska, N. & Evstatiera, L. (2003). *In vitro* cultivation of *Rhodiola rosea* L. *Genetic Breeding*, 32(1-2), 3-6.

Erikson, R. (1994). Phylogeny of the Cyclanthaceae. *Plant Systematic and Evolution*, 190(1-2), 31-47.

Evans, D. (1989). Somaclonal variation - Genetic basis and breeding applications. *Trends in Genetics,* 5(2), 46-50.

Fotopoulos, S. & Sotiropoulos, T. (2004). *In vitro* propagation of the peach rootstock: The effect of different carbon sources and types of sealing material on rooting. *Biologia Plantarum,* 48(4), 629-631.

Fuentes, S. R. L., Calheiros, M. B. P., Manetti-Filho, J. & Viera, L. G. E. (2000). The effect of silver nitrate and different carbohydrate sources on somatic embryogenesis in *Coffea canephora*. *Plant Cell Tissue and Organ Culture*, 60(1), 5-13.

Gao, X. F., Xu, C. H., Liu, J. J., Ma, L. P., Yin, L. P. & Jia, W. (2006). Adventitious root induction and *in vitro* culture of *Panax notoginseng*. *China Journal of Chinese Materials and Medication*, 32(18), 1485-1488.

Gautheret, R. (1985). History of plant tissue culture and cell culture: A personal account. In: Vasil, I. (Ed.), *Cell culture and somatic cell genetics of plants* (Vol. 2, pp. 1-59). New york: Academic press.

George, E. & Hall, M. (2007). *Plant Propagation by Tissue Culture Volume 1. The Background* (3rd ed., pp. 65-75). Dordrecht: Springer.

Godwin, I., Sangduen, N., Kunanuvatchaidach, R., Piperidis, G. & Adkins, S. (1997). RAPD polymorphisms among variant and phenotypically normal rice (*Oryza sativa* var. indica) somaclonal progenies. *Plant Cell Reports,* 16(5), 320-324.

Gopal, J., Minocha, J. L. & Dhaliwal, H. S. (1998). Microtuberation in potato (Solanum tuberosum L.). *Plant Cell Reports*, 17, 794-798.

© COPYRIGHT UPM

Gubis, J. Lajchova, Z., Farago, J. & Jurekova, Z. (2003). Effect of genotype and explant type on shoot regeneration in tomato (*Lycopersicon esculentum* Mill.) *in vitro*. *Czech Journal of Genetic and Plant Breeding*, 39(1), 9-14.

Harling, G. (1958). *Monograph of the Cyclanthaceae* (Vol. 18, pp. 1-428). Lund: Håkan Ohlssons Boktryckeri.

Hilae, A. & Te-chato, S. (2005). Effect of carbon sources and strength of MS medium on germination of somatic embryos of oil palm (*Elais guineensis* Jacq.). *Songklanarin Journal of Science Technology*, 27(3), 629-635.

Holme, I. B. & Peterson, K. K. (1996). Callus induction and plant regeneration from different explant types of *Miscanthus x ogiformis* Honda 'Giganteus'. *Plant Cell, Tissue and Organ Culture*, 45(1), 43-52.

Huang, C. L., Hsieh, M. T., Hsieh, W. C., Sagare, A. P. & Tsay, H. S. (2000). *In vitro* propagation of *limonium werightii* (Hance) Ktze. (Plumbaginaceae), and ethanomedicinal plant, from shoot tip, leaf and inflorescence-node explants. *In vitro Cell and Developmental Biology of Plant*, 36(3), 220-224.

Ikeda, L. N. & Tanabe, M. J. (1998). *In vitro* subculture application for ginger. *Horticultural Science*, 24, 142-143.

Ishag, S., Osman, M. G. & Khalafalla, M. M. (2009). Effects of growth regulators, explant and genotype on shoot regeneration in tomato (*Lycopersicon esculentum* c.v. Omdurman). *International Journal of Sustainable Crop Production*, 4(6), 7-13.

Israeli, Y., Lahav, E. & Reuveni, O. (1995). *In vitro* culture of bananas. In: Gowen, S. (Ed.) *Bananas and plantains* (pp. 147-178). London: Chapman and Hall.

Jarret, R. L. & Gawel, N. (1995). Molecular markers, genetic diversity and systematics in Musa. In: Gowen, S. (Ed.) *Bananas and plantians* (pp. 66-83). London: Chapman and Hall.

Jernigan, K. (2009). Barking up the same tree: A comparison of ethnomedicine and canine ethnoveterinary medicine among the Aguaruna. *J Ethnobiology Ethnomedicine Journal of Ethnobiology and Ethnomedicine,* 5(33), 33-33.

Kallak, H., Reidla, M., Hilpis, I. & Virumae, K. (1997). Effects of genotype, explant source and growth regulators on organogenesis of carnation callus. *Plant Cell, Tissue and Organ Culture*, 51, 127-135.

© COPYRIGHT UPM

Kantia, A. & Kothari, S. L. (2002). High efficiency adventitious shoot bud formation and plant regeneration from leaf explants of *Dianthus chinensis* L. *Science Horticulture*, 96, 205-212.

Khan, M., Heyes, J. & Cohen, D. (1988). Plant regeneration from oca (*Oxalis tuberosa* M.): The effect of explant type and culture media. *Plant Cell, Tissue and Organ Culture,* 14, 41-50.

Khuri, S. & J. Moorby. (1995). Investigation into the role of sucrose in potato cv. Estima microtuber production in vitro. *Annals of Botany*, 75(3), 295-303.

Larkin, P. & Scowcroft, W. R. (1981). Somaclonal variation, a novel source of variability from cell cultures for plant improvement. *Theory of Applied Genetics*, 60, 197-406.

Lawrence, G. (1951). *Taxonomy of vascular plants*. New York: Macmillan.

Lipavska, H. & Kondradova, H. (2004). Somatic embryogenesis in conifers: The role of carbohydrate metabolism. *In Vitro Cell Developmental Biology of Plant*, 40(1), 23-30.

Lo Schiavo, F., Pitto, L., Giuliano, G., Torti, G., Nutironchi, V., Marazziti, D., Vergara, R., Orselli, S. & Terzi, M. (1989). DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. *Theory of Applied Genetics*, 77(3), 325-331.

Lorz, H., Gobel, E. & Brown, P. (1988). Advances in tissue culture and progress towards genetic transformation of cereals. *Plant Breeding*. 100(1), 1-25.

Luziatelli, G., Sørensen, M., Theilade, I. & Mølgaard, P. (2010). Asháninka medicinal plants: A case study from the native community of Bajo Quimiriki, Junín, Peru. *Journal of Ethnobiology and Ethnomedicine, 6*(21), 1-23.

Madhulata, P., Kirubakaran, S. I. & Sakthivel, N. (2006). Effects of carbon sources and auxins on in vitro propagation of banana. *Biologia Plantarum*, 50(4), 782-784.

Mandal, A., Maiti, A., Chowdhury, B. & Elanchezhian, R. (2001). Isoenzyme markers in varietal identification of banana. *In Vitro Cell Development and Biology of Plant*, 37(5), 599–604.

Martin, M., Sarmento, D. & Oliveira, M. M. (2004). Genetic stability of micropropagated almond plantlets as assessed by RAPD and ISSR markers. *Plant Cell Reproduction*, 23(7), 492-496.

© COPYRIGHT UPM

Mukhtar, S., Ahmad, N., Khan, M., Anis, M. & Aref, I. (2012). Influencing micropropagation in *Clitoria ternatea* L. through the manipulation of TDZ levels and use of different explant types. *Physiology and Molecular Biology of Plants,* 18(4), 381-386.

Neibaur, I., Gallo, M. & Altpeter, F. (2008). The effects of auxin type and cytokinin and concentration on callus induction and plant regeneration frequency from immature inflorescence segments of seashore paspalum (*Paspalum vaginatum* Swartz). *In Vitro Cell Developmental Biology*, 44(6), 480-486.

Nickell, L. (1982). *Plant growth regulators: Agricultural uses* (1st ed.). Berlin: Springer-Verlag.

Odonne, G., Valadeau, C., Alban-Castillo, J., Stien, D., Sauvain, M. & Bourdy, G. (2013). Medical ethnobotany of the Chayahuita of the Paranapura basin (Peruvian Amazon). *Journal of Ethnopharmacology,* 146(1), 127-153.

Pan R. & Tian, X. (1999). Comparative effect of IBA, BSAA and 5,6-Cl2-IAA-Me on the rooting of hypocotyls in mung bean. *Plant Growth Regulation*, 27(2), 91-98.

Pan, R. & Zhao, Z., (1994). Synergistic effects of plant growth retardants and IBA on the formation of adventitious roots in hypocotyl cuttings of mung bean. *Plant Growth Regulation*, 14(1), 15–19.

Pawlicki, N., Sangwan, R. & Sangwan-Norreel, B. (1993). Somaclonal variation in carotene content of carrot (*Daucus carota* L.). *Acta Botanica Gallica,* 140(1), 17-22.

Pazos-Navarro, M., Del Rio, J. A., Ortuna, A., Romero-Espinar, P., Correal, E. & Dabauza, M. (2012). Micropropagation from apical and nodal segments of *Bituminaria bituminosa* and the furanocoumarin content of propagated plants. *Journal of Horticulture Science and Biotechnology*, 87(1), 29-35.

Perez-Molphe-Balch, E. & Ochoa-Alejo, N. (1997). *In vitro* plant regeneration of Mexican Lime and Mandarin by direct organogenesis. *Horticultural Science*, 32(5), 931-934.

Peschke, V. & Phillips, R. (1992). Genetic implications of somaclonal variation in plants. *Advances in Genetics,* 30, 41-75.

Peyvandi, M., Noormohammadi, Z., Banihashemi, O., Farahani, F., Majd, A,, Hosseini-Mazinani, M. & Sheidai, M. (2009). Molecular analysis of genetic stability in long-term micropropagated shoots of *Olea europaea* L. (cv. Dezful). *Asian Journal of Plant Science*, 8(2), 146–152.

© COPYRIGHT UPM

Preethi, D., Sridhar, T. M. & Naidu, C. V. (2011). Carbohydrate concentration influences on *in vitro* plant regeneration in *Stevia rebaudiana*. *Journal of Phytology*, 3(5), 61-64.

Preil, W. (2003). Micropropagation of ornamental plants. In *Plant Tissue Culture 100 years since Gottlieb Haberlandt* (1st ed., pp. 115-133). New York: Springer.

Rasheed, S., Fatima, T., Husnain, T., Khurram, B. & Riazuddin, S. (2005). RAPD characterization of somaclonal variation in Indica basmati rice. *Pakistan Journal of Botany*, 37(2), 249-262.

Rout, G. R. & Jain, M. (2004). Micropropagation of ornamental plant – Cut flower. *Propagation of Ornamental Plant*, 4(2), 3-28.

Sahijram, L., Soneji, J. & Bollamma, K. (2003). Analyzing somaclonal variation in micropropagated bananas (*Musa* spp.). *In Vitro Cellular & Developmental Biology - Plant,* 39(6), 551-556.

Seyyedyousefi, S., Kaviani, B., Dehkaei, N. & Salehzadeh, A. (2013). Callus induction in *Alstroemeria* using NAA and BAP. *European Journal of Experimental Biology,* 3(51), 137-140.

Shenoy, V. & Vasil, I. (1992). Biochemical and molecular analysis of plants derived from embryogenic tissue cultures of napier grass (*Pennisetum purpureum* K. Schum). *Theoretical and Applied Genetics,* 83(8), 947-955.

Singh, S., Ray, B. K., Bhattacharyya, S. & Deka, P. C. (1994). *In vitro* propagation of *Citrus reticulata* Blanco and *Citrus limon* Burm. *Horticultural Science*, 29(3), 214-216.

Skała, E. & Wysokińska, H. (2004). *In vitro* regeneration of *Salvia nemorosa* L. from shoot tips and leaf explants. *In Vitro Cellular & Developmental Biology - Plant,* 40(6), 596-602.

Skirvin, R. M., McPheeters, K. D. & Norton, M. (1994). Sources and frequency of somaclonal variation. *Horticulure Science*, 29(11), 1232-1237.

Sridhar, T. M. & Naidu, C. V. (2011). Effect of different carbon sources on in vitro shoot regeneration of *Solanum nigrum* (Linn.) – an important antiulcer medicinal plant. *Journal of Phytology*, 3(2), 78-82.

Sul, W. & Korban, S. S. (2004). Effects of salt formulations, carbon sources, cytokinins and auxin on shoot organogenesis from cotyledons of *Pinus pinea* L. *Plant Growth Regulation*, 43(3), 197-205.

Tabaeizadeh, Z., Plourde, A., & Comeau, A. (1990). Somatic embryogenesis and plant regeneration in *Triticum aestivum* x *Leymus angustus* F1 hybrids and the parental lines. *Plant Cell Reports*, 9(4), 204-206.

© COPYRIGHT UPM

Thiart, S. (2003). Manipulation of growth by using tissue culture techniques. *International Plant Propagator's Society,* 53, 61-67.

Thompson, M. & Thorpe T. (1987). Metabolic and non-metabolic roles of carbohydrates. In Bong, J. M. & Durzan, D. J. (Eds.) *Cell and tissue culture in forestry* (pp. 89-112.), Dardrecht: Martinus Nijhoff Publication.

Ting, I. (1982). *Plant physiology*. Reading, Massachusetts: Addison-Wesley Publication.

Trigiano, R. & Gray, D. (2011). Plant Tissue Culture, Development and Biotechnology. CRC Press.

Tripepi, R. & Merkle, S. (1997). Adventitious shoot regeneration. In Geneve, R. & Preece, J. (Eds.), *Biotechnology of ornamental plants* (pp. 45-71). Wallingford, Oxon, UK: CAB International.

Valadeau, C., Castillo, J., Sauvain, M., Lores, A. & Bourdy, G. (2010). The rainbow hurts my skin: Medicinal concepts and plants uses among the Yanesha (Amuesha), an Amazonian Peruvian ethnic group. *Journal of Ethnopharmacology,* 127(1), 175-192.

Varshney, A., Anis, M. & Aref, I. (2012). Potential role of cytokinin–auxin synergism, antioxidant enzymes activities and appraisal of genetic stability in *Dianthus caryophyllus* L. - an important cut flower crop. *In Vitro Cellular and Developmental Biology - Plant,* 49(2), 166-174.

Vasil, I. K. (1987). Developing cell and tissue culture systems for the improvement of cereal and grass crops. *Journal of Plant Physiology*, 128(3), 193-218.

Vasudevan, R. & Staden, J. V. (2011). Cytokinin and explant types influence in vitro plant regeneration of Leopard Orchid. *Plant Cell, Tissue, and Organ Culture*, 107(1), 123-129.

Vickers, W. & Plowman, T. (1984). *Useful plants of the Siona and Secoya Indians of eastern Ecuador*. Chicago, Illinois: Field Museum of Natural History.

Viegas, J., Rocha, M. T. R., Ferreira-Moura, Rosa, D. L., Souza, J. A., Correa, M. G. S. & Silva, J. A. T. (2006). *Anthurium andraeanum* (Linden ex Andre) Culture: *In vitro* and *ex vitro*. *Floriculture and Ornamental Biotechnology*, 1(1), 61-65.

Weaver, R. (1972). *Plant growth substances in agriculture*. San Francisco: W.H. Freeman.

© COPYRIGHT UPM

Weng, H. & Madhialagan, K. (2014). Effect of Cytokinins on in Vitro Regeneration of Cardamine Hirsuta from Nodal Explants. *Journal of Life Sciences and Technologies,* 2(2), 74-77.

Whetherell, D.F. (1982). Introduction to in vitro propagation. In: *Selection of culture media*. New Jersey, USA: Avery publishing group Inc.

Wightman, F., Schneider, E.A. & Thimann, K.V. (1980). Hormonal factors controlling the initiation and development of lateral roots. II. Effects of exogenous growth factors on lateral root formation in pea roots. *Physiologia Plantarum*. 49(3), 304–31.

Wilder, G. & Harris, D. (1982). Laticifers in *Cyclanthus bipartitus* Poit. (Cyclanthaceae). *Botanical Gazette,* 143(1), 84-84.

Yaseen, M., Ahmad, T., Sablok, G., Standardi, A. & Hafiz, I. (2012). Review: Role of carbon sources for in vitro plant growth and development. *Molecular Biology Reports Mol Biol Rep,* 40(4), 2837-2849.

Yildiz, M., Onde, S. & Ozgen, M. (2007). Sucrose effects on phenolic concentration and plant regeneration from sugarbeet leaf and petiole explants. *JSBR Journal of Sugarbeet Research,* 44(1-2), 1-16.

Yuan, L., Loque, D., Kojima, S., Rauch, S., Ishiyama, K., Inoue, E., . . . Wiren, N. (2007). The organization of high-affinity ammonium uptake in arabidopsis roots depends on the spatial arrangement and biochemical properties of amt1-type transporters. *The Plant Cell Online,* 19(8), 2636-2652.

Zaerr, J. B. & Mapes, M. O. (1982). Action of growth regulators. In: Bonga,J. & Durzan, D. (Eds.), *Tissue Culture in Forestry* (pp. 231-255). Dordrecht: Martinus Nijhoff.

Zhang, J., Gao, W. Y., Wang, J. & Li, X. L. (2011). Effects of explant types and media salt strength on growth and secondary metabolite accumulation in adventitious roots of Periploca sepium Bunge. *Acta Physiologiae Plantarum*, 33(6), 2448-2452.

© COPYRIGHT UPM