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

NUR FAUZANA MOHD KASIM

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

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

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By

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June 2015

Chair: Associate Professor Yahya Awang, PhD

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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. bipartitus. 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 cm² 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
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
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

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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 cm² 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
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.
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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:

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LIST OF ABBREVIATIONS

°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
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 Carludovicoidae; 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 pistillate 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 contaminated. 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
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,
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.
REFERENCES


