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SHOOT REGENERATION FROM COTYLEDON NODES AND PRODUCTION OF ANTHRAQUINONES FROM CELL SUSPENSION CULTURE OF GELENGGANG (CASSIA ALATA LINN.)

SITI SAFURA BINTI JAAPAR

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By

SITI SAFURA BINTI JAAPAR

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Chair: Professor Maziah binti Mahmood, PhD

Faculty: Biotechnology and Biomolecular Sciences

Great demands for plant resources to produce conventional drugs as well as traditional medicine due to their precious bioactive compounds have caused drastic depletion on natural biodiversity including *Cassia alata* L., especially in Malaysia. Plant tissue culture technology has become an option to reproduce the resources of preferred compounds, yet there is a gap of information on the species on *in vitro* cultures. Therefore through this study, the effect of plant growth regulators on seed germination and shoot regeneration using cotyledon nodes of *C. alata* had been determined. Besides, the effect of plant growth regulators on leaf-derived callus induction of the species and methyl jasmonate (MeJA) elicitation effect on cells growth in liquid medium, as well as on anthraquinones production also had been studied. The detection of anthraquinones was carried out using TLC and HPLC. The results showed that BAP at a concentration of 1 mg/L in MS medium gave excellent response in seed germination (77.33%) and MS medium supplemented 1 mg/L BAP and 1.5 mg/L NAA induced the highest number of shoots (2.07 ± 0.54 shoots per explants) with the mean length of 1.3 ± 0.1 cm from cotyledon node of the species. The maximum percentage (33.33%) of leaf derived callus has been obtained in MS medium added with 1 mg/L picloram. Meanwhile MS medium with 1 mg/L picloram and 0.2 mg/L BAP induced the highest percentage (100%) of creamy yellow, globular and friable callus with the highest dried weight (0.8 ± 0.03 g) after 50 days of cultures. Elicitation in suspension culture of MS + 1 mg/L picloram + 0.2 mg/L BAP treated with 0.1 µM MeJA stimulated the highest cells biomass production as 4.46 ± 0.49 grams of dried weight. Through TLC analysis, the retention factor (Rf) values of four anthraquinones were determined; rhein (Rf = 0.85), aloe-emodin (Rf =0.82), chrysophanol (Rf = 0.79) and physcion (Rf = 0.71). Physcion and rhein spots were detected from the crude extract from suspension culture treated with 1.0 µM MeJA. Single spot of aloe-emodin was presented from the crude extract from suspension culture treated with 0.1 µM MeJA. Meanwhile aloe-emodin and physcion spots were detected from the crude extract from

suspension culture treated with 0.01 µM MeJA and without any elicitor treatment respectively. Chrysophanol was only detected in the leaf crude extract. Results from HPLC analysis showed that the highest concentration of aloe-emodin (2.63 ± 0.03 mg/g DW) occurred in the sample extract from cell suspension culture supplied with MS + 1 mg/L picloram + 0.2 mg/L BAP + 0.01 µM MeJA for 10 days culture. Besides, the highest concentration of rhein (4.53 ± 0.10 mg/g DW) was detected in the sample extract from cell suspension cultured in MS + 1 mg/L picloram + 0.2 mg/L BAP + 1 µM MeJA for 10 days culture. There was no chrysophanol detected in every sample extract except from leaf extract. It showed that the MeJA concentration and long duration of elicitation did affect the biomass of the cells, as well as the accumulation of anthraquinones in *C. alata* L. suspension cultures. As the conclusion, the shoot regeneration capability of cotyledon nodes of *C. alata* was determined, the cell suspension culture of this species was established and anthraquinones was produced via MeJA elicitation in cell suspension culture.

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

REGENERASI PUCUK DARIPADA NOD-NOD KOTILEDON DAN PENGHASILAN ANTHRAQUINONES DARIPADA KULTUR AMPAIAN SEL GELENGGANG (*CASSIA ALATA* LINN.)

Oleh

SITI SAFURA BINTI JAAPAR

Mei 2015

Pengerusi: Profesor Maziah binti Mahmood, PhD

Fakulti: Bioteknologi dan Sains Biomolekul

Permintaan yang tinggi terhadap sumber tumbuh-tumbuhan dalam menghasilkan ubat-ubatan konvensional dan juga ubat-ubatan tradisional, disebabkan oleh sebatian bioaktifnya yang bernilai, telah mengakibatkan pengurangan pada kepelbagaian bio semulajadi yang drastik termasuklah *Cassia alata* L., terutama di Malaysia. Teknologi kultur tisu tumbuhan telah menjadi pilihan untuk menghasilkan sumber sebatian pilihan, namun terdapat jurang maklumat tentang kultur *in vitro* bagi spesies ini. Justeru melalui penyelidikan ini, kesan pengawalatur pertumbuhan terhadap percambahan biji benih dan regenerasi pucuk menggunakan nod-nod kotiledon *C.alata* L. telah ditentukan. Selain itu, kesan pengawalatur pertumbuhan terhadap aruhan kalus daripada daun spesies ini dan kesan elisitasi metil jasmonate (MeJA) terhadap pertumbuhan sel-sel di dalam kultur ampaian serta kesan terhadap penghasilan anthraquinones telah dikaji. Pengesanan anthraquinones telah dilakukan dengan menggunakan TLC dan HPLC. Keputusan menunjukkan BAP dengan kepekatan 1 mg/L di dalam medium MS memberi tindak balas yang terbaik dengan menghasilkan 77.33% percambahan biji benih dan medium MS yang ditambah dengan 1 mg/L BAP dan 1.5 mg/L NAA mengaruh kadar penggandaan pucuk yang tertinggi (2.07 ± 0.54 pucuk per eksplan) dengan purata panjang pucuk sebanyak 1.3 ± 0.1 cm daripada nod kotiledon spesies ini. Peratus maksimum (33.33%) kalus daripada daun telah terhasil pada medium MS yang ditambah dengan 1 mg/L pikloram. Manakala medium MS dengan 1 mg/L pikloram dan 0.2 mg/L BAP mengaruh peratus kalus berwarna kuning keputihan, globular dan peroi tertinggi (100%) dengan catatan berat kering kalus tertinggi (0.83 ± 0.03 g) selepas 50 hari pengkulturan. Elisitasi dengan 0.1 µM MeJA pada kultur ampaian sel bagi medium MS + 1 mg/L pikloram + 0.2 mg/L BAP menunjukkan penghasilkan biojisim sel yang tertinggi iaitu berat kering sebanyak 4.46 ± 0.49 g. Melalui kaedah TLC, nilai faktor retensi (Rf) untuk empat jenis anthraquinones telah ditentukan; rhein (Rf = 0.85), aloe-emodin (Rf =0.82), chrysophanol (Rf = 0.79) and physcion (Rf = 0.71). Tompokan kecil physcion dan rhein dikesan daripada ekstrak mentah yang diperoleh daripada kultur ampaian sel yang dirawat dengan 1.0 µM MeJA. Satu tompokan aloe-emodin hadir daripada ekstrak mentah yang diperoleh daripada kultur ampaian sel yang dirawat dengan 0.1 µM MeJA.

Manakala tompokan aloe-emodin and physcion dikesan masing-masing daripada ekstrak mentah yang diperoleh daripada kultur ampaian yang dirawat dengan 0.01 µM MeJA dan daripada kultur ampaian tanpa rawatan MeJA. Chrysophanol hanya dikesan pada ekstrak mentah daun. Keputusan analisis HPLC menunjukkan kepekatan tertinggi aloe-emodin (2.63 ± 0.03 mg/g DW) yang diperolehi daripada ekstrak sampel kultur ampaian yang dibekalkan dengan medium MS + 1 mg/L pikloram + 0.2 mg/L BAP + 0.01 µM MeJA untuk 10 hari pengkulturan. Seterusnya, kepekatan tertinggi rhein (4.53 ± 0.10 mg/g DW) yang diperolehi daripada ekstrak sampel kultur ampaian yang dibekalkan dengan medium MS + 1 mg/L picloram + 0.2 mg/L BAP + 1 µM MeJA selama 10 hari pengkulturan. Tiada chrysophanol dikesan daripada setiap sampel melainkan daripada ekstrak daun. Ini menunjukkan kepekatan MeJA dan tempoh elisitasi yang panjang mempengaruhi biojisim sel dan juga pengumpulan anthraquinones di dalam kultur ampaian sel *C. alata* L. Kesimpulannya, keupayaan nod kotiledon untuk percambahan pucuk telah dapat ditentukan. Selain itu, kultur ampaian sel untuk spesis ini telah dapat dijalankan dan anthraquinones telah dapat dihasilkan melalui elisitasi MeJA di dalam kultur ampaian sel.

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I certify that a Thesis Examination Committee has met on 26 May 2015 to conduct the final examination of Siti Safura binti Jaapar on her thesis entitled "Shoot Regeneration from Cotyledon Nodes and Production of Anthraquinones From Cell Suspension Culture of Gelenggang (*Cassia alata* Linn.)" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science

Members of the Thesis Examination Committee were as follows:

Norhani bt Abdullah, PhD
Professor
Institute of Tropical Agriculture
Universiti Putra Malaysia
(Chairman)

Faridah binti Qamaruz Zaman, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Internal Examiner)

Siti Khalijah bt Daud, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Internal Examiner)

Chan Lai Keng, PhD
Professor
School of Biological Sciences
Universiti Sains Malaysia
(External Examiner)

ZULKARNAIN ZAINAL, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 22 September 2015

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:

Maziah binti Mahmood, PhD
Professor
Faculty Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Abdul Rahman bin Omar, PhD
Professor
Institute of Bioscience
Universiti Putra Malaysia
(Member)

Syahida binti Ahmad, PhD
Senior Lecturer
Faculty Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____
BUJANG KIM HUAT, PhD
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This is to confirm that:

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Signature: _____
Name of Chairman of Supervisory Committee: Prof. Maziah binti Mahmood, PhD

Signature: _____
Name of Member of Supervisory Committee: Prof. Abdul Rahman bin Omar, PhD

Signature: _____
Name of Member of Supervisory Committee: Syahidah binti Ahmad, PhD

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
2-iP	N^6-(2-isopentenyl)adenine
ANOVA	Analysis of variance
B5	Gamborg's B-5 medium
BA	6-Benzylaminopurine
BAP	6-Benzylaminopurine
C. alata L.	*Cassia alata* Linn
C. angustifolia	*Cassia angustifolia*
C. auriculata	*Cassia auriculata*
C. obtusifolia	*Cassia obtusifolia*
$CaCl_2$ $2H_2O$	Calcium chloride
$CoCl_2$ 6H2O	Cobalt chloride
cm	Centimeter
$CuSO_4$ 5H2O	Copper sulfate
DMRT	Duncan's Multiple Range Test
DNA	Deoxyribonucleic acid
DW	Dry weight
DZ	Dihydrozeatin
E.coli	*Escherichia coli*
E. spinosa	*Emex spinosa*
et al.	*et alia (and others)*
F_{254}	Flourescent material that absorbs light at 254 nanometer
FDA	Food and Drug Administration
$FeSO_4$ 7H2O	Ferrous sulfate
FW	Fresh weight
g	Gram
GA3	Gibberellic acid
Globinmed	Global Information Hub on Integrated Medicine
g/m^3	Gram per cubic metre
H_3BO_3	Boric acid
HCl	Hydrogen chloride
HPLC	High Performance Liquid Chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
In vitro	In glass (test tube)
iP	isopentenyladenine
KH_2PO_4	Potassium phosphate
KI	Potassium iodide
KIN	Kinetin
KOH	Potassium hydroxide
KNO_3	Potassium nitrate
L. martagon	*Lilium martagon*
M. citrifolia	*Morinda citrifolia*
MeJA	Methyl jasmonate
mg/g	Milligram per gram
mg/L	Milligram per litre
mg/ml	Milligram per millilitre

MgSO4 7H2O	Magnesium Sulfate
ml	millilitre
mm	Millimetre
$MnSO_4$ 4H2O	Manganese sulfate
MS	Murashige and Skoog
MSO	Murashige and Skoog medium without hormone
MWD	Multiple Wavelength Detector
n	Number of sample
N	Total number of sample
NAA	1-Naphthylacetic acid
NaH_2PO_4	Monosodium phosphate
NaOH	Sodium Hydroxide
Na_2MoO_4 2H2O	Sodium molybdate
Na_2EDTA 2H2O	Ethylenediaminetetra-acetic acid
NH_4NO_3	Ammonium Nitrate
nm	nanometre
NO	Nitrogen monoxide
No.	*Number*
NPCB	National Pharmaceutical Control Bureau
ODS	Octadecylsilane
P	Probability
P. barbatus.	*Plectranthus barbatus.*
P. marsupium	*Pterocarpus marsupium*
pH	Hydrogen ion concentration
picloram	4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid
PGRs	Plant growth regulators
PVP	Polyvinylpyrrolidone
R&D	Research and development
r^2	Correlation coefficient
Rf	Retention factors
RM	Ringgit Malaysia
r.p.m.	Revolutions per minute
R. tinctorum	*Rubia tinctorum*
S. androgynus	*Sauropus androgynus*
S. aureus	*Staphylococcus aureus*
S.D.	*Standard deviation*
S.E.	*Standard error*
SPSS	Statistical Package for the Social Sciences
sp.	species
TDZ	Thidiazuron
TLC	Thin Layer Chromatography
UPM	Universiti Putra Malaysia
USA	United State of America
USP	United States Pharmacopeia
US$	United State of America dollar
UV	Ultraviolet
v/v	Volume per volume
v/v/v	Volume per volume per volume
ver.	Version
w/v	Weight per volume
WHO	World Health Organization

WPM	Woody Plant Medium
Z	Zeatin
$ZnSO_4 \cdot 7H2O$	Zinc sulfate
µg/g	Microgram per gram
µg/ml	Microgram per millilitre
µl	Microliter
µM	Micro molar
µm	Micrometre
°C	Degree Celsius

CHAPTER 1

INTRODUCTION

Medicinal plants have been an integral part of the ethnobotanical aspect of the people around the world since ancient time. For thousands years, nature has been the source of medicine including medicinal plants which have become mainstream in the end of the 20^{th} century (Alsarhan et al., 2014). Malaysia, a country that owns an extent of biological diversity of its rainforest, offers a great reservoir of medicinal plants which are propitious supply of biologically active constituents (Noor Rain et al., 2007) as traditional remedy or modern medication.

One of the medicinal plants that many Malaysian still rely on as traditional remedies is *Cassia alata* L. (*Senna alata*). This species is native to tropical America and now is growing wild throughout Malaysia. It is a shrub with a height of one to 3.6 meters, has bright green and linear-oblong shaped leaves, produces large spikes of yellow flowers (Otto et al., 2014) and widely appreciated as a garden ornamental.

Cassia alata L. comes from *Fabaceae* family (Ahmed et al., 2013) and it is renowned as candle bush, Gelenggang (Malay), ringworm bush, empress candle plant and seven golden candlesticks (Babitha et al., 2010). According to Timothy et al. (2012), *C. alata* L. has been used for centuries as a purgative drug and antifungal remedy to treat fungal skin diseases. Traditionally, ground or crushed young leaves of the plant are applied on the affected region to treat skin infections and skin lesions such as ringworm and discoloration (Mitra et al., 2007).

Pharmacological studies performed so far on *C. alata* L. have discovered that this plant has numerous biological activities, such as antimicrobial (Otto et al., 2014), antifungal (Abubacker et al., 2008), and anti-inflammatory (Chatterjee et al., 2012). Wuthi-udomlert et al. (2010) claimed that anthraquinone compounds which are consistently used as purgative and in the treatment of skin diseases have been discovered in immense content in the foliages of *C. alata* L. Meanwhile Gritsanapan & Mangneesri (2009) reported that aglycone and glycoside form of anthraquinones such as aloe-emodin, chrysophanol, rhein and physcione were found in *C. alata* L. leaves. They also stated that rhein was identified to be an eminent constituent in *C. alata* L. leaf extracts (Gritsanapan & Mangneesri, 2009).

Global demand in the manufacture, utilization and worldwide business in medicinal plants and phytomedicines is stimulating the potential to accelerate quite significantly in the future (Debnath et al. 2006). However, commercial exploitation of plants due to their bioactive properties for drugs development has imperilled 4,000 to 10,000 species of medicinal greenery though several of the species are cultivated (Ramawat et al., 2009). In Malaysia, the area of natural forest had drastically reduced from 1990 to

2010, caused by subsistence farming, commercial agriculture, logging, and forest fires (Hashim et al., 2013). These threaten the survival of diverse medicinal plants, including *C. alata* L. Therefore, some biotechnological methods and feasible practices have been introduced to counter the consequential threats of biodiversity and one of them is through plant tissue culture.

Plant tissue culture, specifically micro-propagation or sometimes known as *in vitro* propagation has several improvements over typical practices of vegetative propagation. It holds excellent competence for the production of prime phytomedicines as it facilitates the production of enormous numbers of pathogen-free new clones in minimal time and place, starting from single plant (Debnath et al., 2006). High competence of shoot regeneration has been able to be accomplished through calli derived from leaf and cotyledon (Agrawal & Sardar, 2006) and nodal explants (Siddique & Anis, 2007) in *Cassia angustifolia*.

An investigation by Parveen et al. (2010) showed an adequate regeneration system for *Cassia siamea* by using cotyledonary nodes where numerous buds were produced on the MS medium supplemented with BA and NAA. They stated 90% of shoot regeneration with 12.20 ± 0.73 of shoots per explants and 6.40 ± 0.07 cm of shoot length were achieved using the medium MS + 1.0 µM BA + 0.5 µM NAA. Besides, this mass production via tissue culture technique is considered to be a very preferable method as it provides a sustainable source of biochemicals independent of plant availability (Namdeo, 2007).

There are some examples of the production of secondary metabolites using plant tissue culture. A study conducted by Tan et al. (2013) revealed that cell culture of *Centella asiatica* (L.) using MS medium supplemented with 3 mg/L 2,4-D and 1 mg/L kinetin produced 390 ± 33 µg/g DW luteolin. Besides, callus cultures of *Hydrocotyle bonariensis* was initiated from the leaf using MS medium added with 2 mg/L of 2,4-D and 1 mg/L kinetin produced the highest flavonoid accumulation (10.77 ± 0.25 mg/g DW) with the presence of proline as precursor (Masoumian et al. 2011).

Some prominent benefits and improvements of cell culture system over the traditional cultivation of the whole plant are the production of secondary metabolites as stated by (Sheludko, 2010). The benefits are that the useful compound can be biologically manufactured under controlled conditions but independent of environmental circumstances including climate or soil condition. It also can be microbes and insects-free cell culture. Additionally, any plant's cell could easily be massively cloned in order to produce their targeted compounds. This automated control of cell growth and rational regulation of metabolite processes would decrease the cost of manpower and accelerate the yield (Namdeo, 2007).

Several common approaches for optimization of secondary metabolite yield in plant cell culture are elicitation and immobilization. These typical strategies allow the

improvement of metabolite productivity by altering the cell metabolism with the stimulation of a number of elicitors (Patel & Krishnamurthy, 2013). One of the common elicitors that are widely used is jasmonate and it has been proven to be very effective in triggering secondary metabolite production capacity (Chen et al., 2006) and among jasmonates, methyl jasmonate (MeJA) has been widely used as an elicitor (Sabater-Jara et al., 2010).

Bauer et al. (2009) reported that 2.8 times higher amount of rosmarinic acid was stimulated by MeJA and resulted in significantly better growth in hairy root cultures of *Coleus blumei.* Abd El-Mawla (2012) also stated that MeJA accelerated anthraquinone yield in *Rubia tinctorum* cell cultures. Meanwhile a study by Abd El-Mawla and Ibraheim (2011) stated that a positive effect of MeJA was discovered on anthraquinones and flavonoids accumulation in cell cultures of *Emex spinosa.*

This species possess a great potential as a medicinal plant due to anthraquinones that have many beneficial properties. However the depletion of forest in Malaysia has endangered the species. Its conventional cultivation is very poor due to the species nature as recalcitrant plant is only worsening the problem (Parveen & Shahzad, 2012). Besides, there is still lack of information on plant tissue culture for *C. alata* L., especially in Malaysia. Therefore, this research has been designed to investigate the response of *C. alata* L. towards plant tissue culture application at several levels; seed germination, plantlet growth, plant regeneration and callus induction. Subsequently the anthraquinones production from suspension culture through MeJA elicitation can be determined.

Objectives

To gain significant results from this research, some objectives have been planned including:

1. To determine the shoot regeneration capability of cotyledon nodes of Gelenggang (*C. alata* L.).
2. To establish the cell suspension culture of Gelenggang (*C. alata* L.).
3. To produce anthraquinones via methyl jasmonate elicitation in cell suspension culture of Gelenggang (*C. alata* L.).

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