In-vitro PROPAGATION AND *Agrobacterium tumefaciens*-MEDIATED TRANSFORMATION OF *Jatropha curcas*

STEPFANIE EVERT JOLE

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITY MALAYSIA SABAH
2015

In-vitro PROPAGATION AND *Agrobacterium tumefaciens*-MEDIATED TRANSFORMATION OF *Jatropha curcas*

STEPFANIE EVERT JOLE

THESIS SUBMITTED IN PARTIAL FULLFILMENT FOR THE DEGREE OF MASTER OF SCIENCE

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITY MALAYSIA SABAH
2015

PUMS 99:1

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN TESIS

JUDUL : _____
_____
_____

IJAZAH : _____
_____

SAYA : _____ **SESI PENGAJIAN** : _____
(HURUF BESAR)

Mengaku membenarkan tesis *(LPSM/Sarjana/Doktor Falsafah) ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:-

1. Tesis adalah hak milik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/)

☐ SULIT (Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di AKTA RAHSIA RASMI 1972)

☐ TERHAD (Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)

☐ TIDAK TERHAD

Disahkan oleh:

_____ _____
(TANDATANGAN PENULIS) (TANDATANGAN PUSTAKAWAN)

Alamat Tetap: _____
_____
_____
_____

_____
(NAMA PENYELIA)

TARIKH: _____ TARIKH: _____

Catatan:
*Potong yang tidak berkenaan.
*Jika tesis ini SULIT dan TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT dan TERHAD.
*Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana Secara Penyelidikan atau disertai bagi pengajian secara kerja kursus dan Laporan Projek Sarjana Muda (LPSM).

DECLARATION

I hereby declare that no part of this report has been previously submitted for a degree in this or any other university. All work were based on my own research except as cited in references and other part of the report where indicated.

24th June 2015

_____

Stepfanie Evert Jole
PB2009-8058

CERTIFICATION

NAME : **STEPFANIE EVERT JOLE**

MATRIC NO. : **PB2009-8058**

TITLE : ***In-vitro* PROPAGATION AND *Agrobacterium tumefaciens*-MEDIATED TRANSFORMATION OF *Jatropha curcas***

DEGREE : **MASTER OF SCIENCE (BIOTECHNOLOGY)**

VIVA DATE : **13th NOVEMBER 2014**

CERTIFIED BY

1. SUPERVISOR

Mr. Wilson Yong Thau Lym Signature

_____

2. CO-SUPERVISOR

Assoc. Prof. Dr. Jualang Azlan Gansau

_____

ACKNOWLEDGEMENT

I wish to express my deepest gratitude and appreciation to my supervisor, Mr. Wilson Yong Thau Lym for his excellent guidance, patience, and providing me with an excellent atmosphere for doing research and writing of this thesis. The good advice, support and encouragement of my co-supervisor, Assoc. Prof. Dr. Jualang Azlan Gansau was invaluable for which I am extremely grateful. I thank Dr. Kenneth Francis Rodrigues for lending me his expertise and intuition to my scientific and technical problems. Thank you to Mdm Grace Joy Chin for her helps in synthesizing primers for some part of my studies.

I cannot forget friends who went through hard times together, cheered me on and share their knowledge with me: Ms Lem Fui Fui, Mr Fernandes Opook, Ms Audrey Rose, Mr Ahmad Zaidi and Ms Thien Vun Yee. It is my pleasure to acknowledge all the laboratory assistant of BRI for their continuous help throughout my research: Mdm Azian Awang Besar, Mdm Vidarita Maikin, Mr Mony Mian and Mr Emran Raga.

Most importantly, none of this would have been possible without the love, prayer and patience of my family. To my parent, thank you for encouraging me in all of my pursuits and inspiring me to follow my dreams. I always knew that you believed in me and wanted the best for me. To my siblings who cheer me up and their words of encouragement throughout my studies and also the one who inspire, encourage and been there for me through my up and down. Your love and support and belief in me were a treasure.

Finally I thank my God, my good Father, for letting me through all the difficulties. I have experienced Your guidance day by day. You are the one who let me finish my master degree. I will keep on trusting You for my future. Thank you, Lord!

Stepfanie Evert Jole
24th June 2015

ABSTRACT

This study consisted of several parts which include development of tissue culture and regeneration system for *Jatropha curcas*, establishment of *Agrobacterium*-mediated transformation system and molecular analysis to confirm the integration of transgene in plant host genome. Observation made on callus induction after 60 days of culturing. Studies showed that MS medium supplemented with 2.0mg/L NAA and 1.0 mg/L BAP with large callus (green and compact structure) resulted the highest mean fresh weight, 4.754 grams. Callus regeneration of *J. curcas* was carried out using MS medium supplemented with combination concentration of BAP and IBA. However, calli were unable to regenerate in any of the treatment media tested after 12 weeks of culturing. Genetic transformation protocol for *J. curcas* callus mediated by *Agrobacterium tumefaciens* was optimized using green fluorescent protein as a reporter gene. *A. tumefaciens* at concentration of OD_{600nm} 0.6, showed the highest virulence on *J. curcas* callus. Two days of pre-culture period and 3 days of co-cultivation period were optimum for *J. curcas* transformations. Result also showed that 45 minutes of immersion and addition of 300 µM acetosyringone gave the highest percentage of positive transformants for *J. curcas* callus. The putative transformants were selected in the presence of hygromycin as a selection agent. Callus was also subjected to GUS histochemical assay analysis. Selected callus was excised and inoculated onto MS media supplemented with different concentrations of BAP and NAA in the range of 0.5mg/L to 5.0mg/L for regeneration. Molecular analyses were carried out to further confirm the expression of transgene in the putative transformants. PCR was carried out using the designed 35S primer and the result showed amplification of 454bp of 35S promoter region from the transformed callus. Real-time PCR was carried out to further confirm the integration of transgene and copy number of gene inserted in the putative transformants callus.

ABSTRAK

KAJIAN PEMBIAKAN In-vitro Jatropha curcas DAN TRANSFORMASI MENGGUNAKAN Agrobacterium tumefaciens

Penyelidikan ini di bahagikan kepada beberapa bahagian iaitu tisu kultur, dan regenerasi Jatropha curcas, transformasi menggunakan Agrobacterium tumefaciens dan analisis molecular untuk mengesahkan kejayaan transformasi. Pemerhatian di jalankan selepas 60 hari pengkulturan. Kajian menunjukkan bahawa pembentukan kalus berwarna hijau padat dengan berat segar tertinggi didapati pada MS yang mengandungi 2.0mg/L NAA and 1.0 mg/L BAP, 4.754 gram. Kajian regenerasi J. curcas dari kalus juga telah di jalankan. Hasil kajian menunjukkan kalus tidak berupaya membentuk pokok kecil pada media yang di uji. Protokol genetik transformasi untuk kalus J. curcas telah di optimumkan menggunakan green fluorescence protein sebagai penanda. A. tumefaciens pada kepekatan OD_{600nm}0.6 menunjukkan tahap penjangkitan yang tertinggi untuk kalus J. curcas. Dua hari jangka masa pre-kultur dan tiga hari jangka masa ko-kultivasi telah di optimumkan untuk transformasi J. curcas. Keputusan juga menunjukkan bahawa rendaman selama 45 minit dengan penambahan 300 µM asetosiringon memberi peratusan tertinggi kepada transforman positif untuk kalus J. curcas. Transforman-transforman putatif telah dipilih dalam kehadiran higromisin sebagai agen pemilihan. Kalus juga diuji berasaskan analisis histokimia GUS. Kalus dipilih dan dipotong, kemudian dipindahkan ke media pertumbuhan kalus yang mengandungi BAP dan NAA pada kepekatan yang berbeza (0.5 sehingga 5.0mg/l) untuk regenerasi. Analisis molekular dijalankan untuk mengesan ekspresi transgen dalam transforman positif pada kalus J. curcas. Keputusan tindak balas rantai polimer menunjukkan amplifikasi 454 bp jujukan promoter 35S daripada kalus yang telah ditransformkan. Analisis real time PCR juga telah dijalankan untuk mengesan integrasi transgen ke dalam genom J. Curcas dan bilangan gen yang telah masuk ke dalam kalus yang di transformkan, juga dengan menggunakan jujukan promoter 35S untuk konfirmasi.

TABLE OF CONTENT

CHAPTER 4 : RESULTS AND DISCUSSION

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

DNA	-	Deoxyribonucleic Acid
PCR	-	Polymerase Chain Reaction
qRT-PCR	-	Quantitative Realtime Polymerase Chain Reaction
DMSO	-	Dimethyl Sulfoxide
mg	-	Milligram
ml	-	Milliliter
µg	-	Microgram
µl	-	Microliter
l	-	liter
µm	-	Micrometer
µM	-	Micromolar
pmol	-	pico molar
mM	-	Millimolar
mm	-	Millimeter
nm	-	Nanometer
kbp	-	Kilobase pair
bp	-	Base pair
CTAB	-	Cetyltrimethylammonium bromide
M	-	Molarity
V	-	Voltage
EDTA	-	Ethylenediamine tetraacetic acid

dNTP	-	Deoxynucleoside triphosphate
LB	-	Luria Bertani
min	-	Minutes
sec	-	Second
%	-	Percentage
°C	-	Degree Celsius
GFP	-	Green Fluorescence Protein
GUS	-	β-glucuronidase
OD	-	Optical density
CT	-	Threshold cycle
Ppm	-	Part per million
Psi	-	Pressure unit
Kg	-	Kilogram
MCS	-	Multiple cloning site
Cm	-	Centimeter
Tm	-	Melting point

LIST OF APPENDIX

CHAPTER 1

INTRODUCTION

Jatropha curcas L., from the genus *Jatropha* classified as a shady woody plants belong to the division Spermatophyla and family Euphorbiaceae with approximately 170 known species (Pax, 1910). This species is also known by common names as Pokok Tangan-tangan, Pokok Jarak Pagar, Pokok Jarak Belanda (Malaysia), Jarak Cina, Tanggang-Tanggang Kvali (Indonesia), Physic nut, Hell oil and Pulza Tartago (English name) (Quattrocchi, 2000). The plant has its native distributional range in Mexico, Central America, Brazil, Bolivia, Peru, Argentina and Paraguay (USDA ANGRPNGRLBM, 2000). It has now been domesticated in Africa and Asia mainly due to its ability to grow in climatic zones in tropical and subtropical regions of the world particularly in marginal lands (Fairless, 2007).

Jatropha curcas grows fast, can survive in dry, semi dry and mining areas. Although *J. curcas* trees can live in dry climates, these plants also need water and nutrients for optimum growth (David *et al*., 2006). This low-growing plant with a height of three meters and has a relatively long life expectancy of more than 50 years (Tokio, 2008). *J. curcas* has 5 to 7 shallow lobed leaves with a length and width of 6 to 15 cm, which are arranged alternately. In the early stages of growth, shoots of *J. curcas* is green, gradually yellow and green when they reach the age of two weeks. The seeds were black, with a length of 2 cm and thickness is 1 cm (Heller *et al*., 1996). *J. curcas* is monoecious and flowers are unisexual (Dehgan and Webster, 1979). The maturity of this plant reached 90 days after flowering (Heller, 1996).

Jatropha curcas is non-edible because of its toxicity (Gubitz *et al*., 1999; Akintayo, 2004). According to Levis *et al*., (2000), children who ingest the seed of

J. curcas will suffer anxiety, vomiting and dehydration. Latex produced by this plant also has an antimicrobial agent to microorganisms such as *Staphylococcus aureus*, *Eschericia coli* and *Klebsiella pneumoniae* that can be used to accelerate wounds healing procedure (Thomas *et al*., 2008). Extracts from this species have been shown to have anti-tumor activity (Lin *et al*., 2003) and the leaves can be used as a remedy for malaria and high fever (Gubitz *et al*., 1999; Henning, 1997). Glycerin is a by-product of the process during the production of biodiesel from transesterification of *J. curcas* seed. In medical, glycerine-based drugs are produced which contain the anticancer substances. In addition, glycerine is used in making cosmetics. Oil product from seed of *J. curcas* can be used to make soap and massage oil (Abdrabbo *et al*., 2008). Additionally, the press cake can be used as a fertilizer and the organic waste products can be digested to produce biogas (CH_4) (Lopez *et al*., 1997; Staubmann *et al*., 1997; Gubitz *et al*., 1999).

Biodiesel is a renewable fuel and is safe to use, environmentally friendly and reduces air pollution. *J. curcas* seeds have high oil content (Wood, 2005). The *J. curcas* trees include 47% lipid, 25% protein, 10% fiber, 5% water and 8% carbohydrate (Akintayo, 2004). Aderibigbe *et al*., (1997) also reporting that the kernel contains 27-32% protein and 58-60% lipid. The seeds content of 30–35% oil can be converted into good quality biodiesel by transesterification (Foidl *et al*., 1996; Mandpe *et al*., 2005). For example, in India, the oil product is used as a main ingredient of biodiesel with an average of more than 20 Mt per year (Tiwari *et al*., 2007).

Since 30 years ago, the method of tissue culture or *in-vitro* culture has been widely used in ornamental plant industry and the conservation of plant genetic resources, particularly for species that have many advantages and in demand. Tissue culture is a method used to protect and propagate the plant cell and organ in the nutrient media under sterile environment that is free of microbial contamination (Hartmann *et al*., 2002). This technique allows preservation of the plant species, especially for rare species, threatened, endangered and endemic. Tissue culture methods have proven to propagate seedlings on a large scale in a short time compared to conventional methods. The largest contribution of tissue culture to the agricultural industry is in the field of plant breeding. Parent plants

are selected and cloned to produce a few more progenies, which was planted on a large scale.

Genetic engineering has many potential applications in fields such as medicine, agriculture and industries. In agriculture, the new application of genetic engineering includes the development of transgenic plants. The transgenic plants carry desirable traits like disease resistance, insect resistance, and herbicide resistance, promote flowering of the plant and so on. *Agrobacterium*-mediated technique is most widely used as it considered more efficient for a stable integration of genes into plant genome. It offers several advantages, including the ability to transfer large segments of DNA with minimal rearrangement, precise insertion of transgenes resulting in fewer copies of inserted genes, and simple technology with lower cost (Binns, 1990). Reporter genes have been used as a marker to visualize gene expression and protein localization *in-vivo* in a wide spectrum of prokaryotes and eukaryotes (Jefferson, 1987). Commonly used reporters include genes encoding chloramphenicol acetyl transferase (CAT), green fluorescent protein (GFP), luciferase (LUC), and β-glucuronidase (GUS). In this study, the *Agrobacterium*-mediated transformation system for *J. curcas* callus was optimized by using GFP as a reporter. Transformation efficiency of a specific plant can be evaluated conveniently with the GFP gene as a result of a simple assay using a fluorescence microscope. GFP gene from the jellyfish (*Aequiorea victoria*), as a reporter gene, has been successfully applied to visual selection of transformed plants (Stewart, 2005).

J. curcas oil demand growing in line with the increased price of diesel fuel. To meet the global demand for fuel, the effort to increase the cultivation and seed production can be done by propagating this plant with *in-vitro* culture techniques. Therefore, this study was done to produce a reproduction procedure by propagating the leaves of *J. curcas* through callus induction and callus regeneration which will increase the number of plants in minimum period of time and not affected by the surrounding environment. The development of an efficient regeneration system amenable to genetic transformation is a prerequisite for plant genetic engineering (Misra and Misra, 1993; Misra *et al*., 1994). Genetic engineering appears to be an effective approach to modify seed oil for higher