UNIVERSITI PUTRA MALAYSIA

CALLUS INDUCTION FROM EXPLANTS OF EURYCOMA LONGIFOLIA JACK (TONGKAT ALI) AND THE PRODUCTION OF 9-METHOXYCANTHIN-6-ONE FROM THE INDUCED CALLUS

ROSLI BIN NOORMI.

FBSB 2005 26

CALLUS INDUCTION FROM EXPLANTS OF *EURYCOMA LONGIFOLIA* JACK (TONGKAT ALI) AND THE PRODUCTION OF 9-METHOXYCANTHIN-6-ONE FROM THE INDUCED CALLUS

ROSLI BIN NOORMI

MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA

2005

CALLUS INDUCTION FROM EXPLANTS OF *EURYCOMA LONGIFOLIA* JACK (TONGKAT ALI) AND THE PRODUCTION OF 9-METHOXYCANTHIN-6-ONE FROM THE INDUCED CALLUS

ROSLI BIN NOORMI

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

June 2005

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

CALLUS INDUCTION FROM EXPLANTS OF *EURYCOMA LONGIFOLIA* JACK (TONGKAT ALI) AND THE PRODUCTION OF 9-METHOXYCANTHIN-6-ONE FROM THE INDUCED CALLUS

By

ROSLI BIN NOORMI

June 2005

Chairman : Professor Maziah Mahmood, PhD

Faculty : Biotechnology and Biomolecular Sciences

A study was carried out to determine 9-methoxycanthin-6-one distribution in intact plants and callus cultures of *Eurycoma longifolia* Jack. Qualitative analysis using TLC revealed that 9-methoxycanthin-6-one was present in leaf, petiole, stem, rachis, tap root, fibrous root, cotyledon and zygotic embryo of intact plant. The quantitative analysis using HPLC showed that the highest concentration of 9-methoxycanthin-6-one content was found in tap root. It was 4.10 mg/g DW tissues.

9-methoxycanthin-6-one was also present in callus tissues derived from different explants. The highest concentration was detected in fibrous root-derived callus (7.12 mg/g DW tissues). From the comparison between the data of callus tissues and intact plant parts, higher concentration of 9-methoxycanthin-6-one, more than 73.7 % was detected in callus tissues.

The ability of the callus to produce 9-methoxycanthin-6-one in different types of basal media (Murashige and Skoog, Gamborg, Schenk and Hildebrandt and White) were examined and identified. A basal MS medium exhibited the highest 9-methoxycanthin-6-one content (3.84 mg/g DW tissues). Hence, MS medium was selected for subsequent studies. The study on the effect of MS medium strenght (quarter, half, full and double strength) on 9-methoxycanthin-6-one production showed that for full strength of MS, 4.97 mg of the compound per DW tissues could be obtained from callus cultured in quarter MS basal media.

The effects of five different carbon sources such as sucrose, glucose, fructose, sorbitol and mannitol [(0, 1.0, 2.0, 3.0, 4.0 and 5.0 % (w/v)] on 9-methoxycanthin-6-one production were studied separately. Two % (w/v) fructose promoted the production of 9-methoxycanthin-6-one (4.59 mg/g DW) and gained the highest yield compared to other carbon sources tested.

A series of studies was also carried out to examine the effects of various concentrations [(0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L in full strength MS medium supplemented with 3 % (w/v) sucrose)] of PGRs (plant growth regulators auxins) (2,4-D, picloram, dicamba, NAA and IAA) on callus growth and 9-methoxycanthin-6-one production. The addition of 3.0 mg/L dicamba increased the 9-methoxycanthin-6-one production (12.3 mg/g DW tissues).

The effects of different initial pH values (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) on growth and 9-methoxycanthin-6-one production of *Eurycoma longifolia* Jack callus cultures was observed. The highest 9-methoxycanthin-6-one production was obtained at pH 5.5 (1.53 mg/g DW tissues).

Feeding of each amino acids (DL-tryptophan, L-phenylalanine and L-tryrosine) at a series of concentrations (0, $1x10^{-4}$, $1x10^{-3}$, $1x10^{-2}$, $1x10^{1}$, 1.0, $1x10^{1}$, $1x10^{2}$, $1x10^{3}$, $1x10^{4}$ μM) was observed to reduce the callus biomass growth as well as the 9-methoxycanthin-6-one production. The production of 9-methoxycanthin-6-one (2.34 mg/g DW tissues) in callus cultures also increased when the medium was supplemented with $1x10^{-1}$ μM phenylalanine.

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

INDUKSI KALUS DARIPADA EKSPLAN *EURYCOMA LONGIFOLIA* JACK (TONGKAT ALI) DAN PENGHASILAN 9-METOKSIKANTIN-6-ON DARIPADA KALUS YANG TELAH DIINDUKSI

Oleh

ROSLI BIN NOORMI

Jun 2005

Pengerusi : **Profesor Maziah Mahmood, PhD**

Fakulti : **Bioteknologi dan Sains Biomolekul**

Kajian telah dijalankan untuk menentukan taburan 9-metoksikantin-6-on di dalam pokok induk dan kultur kalus *Eurycoma longifolia* Jack. Analisa secara kualitatif menggunakan TLC telah menunjukkan bahawa 9-metoksikantin-6-on hadir di dalam daun, petiol, batang, rakis, akar tunjang, akar serabut, kotiledon, dan zigot embrio daripada pokok induk. Analisa secara kuantitatif menggunakan HPLC pula telah menunjukkan bahawa kepekatan tertinggi sebatian 9-metoksikantin-6-on (4.10 mg/g berat kering) telah hadir di dalam pokok induk iaitu di dalam akar tunjang. 9-metoksikantin-6-one juga telah di kesan hadir dalam tisu kalus daripada eksplan yang berbeza. Kepekatan tertinggi (7.12 mg/g berat kering) telah dikesan di dalam kalus akar serabut. Keputusan menunjukkan bahawa kepekatan 9-metoksikantin-6-on telah di dapati lebih tinggi (73.66%) di dalam tisu kalus berbanding daripada pokok induk.

Keupayaan kalus untuk menghasilkan 9-metoksikantin-6-one di dalam jenis media asas MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972), WH (White, 1963) and B5 (Gamborg *et al.*, 1968)) yang digunakan telah diperiksa. Didapati bahawa media asas MS mempamerkan kandungan 9-metoksikantin-6-on tertinggi (3.84 mg/g berat kering). Media MS telah digunakan untuk kajian selanjutnya. Sementara itu, kajian terhadap kesan media MS ke atas kekuatan media (suku, separuh, penuh dan dua kali ganda kekuatan) telah menunjukkan bahawa penghasilan 9-metoksikantin-6-on tertinggi (4.97 mg/g berat kering) telah diperolehi daripada kultur kalus di dalam suku kekuatan media asas MS.

Kesan lima sumber karbon yang berbeza seperti sukrosa, glukosa, fruktosa, sorbitol dan manitol [0, 1, 2, 3, 4, dan 5 % (w/v)] ke atas penghasilan 9-metoksikantin-6-on telah dikaji satu persatu. Pemberian fruktosa pada 2% (berat/isipadu) talah mempamerkan penghasilan 9-metoksikantin-6-on (4.59 mg/g berat kering) dan merupakan penghasilan tertinggi berbanding dengan sumber-sumber karbon yang dikaji.

Kajian juga telah dijalankan untuk memeriksa kesan pelbagai kepekatan [(0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L) dalam media asas MS penuh dibekalkan dengan 3 % (berat/isipadu) sukrosa] pengatur pertumbuhan tumbuhan (PGRs) (2,4-D, pikloram, dikamba, NAA and IAA) pada pertumbuhan kalus dan penghasilan 9-metoksikan-6-on. Penambahan 3.0 mg/L dikamba telah meningkatkan penghasilan 9-metoksikan-6-on (12.3 mg/g berat kering).

Kesan perbezaan pH awal (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) pada pertumbuhan kultur kalus dan penghasilan 9-metoksikan-6-on daripada *Eurycoma longifolia* Jack telah diperhatikan. Penghasilan 9-metoksikan-6-on tertinggi telah berupaya diperolehi pada pH 5.5 (1.53 mg/g berat kering).

Pembekalan setiap asid amino (DL-triptofan, L-fenilalanina and L-tirosina) pada kepekatan (0, $1x10^{-4}$, $1x10^{-3}$, $1x10^{-2}$, $1x10^{1}$, 1.0, $1x10^{1}$, $1x10^{2}$, $1x10^{3}$, $1x10^{4}$ μM) telah diperhatikan untuk meningkatkan pertumbuhan kalus juga penghasilan 9-metoksikan-6-on. Penghasilan 9-metoksikan-6-on (2.34 mg/g berat/isipadu) juga telah meningkat apabila media dibekalkan dengan $1x10^{-1}$ μM fenilalanina.

ACKNOWLEDGEMENTS

In the name of Allah, Most Gracious, Most Merciful. Due to His willingness, the completion of his study was made possible.

I would like to express my deeply appreciation and gratitude to the chairman of my supervisory committee, Prof Hjh. Dr. Maziah Mahmood, for her help, guidance and constant support in making the completion of this thesis a success. I am also grateful to Professor Dr. Chan Kit Lam from School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang for his supports, advice and generous assistance. The help rendered by my supervisory committee, Assoc. Prof. Dr. Nor'aini Mohd. Fadzillah and Dr. Janna Ong Abdullah are greatly appreciated.

I greatly appreciate my parents, Norsiah Bt Maggon and Noormi Bin Hussain for their encouragement. To all of my lab-mates namely Sobri H, Anna, Ramani P, Tee CS, CY, Deswina, Chew, Chia Yee, Wai Sum Y, Sree, Suzita S, Nurkhanani, Sinarwati, Naila, Ali, Wai Sun, Hatta, and Uncle Thong, I thank for theirs strongly support.

Last but not least, my appreciation goes to the members from Dr. Radzali's lab; Ms. Loo Wai Sum and Kak Aishah their support during the whole study period and also others from Dr. Yunus's lab; Arif Khalid for his valuable guidance throughout HPLC analysis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Jack callus cultures by addition of phenilalanine, tryptophan and tyrosine supplemented with B5 vitamins and incubated at pH 5.7, 25 $\pm$ 2 ^{o}C with a photoperiod of 16 hours fluorescent light.

LIST OF ABBREVIATIONS

%	Percentage
C	Degree Celcius
2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-benzylaminopurine
$CHCl_3$	Chloroform
Dicamba	3,6-Dichloro-o-aniscic acid
DW	Dry weight
Fwt	Fresh weight
G	Gram
IAA	Indole-3-acetic acid
L	Litre
Mg	Milligram
NAA	α-Naphthaleneacetic acid
PGR(s)	Plant growth regulator(s)
Picloram	4-Amino-3,5,6-trichloropicolinic acid
R_f	Distance of the substance over distance of the solvent movement
R_t	Retention time (min)
w/v	Weight for volume
µg	Microgram

LIST OF ABBREVIATIONS

%	Percentage
C	Degree Celcius
2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-benzylaminopurine
$CHCl_3$	Chloroform
Dicamba	3,6-Dichloro-o-aniscic acid
DW	Dry weight
Fwt	Fresh weight
G	Gram
IAA	Indole-3-acetic acid
L	Litre
Mg	Milligram
NAA	α-Naphthaleneacetic acid
PGR(s)	Plant growth regulator(s)
Picloram	4-Amino-3,5,6-trichloropicolinic acid
R_f	Distance of the substance over distance of the solvent movement
R_t	Retention time (min)
w/v	Weight for volume
µg	Microgram

CHAPTER 1

INTRODUCTION

It is well known that plants produce a variety of economically important secondary metabolites, with approximately 4,000 new discoveries every year, total up to over 100,000 known compounds (Wei *et al.*, 2002). Some of the plant biochemicals are used in the healthcare, food, flavour and cosmetics industries (Frank and Masanaru, 1995; Pascal and Johan, 2002). Meanwhile, others are used for the production of agrochemicals, fragrances, colours and biopesticides (Ramachandra and Ravishankar, 2002; Verpoorte and Memelink, 2003). Examples of plant secondary metabolites used for the production of pharmaceuticals are dopamine, morphine, codeine, reserpine, and the anticancer drugs such as vincristine, vinblastine and taxol (Frank and Masanaru, 1995).

It was estimated that only 10 - 15 % of the known higher plant species had been investigated for their important bioactive compounds (Za'rate *et al.*, 2001). Natural drug production from plants frequently involves extraction from living plants. This method is often tedious, costly and low yields. Furthermore, the target compounds may only be available seasonally (Kutney, 1998).

Thus, plant cell culture methodologies have the potential to overcome these problems (Gao *et al.*, 2000). Plant tissue culture offers an alternative approach for the production and manufacturing of natural and additional plant secondary products (Oksman –

Caldentey *et al.*, 1994). This technology also offers an attractive source for the production of high-value secondary metabolites (Alfermann and Petersen, 1995; Stockigt *et al*, 1995). This technology has the advantages over the conventional agriculture productions as it is independent on geographical seasonal variations, the continuous supply of products has uniform quality and yield is ensured. In addition, it is possible to produce novel compounds and increase the production efficiency by applying cell culture technology (Ramachandra and Ravishankar 2002).

Moreover, *in vitro* culture enables the possibility to harvest the desired natural products without contamination of pesticides, herbicides or insecticides, and also to overcomes the natural heterogeneity in plant materials and variations in product contents (Taticek *et al.*, 1991). *In vitro* techniques can be an important approach to produce useful secondary products (Aziz *et al.*, 2002). There have been a number of reports on using plant and organ tissues to produce a wide range of different secondary compounds (Rhodes *et al.*, 1990;1997).

The plant material used in this study is *Eurycoma longifolia* Jack (Figure 1), which is also known locally as 'Tongkat Ali' and 'Pasakbumi', in Indonesia. This plant is reputed to increase male virility and sexual prowess and has gained notoriety as a male aphrodisiac (Kuo *et al.,* 2003). Pharmacological evaluations on the various compounds isolated from *Eurycoma longifolia* Jack showed that it also possessed anti-malaria (Kardono *et al.*, 1991); anti-ulcer (Tada *et al.*, 1991); cytotoxic (Morita *et al.*, 1990; Kardono *et al.*, 1991; Itokawa *et al.*, 1992; Morita *et al.*, 1993); antimalarial