UNIVERSITI PUTRA MALAYSIA

OPTIMIZATION OF INVITRO CULTURES AND EFFECTS OF ELICITATION THE FLAVONOID CONTENTS OF PEGAGA (CENTELLA ASIATICA L. URBAN)

ONG MEI KYING

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By

ONG MEI KYING

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

February 2008

Dedicated with love and gratitude to:

My father, Ong Siow Kin

and

My mother, Ng Siew Cheng

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

OPTIMIZATION OF *IN VITRO* CULTURES AND EFFECTS OF ELICITATION ON THE FLAVONOID CONTENTS OF PEGAGA (*CENTELLA ASIATICA* L. URBAN)

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Chairman : Associate Professor Norihan Mohd Saleh, PhD

Faculty : Biotechnology and Biomolecular Sciences

Secondary metabolites in *Centella asiatica*, especially flavonoids is known to possess strong antioxidative activity and widely investigated as a new source of active compound for health benefits. The flavonoids content in field planted *Centella asiatica* is very low, therefore high volume of *Centella asiatica* plant supply is needed to fulfill the increase demand for the active compounds as an important nutraceutical resources. The main aim of this research done was to obtain the most applicable, simplest and effective *in vitro* approaches that can increase the flavonoids content in *Centella asiatica* (CA05). Many research studies done have proved that flavonoids content can be successfully enhanced through elicitation. In this study, elicitation of important flavonoids such as catechin was investigated and their presence in various tissues of *Centella asiatica* (CA05) planted using various cultivation methods were assessed. The initial work involved the optimization of plantlet regeneration and root

culture of *Centella asiatica* (CA05). Regeneration of *Centella asiatica* (CA05) from various explants is necessary in order to produce continuous supply of plant materials for further manipulation. In addition to *in vitro* plantlets and root culture, hydroponically grown plantlets were also used for flavonoids elicitation using biotic elicitors, namely chitosan and yeast extract. Various techniques of sterilization and media formulation using different plant growth regulators such as auxin and cytokinin were applied to regenerate the plant. The explants were sterilized using 30% Clorox (1.58% sodium hypochlorite) for 5 minutes (leaf), 30% Clorox (1.58% sodium hypochlorite) for 15 minutes (stem), 0.05% mercury chloride for 5 minutes and followed by 30% Clorox (1.58% sodium hypochlorite) for 20 minutes (seed) for 56.7% of viable leaf explant, 75.0% of viable stem explant and 48.3% of sterile seedlings, respectively. For plant regeneration, MS supplemented with 2.26µM Indole-3-acetic acid (IAA) and 2.26µM 6-Benzylaminopurine (BAP) exhibited 38.0% of shoot regeneration frequency from leaf explants. MS supplemented with 2.26µM Indole-3-butyric acid (IBA) showed rapid induction of root from stem (63.0%) after 7 days. In this study, the seed of *Centella asiatica* were treated with various techniques to break the seed dormancy. The hard seed were pretreated by scarification and soaking for various time regime. Effect of presoaking in MSO liquid medium for 1 day before culture gave the highest percentage means of germination (81.0%). High pressure liquid chromatography (HPLC) was used to determine the flavonoids in various *Centella asiatica* plant parts (root, stem, leaf) grow*n in vitro* and hydroponics. Catechin is detected to be major flavonoids in *Centella asiatica* in addition to naringin, hesperidin, rutin and myricetin. Result from the study showed that *in vitro* plantlets

contain high total flavonoids (4456.9 ± 287.5 µg/g) compared to that of hydroponics-grown plantlets (2401.0 ± 148.4 µg/g) and that of field plant (2323.5 ± 376.8 µg/g). In general, leaf of *Centella asiatica* was found to contain the highest flavonoids content compared to either root or stem. Effects of both elicitors, namely chitosan and yeast extract on flavonoids production were evaluated. Result of the study showed that root culture treated with 0.5 mg/l chitosan for one week showed the highest flavonoids (2968.2 ± 66.1 µg/g) with 2.28 fold increase of catechin (128.1% higher than control). While, for the elicitation in *in vitro* plantlet with 5 mg/l chitosan in 1 week, the highest total flavonoids amount was recorded at 5530.6 ± 385.5 µg/g with increase of 17.9% or 1.2 fold compared to control. Prolong exposure to elicitors up to four weeks did not show any increase of flavonoids content in all cultivated plant tissues. Therefore, it can be concluded that, flavonoids production was improved with chitosan elicitation, and it can be helpful in increasing the productivity of flavonoids.

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

PENINGKATAN KULTUR *IN VITRO* DAN KESAN PENGARUHAN KANDUNGAN FLAVONOID DALAM PEGAGA (*CENTELLA ASIATICA L. URBAN)*

Oleh

ONG MEI KYING

Februari 2008

Pengerusi : Profesor Madya Norihan Mohd Saleh, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Metabolit sekunder yang dikandungi oleh *Centella asiatica*, terutamanya flavonoids, diketahui mempunyai aktiviti antioksidatif dan telah dikaji dengan meluas sebagai sumber baru sebatian aktif yang baik untuk kesihatan. Disebabkan kandungan flavonoids yang rendah dalam pokok *Centella asiatica* tanaman ladang, bekalan pokok *Centella asiatica* yang tinggi diperlukan untuk memenuhi permintaan yang meningkat terhadap bahan aktif ini sebagai sumber nutraceutika yang penting. Tujuan utama kajian ini dijalankan adalah untuk memperolehi pendekatan secara *in vitro* yang paling mudah, berkesan dan berkeupayaan untuk meningkatkan kandungan flavonoids dalam pokok *Centella asiatica* (CA05). Kebanyakan kajian penyelidikan telah berjaya membuktikan kandungan flavonoids boleh ditingkatkan melalui pengaruhan. Dalam kajian ini, oleh kerana kandungan flavonoids boleh ditingkatkan melalui pengaruhan secara *in vitro*, pengaruhan flavonoids penting seperti catechin telah dikaji dan kewujudannya di dalam pelbagai tisu *Centella asiatica* (CA05) yang

dibiakkan menggunakan pelbagai kaedah penanaman telah dibuat penilaian. Kajian awal melibatkan regenerasi anak pokok dan kultur akar *Centella asiatica* (CA05). Regenerasi anak pokok *Centella asiatica* (CA05) daripada pelbagai eksplan adalah perlu untuk menghasilkan sumber pokok yang berterusan bagi manipulasi seterusnya. Selain kultur akar dan pokok *in vitro*, pokok yang ditanam melalui teknik hidroponik juga digunakan untuk pengaruhan flavonoids dengan menggunakan agen perangsang biotik, seperti chitosan dan ekstrak yis. Pelbagai teknik pensterilan dan formulasi media dengan penggunaan pengawalatur tumbuhan seperti auksin dan sitokinin digunakan untuk meregenerasikan pokok ini. Eksplan daun disteril dengan 30% Klorox (1.58% sodium hipoklorit) selama 5 minit menghasilkan 56.7% eksplan hidup, 75% eksplan batang hidup dihasilkan dengan 30% Klorox (1.58% sodium hipoklorit) selama 15 minit, manakala pensterilan biji benih dengan menggunakan 0.05% merkuri klorida selama 5 minit, diikuti dengan 30% Klorox (1.58% sodium hipoklorit) selama 20 minit adalah perlu untuk menghasilkan 48.3% pokok *in vitro*. Untuk regenerasi pokok, media Murashige and Skoog (MS) yang ditambahkan dengan 2.26 μM IAA dan 2.26 μM BAP telah menunjukkan 38.0% frekuensi regenerasi pucuk daripada eksplan daun. Media Murashige and Skoog (MS) yang ditambahkan dengan 2.26 μM IBA menunjukkan kadar penghasilan akar daripada eksplan batang (63.0%) selepas 1 minggu. Dalam kajian ini juga, biji benih *Centella asiatica* telah dirawat dengan pelbagai teknik memecahkan kedormanan benih. Benih yang berkulit keras ini dipra-rawat dengan sagatan dan pra-rendaman dalam pelbagai masa rendaman. Kesan pra-rendaman benih di dalam MSO medium cecair selama sehari sebelum dikultur memberi peratusan percambahan yang tertinggi iaitu 81.0%.

Kromatografi cecair bertekanan tinggi (HPLC) digunakan untuk menentukan flavonoids yang terkandung dalam pelbagai bahagian tisu *Centella asiatica* (akar, batang, daun) yang dikultur secara *in vitro* mahupun hidroponik. Catechin dalam *Centella asiatica* dikesan paling tinggi selain naringin, hesperidin, rutin dan myricetin. Keputusan kajian ini menunjukkan pokok *in vitro* mengandungi kandungan flavonoids yang tertinggi iaitu 4456.9 $\pm$ 287.5 μg/g berbanding dengan pokok hidroponik (2401.0 $\pm$ 148.4 μg/g) dan pokok tanaman ladang (2323.5 $\pm$ 376.8 μg/g). Secara amnya, daun *Centella asiatica* ditemui mengandungi kandungan flavonoids yang tertinggi berbanding akar mahupun batang. Kesan peningkatan kandungan flavonoids melalui penggunaan agen perangsang biotik seperti chitosan dan ekstrak yis telah dinilai. Keputusan kajian menunjukkan kultur akar yang dirawat dengan 0.5 mg/l chitosan selama seminggu menunjukkan kandungan flavonoids tertinggi iaitu 2968.2 $\pm$ 66.1 μg/g, dengan peningkatan catechin sebanyak 2.28 ganda (128.1 % lebih tinggi berbanding kultur asal tanpa rawatan). Sementara, pengaruhan pokok *in vitro* dengan 5 mg/l chitosan selama seminggu, mencatat kandungan jumlah flavonoids tertinggi sebanyak 5530.6 $\pm$ 385.5 μg/g dengan peningkatan 17.9 % atau 1.2 ganda berbanding kultur asal tanpa rawatan. Pendedahan tumbuhan yang berlanjutan kepada agen pengaruh biotik melebihi empat minggu tidak menunjukkan peningkatan kandungan flavonoids dalam semua tisu *Centella asiatica.* Oleh itu, adalah dapat dirumuskan bahawa producktiviti flavonoids boleh dimajukan dengan pengaruhan chitosan dan ini dapat membantu meningkatkan produktiviti flavonoids.

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I certify that an Examination Committee has met on 5th February 2008 to conduct the final examination of Ong Mei Kying on her Master of Science thesis entitled "Optimization of *In Vitro* Cultures and Effects of Elicitation on the Flavonoids Contents of Pegaga (*Centella asiatica* L. Urban)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Science.

Members of the Examination Committee were as follows:

Janna Ong Abdullah, PhD
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Suhaimi Napis, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Mohd Puad Abdullah, PhD
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Muhammad Nor Omar, PhD
Product Development and Advisory Services
Malaysian Palm Oil Board (MPOB)
Malaysia
(External Examiner)

_____
HASANAH MOHD. GHAZALI, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 26 May 2008

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:

Norihan Mohd. Saleh, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Azizah Abdul Hamid, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 12 June 2008

DECLARATION

I declare that the thesis is my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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ONG MEI KYING

Date: 24 April 2008

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