UNIVERSITI PUTRA MALAYSIA

OPTIMIZATION OF CULTURE PARAMETERS FOR FLAVONOID PRODUCTION FROM CALLUS AND SUSPENSION CULTURES OF THE KARAS TREE (AQUILARIA MALACCENSIS LAM.)

NURUL HAZWANI BINTI DAUD

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By

NURUL HAZWANI BINTI DAUD

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

October 2013

SPECIALLY DEDICATED TO:

My Beloved

Late Father, Mother, Brothers, Sisters

And all my friends

''Who have been a great source of motivation and supported me since the beginning of my studies''

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 CULTURE PARAMETERS FOR FLAVONOID PRODUCTION FROM CALLUS AND SUSPENSION CULTURES OF THE KARAS TREE (*AQUILARIA MALACCENSIS* LAM.)

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Chairman : Associate Professor Rozi Mohamed, PhD

Faculty : Forestry

Plant tissue culture technique has been shown to be efficient for producing flavonoids at higher amount and lesser time period compared to intact plant. This study aimed at producing flavonoids in elevated amounts using callus and cell suspension cultures of *A. malaccensis*. *Aquilaria* trees not only produce secondary metabolites known as agarwood (*gaharu*) but also rich in flavonoids.

To introduce plant materials into tissue culture, different sterilization regimes for leaf, nodes and seeds of *A. malaccensis*, were investigated. Pre-sterilization using 0.2% Benomyl for 15 minutes improved the number of 'sterile and survived' individuals for all types of explants (83-90%), after surface sterilization using mercury chloride (0.1 to 0.2 % $HgCl_2$). The most favorable explants for producing highest flavonoid cell lines

were determined between leaf and nodal explants by using spectrophotometer and HPLC analysis. Result showed that flavonoid content in the leaf explants (8.85 ± 0.76 mg/g dry weight (DW)) was 7-fold higher than that of nodal explants (1.25 ± 0.53 mg/g DW). Leaf contained high amount of quercetin (16.19 ± 1.02 µg/g DW) then rutin (0.21 ± 0.31 µg/g DW). Nodes explants contained quercetin and rutin at 0.94 ± 0.07 µg/g DW and 0.06 ± 0.01 µg/g DW, respectively. Kaempherol was not detected in any of the explants.

Leaf was used as explant for callus induction due to high flavonoid content and placed on different types of plant hormones to indicate the type of hormone use to induce callus. Callus grew best on Murashige and Skoog (MS) medium supplemented with 2.0 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.5 mg/L 6-benzylaminopurine (BAP). Total flavonoid content in MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L was 6- fold higher than in phytohormone- free MS medium. Using this optimal hormone combination, callus growth was observed on different strengths of MS medium, carbon sources and pH. Full-strength MS medium supplemented with 2.0 mg/L 2,4-D and 0.5 mg/L BAP, 20 g/L sucrose, in pH 5.7, significantly yielded the highest biomass (231 ± 0.13 mg DW /culture) of callus. These calli were used to initiate the cell suspension cultures.

To enhance the flavonoid production in callus and cell suspension cultures, examine phenylalanine (Phe) was used as a precursor in ranged of 20 to 100 mg/L. Cell suspension culture added 60 mg/L Phe yielded the highest amount of total flavonoid

(37.92 ± 0.77 mg/g DW), 4.8-fold higher than Phe-free cell culture, and 2-fold higher than that in calli (19.11 ± 0.36 mg/g DW). The suspension culture with 60 mg/L Phe had the highest amount of rutin (17.69 ± 1.37 µg/g DW), quercetin (3.46 ± 0.97 µg/g DW) and kaempherol (2.28 ± 1.67 µg/g DW). When compared the cell suspension culture with 60 mg/L Phe to Phe-free cell culture, only rutin and quercetin were detected and at lower levels, 1/10-fold and 1/4-fold, respectively. This study demonstrated that callus and cell suspension cultures of *A. malaccensis* produced flavonoids, and feeding the cultures with Phe elevated flavonoids levels and induced production of additional compound which is kaempherol.

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

MENGOPTIMUMKAN KULTUR PARAMETER UNTUK PENGHASILAN FLAVONOID DARI KALUS DAN AMPAIAN SEL POKOK KARAS (*AQUILARIA MALACCENSIS* LAM.)

Oleh

NURUL HAZWANI BINTI DAUD

Oktober 2013

Pengerusi : Profesor Madya Rozi Mohamed, PhD

Fakulti : Perhutanan

Tisu kultur tumbuhan telah menunjukkan keberkesanan untuk menghasilkan flavonoid yang tinggi dalam tempoh masa yang lebih singkat berbanding dengan pokok induk. Kajian ini bertujuan untuk menghasilkan flavonoid pada kadar yang tinggi menggunakan kalus dan ampaian sel *Aquilaria malaccensis.* Pokok *Aquilaria* bukan sahaja menghasilkan metabolit sekunder yang dikenali sebagai gaharu tetapi juga kaya dengan flavonoid.

Untuk memperkenalkan bahagian tumbuhan ke dalam tisu kultur, cara pensterilan yang berbeza untuk daun, nodal dan biji benih *A. malaccensis* telah dikaji. Pra- pensterilan menggunakan 0.2% Binomil selama 15 minit telah meningkatkan bilangan individu yang nyah kuman dan sihat untuk semua jenis eksplan (83-90%), setelah pensterilan permukaan menggunakan merkuri klorida (0.1 ke 0.2% $HgCl_2$). Eksplan yang

menghasilkan kandungan flavonoid yang tertinggi telah ditentukan di antara eksplan daun dan nodal dengan menggunakan spektrofotometer dan analisis HPLC. Keputusan menunjukkan, kandungan flavonoid di dalam eksplan daun (8.85 ± 0.76 mg/g berat kering) adalah 7-kali ganda lebih tinggi daripada eksplan nodal (1.25 ± 0.53 mg/g berat kering). Daun mengandungi jumlah kuersetin yang tinggi (16.19 ± 1.02 µg/g berat kering) diikuti dengan rutin (0.21 ± 0.31 µg/g berat kering). Eksplan nodal mengandungi jumlah kuersetin dan rutin yang rendah (masing-masing dengan berat kering 0.94 ± 0.07 µg/g dan 0.06 ± 0.01 µg/g). Kaempferol tidak dikenalpasti di dalam mana-mana eksplan.

Daun telah digunakan sebagai eksplan untuk induksi kalus kerana mempunyai kandungan flavonoid yang tinggi dan diletakkan di dalam hormon tumbuhan yang berbeza untuk mengenal pasti jenis hormon utuk pertumbuhan kalus. Kalus hidup dengan sihat di dalam media Murashige dan Skoog (MS) yang mengandungi 2.0 mg/L asid 2,4-diklorofenoksi asetik (2,4-D) dan 0.5 mg/L 6-benzilamino Purina (BAP). Kandungan flavonoid di dalam media yang dibekalkan dengan 2.0 mg/L 2,4-D dan 0.5 mg/L BAP adalah 6-kali ganda lebih tinggi daripada kalus yang tumbuh di dalam media MS tanpa hormon (kontrol). Menggunakan kombinasi hormon yang optimum ini, pertumbuhan kalus di dalam media MS yang berbeza kepekatan, sumber karbon dan pH dikaji. Media MS dengan kepekatan penuh yang ditambah dengan 2.0 mg/L 2,4-D, 0.5 mg/L BAP, 20 g/L sukrosa dan pH pada 5.7, menghasilkan berat kalus yang tertinggi (231 ± 0.13 mg berat kering /kultur). Kalus tersebut telah digunakan untuk menghasilkan ampaian sel.

Bagi meningkatkan pengeluaran flavonoid dalam kultur kalus dan ampaian sel, fenilalina telah digunakan sebagai prekursor di antara 20 hingga 100 mg/L. Kultur sel ampaian dengan 60 mg/L fenilalanina menghasilkan kandungan flavonoid yang tertinggi (37.92 ± 0.77 mg/g berat kering), iaitu 4.8-kali ganda tinggi daripada ampaian sel tanpa fenilalanina, dan 2- kali ganda tinggi daripada kalus (19.11 ± 0.36 mg/g berat kering). Kultur sel ampaian dengan 60 mg/L fenilalanina mempunyai jumlah tertinggi rutin (17.69 ± 1.37 µg/g berat kering), kuersetin (3.46 ± 0.97 µg/g berat kering) dan kaempferol (2.28 ± 1.67 µg/g berat kering). Apabila sel ampaian yang ditambah dengan 60 mg/L fenilalanina dibandingkan dengan kultur sel ampaian tanpa fenilalanina, hanya rutin dan kuersetin dikesan tetapi masing-masing 1/10 dan 1/4- kali ganda lebih rendah. Kajian ini menunjukkan kultur kalus dan sel ampaian *A. malaccensis* menghasilkan flavonoid, dan penambahan fenilalanina ke dalam kultur meningkatkan paras flavonoid dan menggalakkan penghasilan sebatian flavonoid yang lain iaitu kaempferol.

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I certify that a Thesis Examination Committee has met on 9th October 2013 to conduct the final examination of Nurul Hazwani Binti Daud on her thesis entitled "Optimization of Culture Parameters for Flavonoid Production from Callus and Suspension Cultures of the *Aquilaria malaccensis* Lam." in accordance with 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 degree of Master of Science.

Members of the Thesis Examination Committee were as follows:

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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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Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citation, 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 institutions.

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NURUL HAZWANI DAUD

Date: 9 October 2013

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