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Cik Nursyatina Abdul Raof
Pegawai Sains
Pelantar Sains Fundamental
Pejabat Pelantar Penyelidikan

Encik Mohd Pisol Ghadzali
Pemangku Ketua Pustakawan
Perpustakaan Hamzah Sendut 1

Disampaikan satu salinan laporan akhir projek untuk simpanan Perpustakaan

Puan Ansuya Narhari
Penolong Bendahari
Unit Kumpulan Wang Penyelidikan
Jabatan Bendahari

Sila ambil tindakan menutup akaun projek pada **28 November 2008** dan sila kemukakan satu salinan kewangan terakhir ke pejabat (RCMO)

CMI/liza...laporanakhirsaga/08

ii) Objectives Achieved (Please state the extent to which the project objectives was achieved)

The effects of different supplement (such as elicitors) mixtures, individual treatments and consecutive regimes were thoroughly successful.

iii) Objectives not achieved (Please identify the objectives that were not achieved and give reasons)

Pravastatin was not employed in the study due to the chemical being unusable due to mishandling during the shipment.

C. **Assessment of Research Approach** (Please highlight the main steps actually performed and indicate any major departure from the planned approach or any difficulty encountered)

MATERIALS AND METHODS

3.1. EXPLANT SOURCE (Title in Caps. Do the same for all titles)

Pomelo fruits (*Citrus grandis*) Osbeck were obtained from a plantation in Sungai Gedong, Perak. The fruits were carefully selected direct from the trees and free from insect attacks, disease infection and necrosis.

3.2 . CHEMICALS

Chemicals (analytical grade) for the Murashige and Skoog (MS) medium preparation were purchased from Sigma Chemicals, St. Louis, United States; Koch-light Laboratory, Colbrook, Bucks, England; Fluka, Japan, and Merck, Darmstadt, German. Agar was bought from Bacto Difco Laboratories, Detroit, USA. Plant growth regulators such as 2, 4-dichlorophenoxyacetic acid (2,4-D), and 6-furfurylaminopurine (kinetin) and abscisic acid (ABA) were obtained from Fluka, Japan. Chemicals for media preparation on elicitation studies, such as limonene, linalool, and mevalonic acid lactone were purchased from Fluka, Japan, while yeast extract was purchased from Becton Dickinson & Co, USA.

3.3 MS Media Preparation

Media used in this study was MS (Murashige & Skoog, 1962) basal medium (Appendix 5.1) with 510mg/L phosphate, 3ppm 2, 4-D, 3ppm kinetin and 0.2mg/L ABA..

3.4 Modified MS Media

The media is composed of MS basal media with 510mg/L phosphate, 30g/L sucrose, 3 mg/L 2,4-D, 3 mg/L kinetin, 0.2mg/L ABA and 8g/L agar based on the study by Zarina (2004) and is called Modified MS media for the purpose of this study. The callus grown on the Modified MS media was subcultured at an interval of 4 weeks and maintained for 5 months to determine the content of limonene and linalool (Method 3.12).

3.5 Explants Sterilization and Viability

3.5.1 A study on the effect of concentration of sodium hypochlorite (Chlorox) and sterilization time on *C. grandis*

The sterilization study was done using Chlorox®, a commercial disinfectant which contains 5.75% sodium hypochlorite inside a sterilized laminar flow hood.

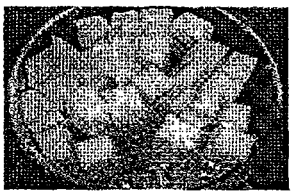


Plate 3.1: Cut albedo tissues in a Petri plate

3.5.2 Study on the Viability of Sterilized Albedo Tissues

After four weeks of incubation, the albedo tissues sterilized with 10 different methods (A to J) were observed for contamination and on viability.

3.6 Determination of Moisture Content

3.6.1 Determination of Fresh *C. grandis* Fruit Moisture Content

The fruit used for this study was a fresh, green-colored fruit which was 6cm in diameter. The fruit was first cut and separated into skin, flavedo, albedo, and juice vesicles. These parts were weighed and recorded as wet weight. Then these parts were dried individually in an oven at 50±2°C for 8 hours.

3.6.2 Determination of Callus Moisture Content

In the present study, moisture content was determined to ascertain the calli to be alive and growing. content was then calculated using the Eq. 3.1. A total of three samples were analyzed to determine the average percentage of moisture content.

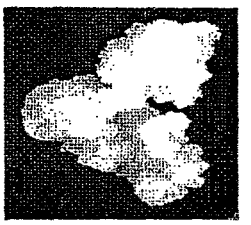


Plate 3.2: Sixty-day old callus culture grown on Modified MS media.

3.7 Determination of Growth Period of Callus Culture

Weight of the callus cultures formed from the albedo tissues was measured to determine the time period for maximum callus growth.

3.8 A Study on the Effect of Subculturing Callus

In the present study, the effect of subculturing callus in the MS media (Method 3.4) on the callus growth rate was determined. In the present study, callus was grown for 28 days in MS media before subculturing further for another 28 days.

3.9 Observations of Callus Grown on Modified MS media using Scanning Electron Microscope (SEM)

3.10 Studies on the Accumulation of Limonene and Linalool in Callus and Albedo Tissue

Callus tissues used for this study were 1, 2, 3, 4 and 5 month old. Albedo tissues, however, were only 7, 14, 21, 28 and 35 days old. Both the callus and albedo tissues were maintained on the Modified MS media

before extraction was carried out for the appropriate culture periods mentioned above for the individual tissues studied.

3.10.1 Extraction using Soxhlet Apparatus

The most conventional extraction technique employed is Soxhlet method. This process is very efficient to extract from solid samples.

3.11 Gas Chromatography method for quantification of Limonene and Linalool

A simple gas chromatographic method was developed for the quantification of limonene and linalool in the plant tissue samples.

3.11.1 Instrumentation

The gas chromatography (GC) system used in this experiment was Shimadzu, GC-17A from Japan.

3.11.2 Chromatography Conditions

Analysis of gas chromatography had been carried out to determine the quantity of limonene and linalool in the samples.

3.11.3 Preparation of stock and calibration standards of Limonene and Linalool

Stock standard solutions of limonene and linalool at 100 ppm were prepared by dissolving appropriate amount of the compounds in methanol.

3.11.4 Method validation

The linearity of limonene and linalool was conducted using six calibration standards at concentrations of 0.2 ppm, 1.0 ppm, 2.0 ppm, 3.0 ppm, 4.0 ppm and 6.0 ppm. The peak area of limonene and linalool was plotted against the corresponding concentration to obtain the calibration curve.

3.11.5 Quantitative Analysis of Limonene and Linalool in Tissue Samples

Analysis of limonene and linalool content in various tissue samples (planted in Modified MS media) that were 1 to 5 months old, with a one month interval in between (for callus cultures), and 7 to 35 days old, with a 7 day interval in between (for albedo tissue cultures) were determined by comparing their retention time with the retention time of the standard samples, using the above developed GC-FID method.

3.12 Study on the Effect of Elicitation using Yeast Extract on Albedo Tissue

The objective of this study was to determine the effect of elicitation on the albedo tissue cultures in Modified MS media using yeast extract as elicitor.

3.12.1 Media Preparation of Yeast Extract

Yeast extract purchased from Becton Dickinson & Co., USA was used in this study. Yeast extract was directly added into the Modified MS media (Method 3.4) and the pH was adjusted to 5.7.

3.12.2 Study on the Effect of Yeast Extract on Albedo Tissue Fresh Weight and Accumulation of Limonene and Linalool

This experiment was conducted using factorial design of yeast extract concentrations of 0, 50, 100, 150, and 200 mg/L and culture periods from 1 to 5 weeks. A total of 30 treatments were carried out.

3.13 Studies on the Effect of Exogenous Precursor on Albedo Tissue

Linalool and mevalonic acid (MVA) were used as precursors and supplied directly to the Modified MS media (Method 3.4). Linalool concentrations used were 0.056mM, 0.279mM, 0.559mM, 0.838mM and 1.117mM respectively.

3.13.1 Media Preparation with Linalool

Linalool was purchased from Fluka, Japan. It was in a liquid form with ~97% purity. 10, 50, 100, 150, and 200µL of linalool were supplied to 1L Modified MS media (Method 3.4), where different concentrations of linalool (0.056 mM, 0.279mM, 0.559mM, 0.838mM and 1.117mM) were obtained.

3.13.2 Study on the Effect of Linalool Supplementation on Albedo Tissue Fresh Weight and Limonene Accumulation

A factorial designed experiment was conducted. Albedo tissues were extracted to determine limonene concentration at different culture periods (7, 14, 21, 28 and 35 days). Subculturing was carried out after 28 days so that nutrient depletion does not occur.

3.13.3 Media Preparation with MVA

Aqueous solution of MVA was prepared by dissolving mevalonic acid lactone (Fluka, Japan) in distilled water. The solutions were prepared in concentrations of 5, 10, 50, 100, 150, and 200mg/L and were filter sterilized in Nalgene® disposable sterile filterware and kept as a stock stored in the refrigerator at 4°C.

3.13.4 A Study on the Effect of MVA Supplementation on Albedo Tissue Fresh Weight and Limonene and Linalool Accumulation

This study was conducted using a factorial combination of different concentrations of MVA at 0.077, 0.384, 0.768, 1.152, and 1.537mM and culture periods of week 1 to week 5.

3.14 A Study on Combined Effect of Precursors and Elicitor on Limonene/Linalool Accumulation and Albedo Tissue Fresh Weight

The combined effect of the following precursor/elicitor was studied:-

(a) MVA and yeast extract (simultaneous treatment)

(b) MVA and linalool (consecutive treatment)

(c) Yeast extract and linalool (consecutive treatment)

(d) MVA, linalool, and yeast extract

Albedo tissues were grown on these precursor/elicitor media at optimum concentrations in various combinations.

3.14.1 Media Preparation for Simultaneous Supplementation of MVA and Yeast Extract and the Effect on Linalool/Limonene Accumulation and Albedo Tissue Fresh Weight

Media used in this study consisted of Modified MS media added with 100mg/L yeast extract together with 0.077mM of MVA. Preceding experiment on the effect of yeast extract and MVA showed that these concentrations were the optimum for limonene and linalool accumulation.

3.14.2 Media Preparation for Consecutive Supplementation of MVA and Linalool and the Effect on Limonene Accumulation and Albedo Tissue Fresh Weight

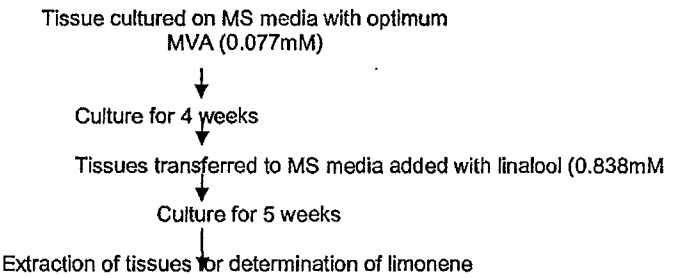
In the present study, combined effect of MVA and linalool on the tissue growth and accumulation of limonene was determined. Concentrations selected from previous studies were 0.077mM of MVA and 0.838mM of linalool, as these concentrations produced the best result individually by producing the highest level of limonene compared to all the concentrations studied.

a) Culture on MVA media

Albedo tissues were cultured on Modified MS media added with 0.077mM of MVA and maintained for 4 weeks. Five explants were used for each treatment and cultures were maintained in the dark throughout the culture period at room temperature (25± 2°C).

b) Culture on linalool media

After 4 weeks, tissues were transferred from MVA added media into linalool added media. Five tissues were used for this work. Cultures were maintained in the dark throughout the culture period at room temperature (25± 2°C).



3.14.3 Media Preparation with Consecutive Supplementation of Yeast Extract and Linalool and Determination of the Effect on Limonene Accumulation

Media used in this study consisted of Modified MS media added with100mg/L yeast extract, followed by 0.838mM of linalool. Preceding experiments of yeast elicitor and linalool on secondary metabolite accumulation showed that these were the optimum concentrations required for limonene accumulation.

3.14.4 Media Preparation with Consecutive Supplementation of MVA, Yeast Extract and Linalool and the Effect on Limonene Accumulation and Tissue Fresh Weight

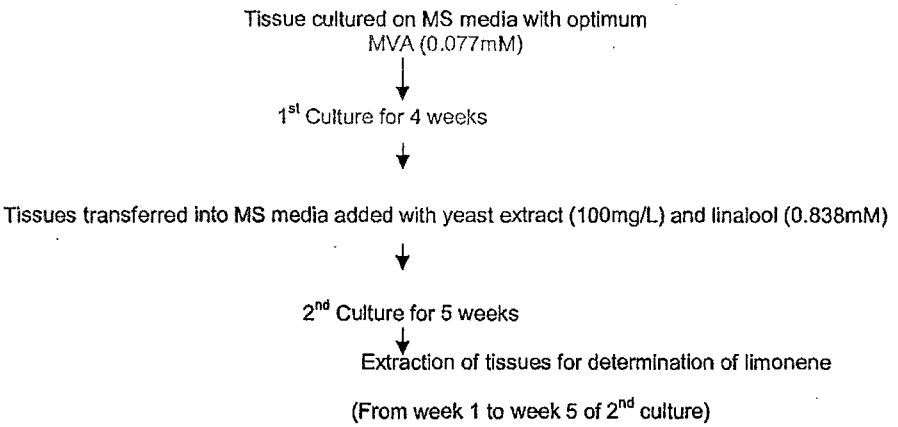
In the present study, combined effect of MVA, yeast extract, and linalool on the tissue growth and accumulation of limonene was determined. Concentrations of MVA, yeast extract and linalool used were selected based upon previous studies.

a) Culture on MVA media

Albedo tissues were cultured on Modified MS media added with 0.077mM of MVA and maintained for 4 weeks. Five explants were used for each treatment and cultures were maintained in the dark throughout the culture period at room temperature (25± 2°C).

b) Culture on yeast extract and linalool media

After 4 weeks, tissues were transferred from MVA added media into media where both yeast extract (100mg/L) and linalool (0.838mM) were added. Five tissues were used for this work. Cultures were maintained in the dark throughout the culture period at room temperature (25± 2°C).



3.15 Statistic Analysis

The results were treated statistically using one-way analysis of variance (ANOVA). When there was a statistically significance difference, post-hoc Tukey's HSD was then performed. A statistically significant difference was considered at $p<0.05$.

D. Assessment of the Project Schedule (Please make any relevant comment regarding the actual duration of the project and highlight any significant variation from plan)

Everything went as planned except the use of pravastatin as explained above

E. Assessment of Project Costs (Please comment on the appropriateness of the original budget and highlight any major departure from the planned budget)

Everything was alright

F. Benefits of the Project

i. Technical contribution of the project :

a. What was the achieved direct output of the project :

Basic Research Project

- ☐ Algorithm
- ☐ Structure
- ☐ / Data
- ☐ Other, please specify : _____

b. How would you characterize the quality of this output?

[ ] Significant breakthrough

[ ] Major Improvement

[/] Minor Improvement

ii. Contribution of the project to knowledge

a. How has the output of the project been documented?

[ ] Detailed project report

[/] Product/process specification documents

[ ] Other, please specify: _____

ii) How significant are citations of the results?

[ ] / Citations in national publications

[ ] Citations in international publications

[ ] None yet

[ ] Not known

G. REPORTS, PAPERS AND PUBLICATIONS

i. List of reports and conference/seminar papers written:

_____**MSc Thesis**_____

ii. List of scientific publications (including name(s) of co-author(s), date of publication, location and name of publisher. Please attach pre-print copies of the publications)

_____

I. KEY PERFORMANCE INDICATORS ACHIEVEMENT

i) Scientific Knowledge Creation

No. of Publications (with impact factors)
*(**Please list down all related publications since project started and attach the hardcopy)*

No	Type of Publication	No. of publication/s	Title	Impact factor
1	**Journal** i) International *[High impact or cited in Science Citation index (SCI) or Current Contents (CC)]*		In the process of preparation	
	ii) Local			
2	**Papers (seminar/ conference/ workshop/Inaugural Lecture/Keynote)** i) International			
	ii) Local			
3	**Chapter in Scientific books/ Monographs** i) International			
	ii)Local			
4	**Electronic Journal (Peer reviewed/ with impact factors)** i) International ii)Local	In the process of preparation		

ii) Technology Creation

(Relevant documents to be attached)

No	Technology creation	
1	Major scientific discoveries & new inventions	i) ii) iii)
2	No. of patents filed	i) ii) iii)

3	No. of patents attained	i) ii) iii)
4	No. of technology platforms created and transferred	i) ii) iii)
5	No. of technology platforms acquired & applied	i) ii) iii)

J. ORGANISATIONAL OUTCOMES OF THE PROJECT ((Please describe as specifically as possible the organizational benefits arising from the project and provide an assessment of their significance)

i. Contribution of the project to expertise development

a. How did the project contribute to expertise?

- [ ] PhD degrees — How many: _____
- [/] MSc degrees — How many: one
- [/] Research staff with new specialty — How many: 2
- [ ] Other, please specify: _____

b. How significant is this expertise?

- [ ] One of the key areas of priority for Malaysia
- An important area, but not a priority one [/]

ii. Economic contribution of the project? (only fill if relevant)

a. How has the economic contribution of the project materialized?

- [ ] Cost savings
- [ ] Time savings
- [ ] Other, please specify: Possible to scale up limonene production using exogenous compounds

b. How important is this economic contribution?

- [ ] High economic contribution — Value: RM _____
- [/] Medium economic contribution — Value: RM NA
- [ ] Low economic contribution — Value: RM _____

c. When has this economic contribution materialized?

- [ ] Already materialized
- [ ] Within months of project completion
- [ ] Within three years of project completion

L.NATIONAL IMPACTS OF THE PROJECT

i. Contribution of the project to organizational linkages

a.. Which kinds of linkages did the project create?

- [ ] Domestic industry linkages
- [ ] International industry linkages
- [ ] Linkages with domestic research institutions, universities
- [/] Linkages with international research institutions, universities

b. What is the nature of the linkages?

- [ ] Staff exchanges
- [ ] Inter-organizational project team
- [ ] Research contract with a commercial client
- [/] Informal consultation
- [ ] Other, please specify: _____

ii. Social-economic contribution of the project

a. Who are the direct customer/beneficiaries of the project output?

Customers/beneficiaries:	Number:
_____	_____
_____	_____

b. How has/will the socio-economic contribution of the project materialized?

- [ ] Improvements in health
- [ ] Improvements in safety
- [ ] Improvements in the environment
- [ ] Improvements in energy consumption/supply
- [ ] Improvements in international relations
- [/] Other, please specify: Limonene as a 'green product' production on a commercial basis

iii. **How important is this socio-economic contribution?**

- [ ] High social contribution
- [ ] Medium social contribution
- [/] Low social contribution

iv. **When has/will this social contribution materialized?**

- [ ] Already materialized
- [ ] Within three years of project completion
- [ ] Expected in three years or more
- [/] Unknown

Signature: Date:

Official Stamp:

Endorsed by:
Research Management Centre
Official Stamp: