THE OPTIMISATION OF GROWTH REGULATORS, PRECURSORS AND ELICITORS SUPPLEMENTATION FOR MAXIMUM LIMONENE AND LINALOOL ACCUMULATION IN CELL CULTURES OF *Citrus grandis* (L.) OSBECK

by

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DEDICATION

This thesis is dedicated to my beloved husband, Mohd Azlan Mohd Ishak, who has been very patient and understanding for the past four years, and my son and daughters, Muhammad Afiq, Nur Iffah, Nur Izzah and Nur Husna; without them I would not have had the strength and determination to complete the study.

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LIST OF ABBREVIATIONS

2,4-D :	2,4- Dichlorophenoxyacetic acid
ABA :	Abscisic acid
DMAT:	Dimethylallyl transferase
HMGR:	3-hydroxy-3-methylglutaryl CoA reductase
IAA :	Indole-3-acetic acid
IBA :	Indole-3-butyric acid
Kinetin:	6- Furfurylaminopurine
MS :	Murashige and Skoog
MVA :	Mevalonic acid lactone (3,5-Dihydroxy-3-methylvaleric acid)
MVAK :	Mevalonic acid kinase
MVAPK:	Mevalonic acid phosphate kinase
TEM :	Transmission Electron Microscopy

ABSTRACT

The study on *Citrus grandis* (L.) Osbeck encompasses tissue culture production and the extraction of limonene and linalool. The tissue culture methodology comprises of sterilisation, induction and maintenance of callus on media favouring cell growth, to favour production of limonene. Explants sterilisation attained high yields of sterile explants (≈ 90%) in treatment with 20% Chlorox® (1% sodium hypochloride). Callus growth is favourable from explants originated from 5-week-old fruits and cultured on modified MS media with 510 mg/l phosphate, 3 mg/l each 2,4-D and kinetin and 0.2 mg/l ABA. Limonene was first detected from 7- month-old callus up to10-month-old cultures which yields the highest limonene concentration before slightly down at the 11^{th} month. The amount of linalool extracted was highest from 7-month-old callus and decreased as the age of callus cultures increased. Media 6 with supplementation of 510 mg/l phosphate, 0.2 mg/l ABA and 3 mg/l 2,4-D and kinetin respectively, gave maximum concentration of limonene, which is 0.0031 mg/g fresh weight of callus. The effects of supplementation various concentrations of exogenous mevalonic acid (MVA) and linalool to *C. grandis* callus cultured at varying periods were studied. Callus growth was found to be proportional to the concentrations of MVA and linalool especially during the first 6 weeks. Slow in callus growth was observed after week 7 in the cultures. Limonene accumulation was detected as early as week 4 and continued to increase until week 7 in added cultures, whereas no limonene was traced in cultures which were no MVA or linalool added. The highest limonene accumulation, 0.0030 mg/g and 0.0032 mg/g was obtained from cultures added with 0.077 mM MVA and 0.838 mM linalool respectively after the 7^{th} week of the culture period. Linalool accumulation improved when 0.384 mM and higher concentrations of MVA was added

and reduced commencing from the 4th to the 7th week in the cultures. The consecutive supplementation of MVA followed by linalool had shot up the limonene accumulation to 0.0058 mg/g. Mevalonic acid supplied at 0.077 mM demonstrated an inverse relationship between the callus growth and the limonene accumulation rates in the first 31 days, whereas cultures supplied with 0.384 mM MVA showed a direct relationship between the callus growth and the limonene accumulation rates throughout the culture period. Supplementation of 0.559 mM linalool to the cultures increased the limonene accumulation rate for the first 37 days and decreased thereafter. On the contrary, the callus growth rate continuously increased throughout the culture period. With the supplementation of 0.838 mM linalool, the limonene accumulation rate rapidly increased while the callus growth rate dropped after 39 days. Observation on TEM showed that there was no change at the ultrastructure level for added callus in comparison to cultures without the supplementation of MVA or linalool. Yeast extract which was used in the elicitation study revealed that a concentration of 100 mg/l resulted in maximum callus growth. The elicitation effect of chitosan on the limonene accumulation was based on media conductivity where chitosan was found effective only when used between 0.5 to 1.0 mg per g fresh wt. callus. Yeast extract and chitosan as elicitors enhanced the limonene accumulation in the callus cultures. Maximum limonene accumulation at a concentration of 0.0028 mg/g and 0.0036 mg/g was achieved when induced with 100 mg/l of yeast extract and 1.0 mg/g fresh wt. chitosan respectively. A high concentration of yeast extract and chitosan, 150 mg/l and 2.0 mg/g fresh wt. respectively, resulted in low linalool accumulation. Cultures left for a longer period of more than 5 weeks with yeast extract and 2 hours with chitosan also accumulated low linalool. Combined linalool-yeast extract cultures accumulated maximum limonene at concentrations of 0.0051 mg/g in the 5th week of the culture period.

Pengoptimuman Penambahan Bahan Pengawalatur Tumbesaran, Pelopor dan Elisitor Bagi Pengumpulan Limonena dan Linalool Yang Maksima Dalam Kultur Sel *Citrus grandis* (L.) Osbeck

ABSTRAK

Kajian ke atas *Citrus grandis* (L.) Osbeck melibatkan penghasilan kultur tisu dan pengekstrakan limonena dan linalool. Kaedah kultur tisu terdiri daripada pensterilan, induksi dan pengekalan kalus dalam media yang menyokong pertumbuhan sel, yang juga menyokong penghasilan limonena. Pertumbuhan kalus adalah paling sesuai menggunakan eksplan yang terdiri daripada buah yang berumur lima minggu yang dikultur dalam media MS yang diubahsuai dengan 510 mg/l fosfat, 2,4-D dan kinetin yang masing-masing 3 mg/l dan ABA sebanyak 0.2 mg/l. Limonena dikesan bermula pada kalus berumur 7 bulan sehingga bulan ke-10 yang memberikan penghasilan limonena yang tertinggi sebelum menurun sedikit pada bulan ke-11. Jumlah linalool yang diekstrak adalah tertinggi pada kalus berumur 7 bulan dan menurun dengan meningkatnya umur kalus. Media bernombor 6 yang mengandungi 510 mg/l fosfat, 0.2 mg/l ABA dan 3 mg/l 2,4-D dan kinetin masing-masing, telah menghasilkan limonena yang tertinggi sebanyak 0.0031 mg/g berat basah kalus. Kesan penambahan secara eksogenus pelbagai kepekatan asid mevalonik (MVA) dan linalool ke atas kultur *C. grandis* yang dieram pada pelbagai tempoh pengeraman telah pun dikaji. Pertumbuhan kalus adalah berkadaran dengan kepekatan MVA dan linalool yang ditambah terutama pada 6 minggu yang pertama. Pertumbuhan yang perlahan pada kultur telah diperhatikan selepas minggu ke-7. Limonena dapat dikesan seawal 4 minggu dan paras limonena terus meningkat sehingga minggu ke-7 pada kultur yang

ditambah pelopor. Tiada limonena yang dikesan bagi kultur tanpa tambahan MVA atau linalool. Pengumpulan limonena maksimum adalah 0.0030 mg/g dan 0.0032 mg/g daripada kultur ditambah dengan 0.077 mM MVA dan 0.838 mM linalool masing-masing, selepas 7 minggu. Pengumpulan linalool telah meningkat dengan penambahan MVA sebanyak 0.384 mM dan lebih tinggi ke dalam kultur dan menurun dalam semua kultur yang ditambah MVA bermula pada minggu ke-4 hingga minggu ke-7. Penambahan MVA ke dalam kultur kalus dan diikuti dengan linalool menghasilkan pengumpulan limonena yang mendadak sebanyak 0.0058 mg/g. Asid mevalonik yang ditambah pada kepekatan 0.077 mM telah memperlihatkan profil kinetik yang bertentangan di antara kadar pertumbuhan dan kadar pengumpulan limonena dalam tempoh 31 hari yang pertama. Sebaliknya, kinetik bagi pertumbuhan dan pengumpulan limonena pada kultur yang ditambah dengan 0.384 mM MVA telah memperlihatkan hubungan yang berkadar terus di sepanjang tempoh kultur. Linalool yang ditambah pada kepekatan 0.559 mM telah meningkatkan kadar pengumpulan limonena sehingga hari ke-37 dan menurun selepas itu. Sebaliknya, kadar pertumbuhan kalus terus meningkat di sepanjang tempoh kultur. Dengan penambahan 0.838 mM linalool, kadar pengumpulan limonena telah meningkat dengan cepat sementara kadar pertumbuhan kalus jatuh selepas 39 hari. Pemerhatian pada TEM menunjukkan tiada perubahan yang berlaku pada tahap struktur ultra kalus jika dibandingkan dengan kultur yang tidak ditambah MVA atau linalool. Ekstrak yis yang digunakan ke atas kajian elisitasi telah menunjukkan kepekatan 100 mg/l telah memberikan pertumbuhan kalus yang maksimum. Kesan elisitasi chitosan ke atas pengumpulan limonena adalah berasaskan konduktiviti medium di mana penambahan chitosan hanya didapati berkesan apabila digunakan pada kepekatan 0.5 hingga 1.0 mg/g berat basah kalus. Ekstrak yis dan chitosan sebagai elisitor telah meningkatkan pengumpulan limonena dalam kultur kalus.

Pengumpulan limonena yang maksima iaitu 0.0028 mg/g dan 0.0036 mg/g telah diperolehi berikutan dengan penambahan masing-masing 100 mg/l ekstrak yis dan 1.0 mg chitosan/g berat basah kalus. Kepekatan ekstrak yis dan chitosan yang tinggi iaitu 150 mg/l dan 2.0 mg/g berat basah kalus, masing-masing, telah menghasilkan ekstrak linalool yang rendah. Kultur yang dieram untuk tempoh yang lebih lama iaitu selepas 5 minggu dengan ekstrak yis dan 2 jam dengan chitosan juga membawa kepada pengumpulan linalool yang rendah. Gabungan linalool-ekstrak yis dalam kultur telah mengumpul kepekatan limonena yang tertinggi iaitu 0.0051 mg/g pada minggu ke-5 tempoh kultur.

CHAPTER 1: INTRODUCTION

1.1 The Production of Limonene and Linalool Via Plant Cell Culture Manipulation

Limonene, a monocyclic monoterpenoid is the major constituent of citrus essential oils and a by-product of the citrus processing industry. It is the most readily available monoterpene in nature and present in abundance, ranging from 3.6 – 95% in various *Citrus* species. Linalool, an acyclic monoterpenoid contains about 1.85% of the total volatile oils extracted from *Citrus* species. Even though both compounds were considered as low cost materials, it is a fact that their usage is tremendously high especially in the flavour and perfume industries (Gabrielyan et al., 1992). Furthermore, an increase in consumer preference for food containing natural rather than artificial flavour has stimulated increase in demand and utilisation of natural flavour including limonene and linalool (Dörnenburg & Knorr, 1995; Bourgaud et al., 2001).

The past few decades have seen intense scientific interest in the development of the production-valuable phytochemicals technology (Whitehead & Threlfall, 1992). Information on research done on limonene and linalool production via tissue culture manipulation is very limited compared to the research on other compounds such as alkaloids, shikonin, ginseng and taxol (Fukui et al., 1983; Srinivasan et al., 1995; Lu et al., 2001). One possible reason is limonene and linalool at present can be obtained in abundance at low price (Braddock & Cadwallader, 1992). However, we have learnt from history that a supply of natural products via conventional methods always faces a reduction in production although there is an increase in demand. As such, there is a need

to use a biotechnological process to ensure a continuous supply of those compounds. In Malaysia, citrus has been chosen as one of the 16 fruit types for commercial production under the Malaysian National Agricultural Policy. *Citrus grandis* or pomelo has been selected as the most common type of citrus cultivated in the lowlands. Flavouring and peel oils from volatile citrus flavour components and strongly flavoured oils or essential oils are among the major products derived from citrus fruits. *Citrus grandis,* for example, contains exclusively about 92.6% limonene from the peel and based on these factors it can serve as a great potential source to be used in the study of limonene and linalool accumulation.

1.2 Plant Growth Regulators

The growth and development of higher plant tissues in vitro is controlled by gradients of endogenous plant growth substances. Studies on plant growth regulators had documented that exogenous plant growth regulators can be added to supplement endogenous levels in the culture medium to induce some physiological aspects (Dodds & Roberts, 1995). Combinations of plant growth regulators, especially auxin and cytokinin, in many cases have proven beneficial to improve cell growth and secondary metabolite production. For example, the combination of 2,4-D and kinetin in the media had given satisfactory growth of a wide range of dicotyledonous and monocotyledonous species (Dixon, 1985). These include shoot and root formation, somatic embryogenesis and organogenesis that can be regulated by supplementing a certain level of auxin and cytokinin.