ELICITOR AND PRECURSOR ENHANCED PRODUCTION OF LIMONENE IN *CITRUS GRANDIS (L.)* OSBECK ALBEDO TISSUE CULTURES

by

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Thesis submitted in fulfillment of the requirements for the degree of Master of Science

JUNE 2008

ACKNOWLEDGEMENTS

I would like to express my utmost and deepest gratitude and appreciation to my dear supervisor, Professor Madya Dr. Nik Norulaini Nik Abdul Rahman for her continuous effort, patience and guidance in providing invaluable ideas, encouragement and exceptional role she has played in making this project a reality. I am most grateful to her for giving me the opportunity to carry out my study as she provided a lot of inspiration and zeal for me to work hard.

I would also like to thank Professor Ir. Dr. Mohd. Omar Ab. Kadir for his encouragement and guidance over the years. My appreciation is also forwarded to Dr. Norli for her guidance and support whenever I needed it most.

I am most grateful to all the staffs of Technology Industry School and Distance Education School for giving me the help needed throughout my study period in University Science Malaysia. I would also like to thank USM for giving me the opportunity that made my study possible.

My utmost gratitude is forwarded to my beloved parents, Mr. & Mrs. Mayakrishnan, my loving siblings, Ananda Raj and Sashikala and to my dear husband, Thakur Raghu Raj Singh, for their love, support and encouragement during this period.

Last but not least, my warmest appreciation to all my friends especially Yu Lang, Wong, Fatehah, Liyana, Ida, Najwa, Hazana, Shalima, Sabariah, Sabrina, Saidi, En. Azizi, Pak Wahyu, En. Abu, Leng Keng, Kak Fera and Kak Harlina for their invaluable friendship and encouragement all these years.

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LIST OF ABBREVIATIONS AND SYMBOLS

2,4-D	2,4-Dichlorophenoxyacetic acid
%	Percent
ABA	Abscisic acid
Acetyl-CoA	Acetyl coenzyme A
ANOVA	Analysis of Variance
$C_{std.}$	Concentration of standard solution
C.V.	Coefficient Variation
cv	cultivar
DMAPP	Dimethylallyl pyrophosphate
DMAT	Dimethylallyl transferase
FPP	Farnesyl pyrophosphate
GC	Gas chromatography
GC-FID	Gas chromatography-Flame Ionization Detector
GC-MS	Gas chromatography-mass spectrometry
GGPP	Geranyl geranyl pyrophosphate
GPP	Geranyl pyrophosphate
HMG-CoA	3-hydroxy-3-methyl glutaryl coenzyme A
HMG-CoA R	3-hydroxy-3-methyl glutaryl coenzyme A reductase
IAA	Indole acetic acid
IBA	Indole-3-butyric acid
IPP	Isopentenyl pyrophosphate
K	Kelvin
kg	Kilogramme
LOQ	Limit of Quantification
mg/L	Milligramme per litre
mM	MilliMolar
MS	Murashige and Skoog
MVA	Mevalonic acid lactone (3,5-Dihydroxy-3-methylvaletic acid)
NAA	Napthaleneacetic acid
NPP	Neryl pyrophosphate
ppm	Parts per million
psi	Pounds per square inch
PTFE	Polytetrtafluoroethylene
R^2	Correlation coefficient
SEM	Scanning Electron MIcroscope
SFE	Supercritical Fluid Extractor
SPSS	Statistical Procedures for Social Sciences
UV	Ultra-violet
v/v	Volume per volume

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LIST OF PRESENTATIONS

1 A research paper entitled "**Influence of yeast extract in elicitation of limonene and linalool in *Citrus grandis in vitro* culture**" for a colloquim organized by the School Of Distance Education, University of Science Malaysia, in August 2006.

2 A research paper entitled "**Callus Formation and Cell Suspension of *Citrus Grandis* for limonene and linalool extraction using Elicitors"** for a colloquim organized by the School Of Distance Education, University of Science Malaysia (USM) in 8-9 June, 2005.

3 Poster presentation entitled "**Effect of yeast extract as elicitor and linalool as precursor to produce limonene and linalool in tissues of *Citrus grandis* Osbeck**" in Asia Pacific Conference on Plant Tissue Culture and Agribiotechnology (APaCPA) 2007, on 17-21 June, 2007.

PENINGKATAN PENGHASILAN LIMONENE DALAM TISU KULTUR ALBEDO *CITRUS GRANDIS* (L.) OSBECK MENERUSI PENGGUNAAN ELISITOR DAN PREKURSOR

ABSTRAK

Kesan ekstrak yis sebagai elisitor bersama linalool dan asid mevalonik (MVA) sebagai prekursor telah dikaji pada kepekatan dan masa pengkulturan yang berbeza untuk menentukan pertumbuhan tisu dan penghasilan limonene/linalool ke atas tisu albedo *Citrus grandis* Osbeck. Rawatan tisu albedo dengan ekstrak yis dan linalool telah membantutkan pertumbuhan tisu kerana tisu yang tidak dirawat telah mencapai berat basah sebanyak 0.92g, manakala hanya 0.32g dan 0.42g berat basah dicapai oleh tisu yang dirawat oleh ekstrak yis dan linalool masing-masing selepas 4 hingga 5 minggu masa pengkulturan. Asid mevalonik (MVA) pula tidak menunjukkan kesan perencatan terhadap pertumbuhan tisu. Penghasilan limonene yang tertinggi selepas dielisitasi dengan ekstrak yis adalah sebanyak 1.8ppm pada hari ke-28, iaitu selepas dirawat dengan yis pada kepekatan 100mg/L, manakala linalool yang terhasil adalah 5.33ppm pada hari ke-7. Keputusan terbaik bagi linalool yang bertindak sebagai prekursor tercapai apabila 0.838mM digunakan, di mana tahap kepekatan limonene yang adalah 0.97ppm pada hari ke-28. 0.077mM MVA turut menghasilkan kepekatan limonene dan linalool yang terbanyak. Pada hari ke-35, tahap limonene adalah 1.50ppm sementara linalool mencapai tahap kepekatan tertinggi pada hari ke-7, iaitu sebanyak 2.88ppm. Kajian selanjutnya yang melibatkan rawatan kultur tisu dengan 100mg/L ekstrak yis, diikuti 0.077mM MVA telah menghasilkan 3.08ppm limonene pada hari ke-28. Pertumbuhan tisu albedo pada masa itu adalah maksima pada 0.69g. Linalool yang terhasil turut berada pada takat maksima pada hari ke-7, iaitu sebanyak 4.11ppm. Penambahan linalool pada kepekatan 0.838mM dan MVA pada 0.077mM telah menghasilkan pertumbuhan tisu

yang tertinggi selepas 28 hari, iaitu pada 0.74g sebelum menurun ke 0.68g pada minggu yang berikutnya. Jumlah limonene yang diekstrak juga adalah tertinggi pada hari ke-28, iaitu sebanyak 2.81ppm. Penambahan linalool pada 0.838mM dan ekstrak yis pada 100mg/L telah menghasilkan 3.67ppm limonene pada hari ke-28. Pertumbuhan tisu maksima telah dicapai selepas tisu dirawat pada kepekatan ini, iaitu 0.77g pada jangka masa pengkulturan yang sama. Penggabungan ekstrak yis (100mg/L), linalool (0.838mM), dan MVA (0.077mM) pada kajian yang seterusnya telah mencetuskan penghasilan limonene yang paling tinggi selepas hari ke-28, pada 3.27ppm. Berat tisu maksima yang dicapai adalah 0.71g selepas 35 hari. Kajian yang melibatkan penggabungan rawatan elisitor dan prekursor ini telah berjaya kerana kepekatan limonene yang tertinggi dapat dihasilkan pada masa yang singkat, iaitu 28 hari.

ELICITOR AND PRECURSOR ENHANCED PRODUCTION OF LIMONENE IN *CITRUS GRANDIS* (L.) OSBECK ALBEDO TISSUE CULTURES

ABSTRACT

The effects of yeast extract as an elicitor together with linalool and mevalonic acid (MVA) as precursors were studied at various concentrations and time period to determine tissue growth and limonene/linalool production on *Citrus grandis* Osbeck albedo tissues. Treatment of albedo tissues with yeast extract and linalool ceased tissue growth as untreated tissues gained fresh weight of 0.92g compared to only 0.32 to 0.42g for tissues treated with yeast and linalool respectively after 4 to 5 weeks. Mevalonic acid, however, did not inhibit tissue growth. The highest production of limonene after elicitation with yeast extract was 1.8ppm on day 28 when tissues were treated with 100mg/L of yeast, while linalool concentration reached maximum of 5.33ppm on day 7. The best result for linalool was achieved with 0.838mM linalool, where the limonene level achieved was 0.97ppm on day 28. 0.077mM MVA produced the best result for both limonene and linalool accumulation. At day 35, limonene was at 1.50ppm while linalool reached its highest concentration on day 7 at 2.88ppm. Consecutive study by first treating cultures with 100mg/L yeast extract and MVA at 0.077mM produced 3.08ppm of limonene at day 28. Tissue growth was also highest during this time at 0.69g. Linalool produced was maximum on day 7 at 4.11ppm for this study. Treatment with linalool at 0.838mM and MVA at 0.077mM produced maximum tissue growth after 28 days at 0.74g before decreasing to 0.68g after another week. Limonene accumulation was at its peak on day 28 at 2.81ppm. Adding linalool at 0.838mM and yeast at 100mg/L

produced 3.67ppm of limonene on day 28. Highest tissue growth obtained with this treatment was 0.77g during the same time period. Combining yeast extract (100mg/L), linalool (0.838mM) and MVA (0.077mM) in a successive study produced limonene at its peak after 28 days at 3.27ppm. Maximum tissue weight gained was 0.71g after 35 days. This combined treatment of elicitor and precursor was successful as the highest concentration of limonene was obtained at such a short time period, which is within 28 days.

CHAPTER 1

INTRODUCTION

1.0 THE CITRUS FRUIT

Citrus fruits belong to the family of *Rutaceae.* The special feature of citrus fruits is the presence of juice sacs in them. Citrus fruits lack a firm pulp. The juicy pulp is either made into beverages or sucked.

The citrus fruit contains secondary metabolites, limonene and linalool, a special kind of berry (hesperidium) which is filled with stalked spindle-shaped pulp vesicle. The fruits are covered by dark green-coloured skin. White, spongy tissues, known as flavedo, grow inside the skin, whereas another section called albedo grows in the inner section of the fruit close to the peel (Plate 1.1). Albedo tissues are white and spongy that surrounds the juice vesicles. These tissues are chosen for the study as they are more uniform compared to juice vesicles. Besides that, these tissues are found in the inner part of the fruit. Thus, it is believed to be less exposed to dirt and microorganisms from the external environment compared to flavedo.

The peel has numerous oil glands, also known as juice vesicles, which turns yellow or orange at full maturity. Seeds of these fruits contain one or many embryos.

Plate 1.1: A transverse section of the young pomelo fruit.

In vivo study on citrus cultivars was explored for multiple shoot induction and root regeneration by Usman *et al.* (2005). On the other hand, *in vitro* studies have been conducted on citrus tissue cultures such as callus cultures. Bud formation, root induction and callus initiation studies were also carried out by Moreira-Dias *et al*. (2000) by using various types and concentrations of growth regulators to obtain high growth rate.

1.1 LIMONENE AND LINALOOL IN CITRUS SPECIES

There has been an increase in the study of plant secondary metabolism in the last 50 years and some of these metabolites are important sources of pharmaceuticals. According to Bourgaud *et al.* (2001), there are different strategies, using *in vitro* system, which has been extensively studied to enhance the production of secondary plant compounds. Cultures such as root, callus and other organs have been examined.

In citrus species, one of the major secondary metabolites is limonene. In the mevalonic acid pathway, leading to the production of limonene, linalool is the immediate precursor to limonene. Limonene is a monocyclic monoterpenoid and is the major constituent of citrus essential oils and a by-product of the citrus processing industry. Linalool, on the other hand, is an acyclic monoterpenoid which contains about 1.85% of the total volatile oils extracted from *Citrus* species. Linalool is found in the essential oils of *Aniba rosaeodora* (rosewood), *Coriandrum sativum L, Bursera delpechiana, Citrus spp, Citrus aurantium subsp. amara L, Laurus nobilis L, Cinnmamomun camphora, Cinnmamomun verum L, Matricaria chamomilla L, Salvia sclarea L., Lavandula officinalis Chaix* and *Ocimum basilicum* (Maia *et al.*, 2006).

Juice vesicles of the citrus species contain an enzyme system which can phosphorylate mevalonic acid and form both isopentenyl pyrophosphate and dimethylallyl pyrophosphate. These products will eventually turn into the monoterpene, linalool. Intact fruits and leaves convert linalool to limonene. Figures 1.1 and 1.2 below show the structures of limonene and linalool respectively (Vickery & Vickery, 1981).

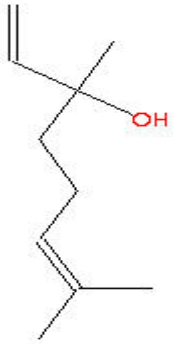

Figure 1.1: Chemical structure of linalool

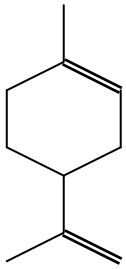

Figure 1.2: Chemical structure of limonene

Monoterpenoids are volatile and produce odours in many plants. They are constituents of the essential oils of plants, many of which are important to the perfume, flavouring and pharmaceutical industries (Vickery & Vickery, 1981). Besides that, consumers prefer food containing natural flavour rather than artificial flavour, thus increasing the demand and utilization of natural flavouring which includes limonene and linalool (Dornenburg & Knorr, 1995).

Research done on limonene and linalool production via tissue culture manipulation is very limited according to Zarina (2005). Much research work had been carried out on other compounds such as alkaloids, shikonin, ginseng and taxol compared to limonene and linalool. Nabeta *et al*. (1983) have worked on limonene and linalool production using callus tissues and suspension cultures of *Perilla frutescens*.

Limonene and linalool are citrus peel extracts that contain insecticidal compounds (Prates *et al*., 1998) that kill many insect pests, like fire ants and fleas. However, these extracts have low toxicity level to mammals. The two most effective insecticidal compounds are d-limonene, a terpene that constitutes about 90% of crude citrus oil and linalool, a terpene alcohol. Terpenes are hydrocarbons found in

essential oils. They are used as solvents, fragrances and flavors in cosmetics and beverages.

1.2 SUPPLEMENTATION OF ELICITORS AND PRECURSORS IN PLANT TISSUE CULTURES TO YIELD SECONDARY METABOLITES

Secondary metabolites are sometimes very low or not detectable in dedifferentiated cells such as callus tissues or suspension cultured cells. For plant cell culture to be economically feasible, certain methods have been discovered and developed that would allow for consistent generation of high yields of secondary metabolites from cultured cells. In order to obtain the desired products at a high enough concentration for commercial purposes, biosynthetic activities of cultured cells were stimulated or restored using various methods.

One of the methods used for obtaining desired secondary metabolites from plants is optimization of cultural conditions like medium, temperature, pH, light and oxygen supply (Misawa, 1994). Several secondary metabolites were found to be secreted in cultured cells at a higher level than those in native plants through these techniques. Manipulation of physical aspects and modification of nutritional elements in a culture is perhaps the most essential approach for optimization of culture productivity. For example, rosmarinic acid by *Coleus bluemei* were accumulated in much higher levels in cultured cells than in intact plants due to medium optimization as discovered by Ulbrich *et al.* (1985).

Other methods employed are selection of high-producing strains, addition of precursors, biotransformation, elicitor treatment, application of immobilized cells and product secretion into the culture media (Misawa, 1994).

The need for selecting high-producing strains has arisen because of the physiological characteristics of individual plant cells is not always uniform. Yamada & Sato (1981) repeated cell cloning using cell aggregates of *Coptis japonica* and obtained a strain which grew faster and produced a higher amount of berberine and cultivated the strain in a 14 L bioreactor. The selected cell line increased growth about 6-fold in 3 weeks and the highest amount of the alkaloid produced was 1.2 g/L of the medium. The strain was very stable, producing a high level of berberine even after 27 generations.

Precursors are naturally occurring compounds, intermediates originating from biosynthetic pathways, or related synthetic compounds (Pras, 1992). Addition of these precursors or related compounds to the culture media sometimes tends to stimulate secondary metabolite production. This approach is advantageous if the precursors are inexpensive.

Phenylalanine is an example of a biosynthetic precursor for rosmarinic acid. Adding this precursor to *Salvia officialis* suspension cultures stimulated the production of rosmarinic acid and shortened the production time as well (Misawa, 1994). Amino acids have also been used as a precursor in cell suspension culture media for production of tropane alkaloids, indole alkaloids and ephedorin. Some stimulative effects have been observed. Perhaps, they affected not only alkaloid biosynthesis

directly as precursors, but also indirectly through other metabolic pathways in the cells (Chan and Staba, 1965).

Biotransformation using intact cells or immobilized cells is an alternative way of producing a product by adding precursors into the culture media. Biotransformation can be used to increase the production of secondary metabolites through the application of enzyme activities present in plant cells for modification of added precursors into more valuable products. This method was used by Demyttenaere *et al.* (2000) to convert geraniol, nerol and citral from liquid cultures *Aspergillus niger* to obtain linalool and α-terpineol.

Elicitation is also one of the most successful methods employed in the induction of secondary metabolite products. This method uses biotic or abiotic molecule treatment. Several types of products have been successfully elevated by elicitation products such as indole alkaloids from *Catharanthus roseus* cultures, cryptotanshinone from *Salvia miltiorrhiza* cultures, paclitaxel from *Taxus* cultures, sesquiterpenes from *Hyoscyamus muticus* root cultures and saponin from *Panax ginseng* cultures (Chong *et al.,* 2005). Exogenous methyl jasmonate was treated on cotton cv. Deltapine by Saona *et al.* (2001). These plants emitted elevated levels of terpenes, linalool being one of them.

The major problem with the use of elicitor molecule to induce secondary metabolite formation is the necrosis effect of elicitor preparation, called hypersensitivity (Buchner *et al*., 1982). Hence, induction of secondary metabolites with elicitor may

cause an increase in the amount of the metabolites but also a reduction in the cell mass (Heinstein *et al*., 1992).

Effective elicitation has been achieved by Chong *et al.* (2005) in *Morinda elliptica* cell suspension culture using yeast extract, jasmonic acid, chitosan and glucan at different cell growth cycle. They discovered that different concentration of different elicitors used exerted different effect in cell growth and secondary metabolite, namely antraquinone production. The production was also dependant on the stage of cell growth. For example, Zarina (2005) found than when linalool was supplemented to callus cultures of *C. grandis* Osbeck, the rate of limonene started increasing steadily from day 21 till day 49. Bourgaud *et al.* (2001) reported that most secondary metabolites are produced during the plateau (log) phase, as during lag phase, carbon allocation is mainly used up for primary metabolism, such as building of cell cultures and respiration.

Albedo tissues were cultured and treated with elicitor (yeast extract) and precursors (MVA and linalool) in combinational studies to determine the level of limonene and linalool produced based on the concentration of elicitor and precursors used and time period of culturing.

1.3 OBJECTIVES

The main objectives to be achieved in this study are:

i) To assess the addition of elicitor such as yeast extract at different concentrations and time periods in albedo tissue cultures and its influence on limonene and linalool accumulation.

ii) To determine the effect of precursors like MVA and linalool at different concentrations and time periods in albedo tissue cultures on limonene accumulation.

iii) To evaluate the effect of

a) yeast extract and MVA

b) yeast extract and linalool

c) linalool and MVA

d) yeast extract, MVA and linalool

in albedo tissue cultures at a predetermined concentration and time period to determine limonene and linalool accumulation.

CHAPTER 2

LITERATURE REVIEW

2.0 INTRODUCTION

Citrus is a common term and genus of flowering plants belonging to the family of *Rutaceae*, originating in tropical and subtropical Southeast Asia. Citrus fruits are notable for their fragrance, partly due to flavonoids and limonoids (which in turn are terpenes) contained in the rind and most are juice-laden.

The most common citrus fruits are mandarin (*C. reticulate Blanco*), pomelo (*C. grandis Osbeck*), sour orange (*C. aurantium L.*), sweet orange (*C. sinensis Osbeck*), lime (*C. aurantifolia Christm.*), citron (*C. medica L.*), lemon (*C. limon L. Burn. f.*) and grapefruit (*C. paradise Osbeck*) (Spiegel-Roy & Vardi, 1984). The genus *Citrus* and its wild relatives are members of the *Rutaceae. Citrus* is divided into six genera and two subgenera. The six generas are *Eremocitrus, Poncirus, Clymenia, Fortunella, Microcitrus* and *Citrus*. The subgeneras consist of *Eucitrus* and *Papeda. Papeda* fruits are non-edible. All the genera have unfoliated leaves except for *Poncirus*, which has trifoliate deciduous leaves.

Citrus fruits are economically important with a large scale production of both the fresh fruit and processed products. One way of consuming orange as fresh fruit is processing them to obtain value-added products. One of the most important by-products of citrus is the essential oil extracted from the peel (Mira *et al.*, 1999). Orange essential oil is used to give the aroma and flavor of orange to many products, such as carbonated drinks, ice-creams, cakes, air-fresheners, perfumes and so on. Carotenoid pigments found in orange extracts have nutritional value as precursors of

vitamin A, certain anti-carcinogenic properties and antioxidant potential. Compounds, such as β-carotene and canthaxanthin are used in food coloring. Citrus essential oils are present in fruit flavedo in great quantities (Caccioni *et al.,* 1998).

The volatile flavour components of orange juice obtained from the cv. Kozan oranges were investigated by Selli *et al.* (2004). They extracted, identified and quantified thirty-four components, including seven esters, two aldehydes, five alcohols, five terpenes, twelve terpenols and three ketones. The main flavour components comprise of linalool, limonene, β-phellandrene, terpinene-4-ol and ethyl 3-hydroxy hexanoate.

2.1 ORIGIN OF *CITRUS GRANDIS* OSBECK (POMELO)

Pomelo (*Citrus grandis*), the largest of citrus fruits, can grow as large as a foot in diameter and weigh up to 25 pounds (11.36 kg), belongs to the family *Rutaceae*. It is also known as Shaddock. The tasty fruit is popular locally for its taste and features significantly in the Chinese new-year celebrations. Pomelo was named after an English sea captain who brought it from Batavia (Jakarta) in Java during the 17th century. The name 'pomelo' (or pommelo) may be derived from the Dutch pompelmoes (grapefruit) or from the obsolescent 'pumplenousse' (Tate, 1999). Variant names for the pomelo fruits is given below in Table 2.1.

Table 2.1: A variety of names for pomelo fruits

Scientific name	*Citrus grandis*
Common name	Pomelo
Malay names	Limau bali, limau besar, limau tambun, limau abong (Malaysia), jeruk bali, jeruk adas, jeruk machan, limau kibau, limau balak, limau besar, limau betawi, jambua (Indonesia).
Other common names	Shaddock, Batavia lemon

Pomelo, believed to be an ancestor of the grapefruit, is native to the Southeast Asian and the Indo-China regions. The exact place of origin is unknown. It is most likely from Malaysia, Thailand and Indonesia where it is found in the wild. In 1884, a variety of pomelos, like *limau bali*, was imported into Malaya from Indonesia by Sir Hugh Low and it was grown in Penang and Perak. In Southeast Asia, it is grown as a cultivable crop in Indonesia, Thailand and Malaysia. In Malaysia, pomelo is widely grown in Perak, Kedah, Melaka, Kelantan and Johor. It is also grown commercially in parts of the USA, Israel, China and Japan and is found growing non-commercially in India, Jamaica and the Middle East (Thujala, 2003).

Plate 2.1: Photo showing a pomelo tree, with young fruits hanging down from one of the branches

The pomelo tree (Plate 2.1) is a large, thorny and highly branched tree growing to around 5 to 15 m in height. It produces fruits all year round. Its bark is brownish yellow in colour and thick. The leaves grow to about 2 to 12 cm wide. Leaves are covered with small spots, which are actually oil glands and this gives the dark green leaves a shiny appearance. When crushed, they give off a strong smell. The flowers are yellowish white or plain white in colour. They produce a pleasant fragrance and grow in a single form to around 2.5 cm wide. The outer skin of pomelo fruit is rough and easy to peel. It is light green to yellow and dotted with oil glands. The fruit is either round or oblong with white thick spongy pith that encloses the edible portion of the fruit. Each fruit consists of 9 to 14 segments covered with paper-thin skin. The flesh of the fruit is white, light yellow, pink or rose-red, juicy with a sweet sour or spicy sweet taste. Some fruits leave a bitter taste in the mouth. The seeds are few in number, yellowish, white and large. (Thujala, 2003). The interior pulp of the fruit is

covered with a spongy, white rind. The segments are covered with a membrane which is removed when eaten (Esme & Eiseman, 1988). Volatile flavour constituents of these fruits were limonene, γ-terpinene, myrcene, nootkatone, decanal, 6-methylheptyl acetate, octyl acetate, neryl acetate, linalool and citronellal. (Sawamura & Kuriyama, 1988).

The pomelo has many traditional medicinal uses. The Malays use its leaves to make lotions to apply on sores and swellings. The Filipinos use its leaves as sedatives for certain nervous complaints. The Chinese also prepare medicaments from its leaves, fruits and flowers together with other ingredients. The fruit is said to cure hangover if chewed slowly. A paste of pomelo rind and ginger applied to joint brings relief from rheumatic pains (Tate, 1999). Table 2.2 shows the list of ingredients and nutrition contained in a 100g pomelo fruit.

Table 2.2: Composition of pomelo fruit (per 100g of edible portion).

Ingredients	**Quantity/100g**
Moisture content (%)	88.90
Protein (g)	0.70
Fat (g)	0.30
Total carbohydrate (g) (including fiber)	9.50
Fiber (g)	0.40
Ash (g)	0.60
C (mg)	27.00
P(mg)	22.00
Fe (mg)	0.50
Na (mg)	1.00
K(mg)	235.00
B-carotene equivalent microgram	30.00
Vitamin B_1 (mg)	0.05
Vitamin B_2 (mg)	0.02
Vitamin C (mg)	53.00

(Source: Yaacob, 1980)

2.2 ESSENTIAL OILS

There are two kinds of oil. One is the essential, volatile or distilled oils, while the second kind is the fatty, non-volatile, expressed or fixed oils. However, the present study is concentrated on essential or volatile oils. Essential oils evaporate or volatilize when they get in contact with air. These kinds of oils are also known as 'volatile oils'. They can be readily removed from the plant tissues without any change in their composition. This characteristic is common in 'distilled oils'. They possess a pleasant taste, have a strong, aromatic odour and maybe coloured. They are typically in the form of liquid (Seth, 2004).

Essential oils are very complex in their chemical composition. The two principal groups of these essential oils are high-volatile (terpenes/hydrocarbons and oxygenated compounds) and non-volatile (pigments and waxes) compounds.

The terpene fraction can constitute from 50% up to more than 95% of the oil. However, this fraction does not give flavour and fragrance to the oil. Terpenes are also present as free glycosylated conjugates amongst the secondary metabolites of certain wine grape varieties of *Vitis vinifera* (Carrau *et al.*, 2005).

Since terpenes are usually unsaturated compounds, they can be easily decomposed by heat, light and oxygen to unpleasant off-flavors and aromas. Due to this, some of the terpenes are removed, while the extraction of oxygenated compound is more of interest to the industries. This procedure is known as "deterpenation" or "folding" and is done to improve oil stability, increase oil solubility and to reduce storage and transportation costs. Deterpenation is done by distillation, solvent extraction or chromatographic separation. However, these conventional methods have major

drawbacks such as low yields, formation of thermally degraded undesirable by-products and/or solvent contamination of the products. Therefore, an alternate refining process is needed to overcome these problems. Thus, extensive research in the field of supercritical extraction was carried out to extract essential oils (Raeissi & Peters, 2005).

The oxygenated compounds are mainly responsible for the characteristic flavor and fragrance. These oxygenated terpenes consist mainly of alcohols, aldehydes and esters such as linalool, citral and linalyl acetate (Kondo *et al*., 2002).

Essential oils are by-products of carbohydrate and fat metabolism. They occur in small concentrations, from minute traces to as much as 1 to 2% or even more, in specialized cells, glands or ducts. They occur in either one particular organ of the plant or distributed over many parts. They are present in flowers (roses), fruits (oranges), leaves (eucalyptus), bark (cinnamomum), roots (ginger), woods (cedar) or seeds (cardamom) and many resinous exudations (Seth, 2004).

The usage of essential oils to the plant itself is vague. The characteristic aroma and flavor they impart to flowers, fruits and seeds probably attract insects and other animals, which play an important role in pollination and/or in the dispersal of fruits and seeds. When essential oils are present in high concentrations, the unpleasant odor repels enemies like parasites, animals and insects. The essential oils have antiseptic and bactericidal properties that act as wound fluid. They affect transpiration and other physiological processes by minimizing the effect of heat on transpiration. They

play a vital role as hydrogen (H) donors in oxidation-reduction reactions as potential sources of energy (Aldrich, 1988).

Essential oils are used in many ways by humans because of their odor and high volatility. They are used in the manufacture of perfumes, sachets, soaps and other toiletry preparations. The perfumes are stored in closed, compactly filled containers since they deteriorate due to oxidization and polymerization when they come into contact with air. In confectionary and aerated waters, they are used as flavoring materials or essences for ice-creams, candies, cordials, liqueurs, non-alcoholic beverages, tobacco and so on. They are valuable in medicine, dentistry and pharmaceuticals because of their therapeutic, antiseptic and bactericidal properties. They are used as insecticides and deodorants, as solvents in paint and varnish industries and in the manufacture of several synthetic odors and flavors, such as attars and scents. Some of the essential oils, clove oil for example, are used as clearing and cleaning agents in histological work. They are also used in diversified products such as chewing gum, toothpaste, dhoop, incense, shoe polish, library paste and fish glue (Seth, 2004).

A study conducted by Yoshida *et al.* (2005) found that (R)-(+)-citronellal and (S)-(-)-β-citronellol, essential oils extracted from citrus herbs, are effective inhibitors of P-glycoprotein (P-gp). P-gp is a transmembrane protein which acts as a multidrug resistance factor in tumor cells. Thus, by inhibiting this protein using essential oil, the problem of reduced anticancer agent reaching the desired cancer cell can be conquered. Table 2.3 (Seth, 2004) shows some of the most commonly used essential oils.

Table 2.3: Essential oil and its usage

Common name	Genus & Species	Remarks
Mandarin oil	*Citrus reticulata*	Oils from peels-confectionery, toilet products and pharmaceutical preparations.
Petitgrain oil	*Citrus* spp in India, *C. aurantium, C. limettoides*	Oil extracted from leaves and twigs-scents, cosmetics, skin creams and soaps.
Orange oil	*Citrus* spp: *C. aurantifoli, C. reticulate*	Oil from ripe peel-scents, cosmetics, skin creams and soaps.
Neroli Portugal	*C. sinensis*	Oil extracted from flowers of the sweet orange.
Neroli: true oil of neroli or neroli bigarade	*C. aurantium*	Oil is extracted from flower of the sour orange.
Bergamot	*C.aurantium*,subsp. Bergamia	Greenish oil extracted from ripe peels has a soft, sweet odor and is used for scenting toilet soaps, in mixed perfumes and as a clearing agent.

2.2.1 ANTIMICROBIAL ACTIVITIES OF ESSENTIAL OIL

Phytoalexins are antimicrobial compounds known to have accumulated after exposure to microorganisms and infection. These compounds are synthesized in a highly localized area around the site of infection and are either absent or present in a small quantity in healthy tissues (Whitehead & Threlfall, 1992). Many research works were carried out to study the antimicrobial activity of these valuable compounds (Vila *et al.,* 1999; Wu *et al.*, 1988). For example, a study was conducted by Kim & Shin (2004) to examine volatile substances of *Callicarpa japonica* Thunb for their antibacterial activities against six food-borne microorganisms. Extracts of *C. japonica* were obtained by simultaneous steam distillation and solvent extraction and found to be effective to kill the six food-borne microorganisms studied.

Duru *et al.* (2004) tested the essential oil of *Micromeria cilicica (Labiatae),* namely pulegone, for antimicrobial activities. They found that the extracts exhibited a significant antibacterial and antifungal activity, particularly against *Candida albicans* and *Salmonella typhimurium*.

The essential oil extracted from *Curcuma zedoaria (Berg) Rosc* showed a high content of epicurzerenone and curdione. The essential oil was proven to be effective against antimicrobial activity. This oil could also inhibit the proliferation of human promyelocytic leukemia HL-60 cells (Lai *et al.*, 2004).

The essential oil compounds obtained from *Nigella damascene* plants and seeds were screened for biological activity by Fico *et al.* (2004). They found that the essential oil was only active against Gram positive bacteria.

A study was conducted by Caccioni *et al*. (1998) on the effect of volatile components of citrus fruit essential oils on *Penicillium digitatum* and *P. italicum* growth. The antifungal efficacy of the oils showed that a positive correlation existed between monoterpenes other than limonene and sesquiterpene and the pathogenic fungi inhibition. The best results were obtained from citrange oils and lemon. *P. digitatum* was found to be more sensitive to the inhibitory action of the oils compared to *P. italicum*.

Nonvolatile residue of orange essential oil was isolated for three permethoxylated flavones, dehydroabietic acid and linoleil monoglyceride. All of them proved to have

antifungal activity against phytopathogenic species and food contaminants (Vargas *et al.*, 1999).

Differentiation and maturation of tissues are important for the production of certain phytoalexins. Laticiferous cells, for example, are storage compartments of alkaloids. Alkaloids accumulate as salts with organic acids in vacuoles of these cells. Dimeric indole alkaloids of *Catharanthus roseus* are biosynthesized only in specifically specialized tissues (Datta & Srivastava, 1997).

2.2.2 LIMONENE AND LINALOOL

(R)-(+)-limonene is the main compound in essential oils of citrus fruits, where it occurs in concentrations of more than 90% and in enantiomerically pure form (Adams *et al.*, 2003). A study was carried out by Adams *et al*. (2003) to produce α–terpineol from limonene through bioconversion using *Penicillium digitatum*. Study of the physical behavior of limonene proved that limonene is non-polar, thus not being soluble in water (solubility 13.8 mg/L at 25°C). To increase the solubility of limonene in water, a co-solvent, ethanol was used (Adams *et al.,* 2003). D-limonene is very effective in the germicide treatment of waste waters (Mira *et al.*, 1999). It is also undergoing clinical evaluation in cancer patients together with perillyl alcohol (Hardcastle *et al*., 1999).

Limonene has higher solubility than linalool in supercritical with ethane, with the difference in solubility increasing with temperature (Raeissi & Peters, 2005). D-limonene is a biodegradable solvent and degreaser, occurring naturally as the main component in citrus peel oil (95%). D-limonene can be used safely and effectively in

a wide range of products and applications due to its high solvency, attractive citrus odor, adaptability and GRAS rating (Generally Recognized As Safe) from the US FDA (Carlson *et al.*, 2005).

Limonene, a major constituent of terpenes is found to be a superior solvent for polystyrol in chemical recycling processes (Kondo *et al.*, 2002). Insects such as in the genera *Podisus, Alcaeorrhynchus* and *Zicrona* have huge pheromones secreted in their dorsal abdominal glands such as R-(+)-α-Terpineol (V) and S-(+)-linalool (III). Thus, a formulation of artificial pheromones containing (E)-2-hexenal and (±)-linalool proved highly attractive to *P. fretus* species (Aldrich, 1988).

Carrau *et al*. (2005) found that higher concentration of assimilable nitrogen by *Saccharomyces cerevisiae* wine yeasts increased linalool and citronellol accumulation. The amount of linalool produced by some strains of *S. cerevisiae* could be the determinant factor of wine quality.

2.3 PLANT TISSUE CULTURE

Plant tissue culture is a method to propagate plant tissue in aseptic condition using suitable nutrient media. Tissue and cell culture are the main techniques used in crop selection and improvement, in the generation of disease-free plants and in the production of secondary metabolites under controlled conditions. There is a possibility of new products formation either by feeding specific precursors of a biosynthetic pathway or by manipulating or deleting key enzymes to alter the nature of the end product (Deans & Svoboda, 1993).

Plants still remain as an important source for producing compounds used in the pharmaceutical, food, flavor and perfume industries, although synthetic compounds were also found. This is because synthetic compounds are difficult and costly to produce and complex mixtures, such as rose oil, cannot be formed successfully by man. Chemically synthetic compounds fail to satisfy as a safe, consumable product in the food and drink industry. So, natural source from plants is the best alternative to overcome this problem. Besides that, chemical synthesis may, depending on the compound, result in a mixture of isomers which are not able to be separated on a commercial scale. This is of obvious importance when the major desirable property resides with one isomer (Yeoman *et al*., 1980).

Most of the work of plant tissue culture to extract desired products has been performed with callus or cell suspension cultures (Kartnig *et al.,* 1993), although hairy root cultures have also been used (Deans & Svoboda, 1993).

2.3.1 MEDIA COMPOSITION

One of the most commonly used media for plant tissue cultures is Murashige and Skoog (MS) (1962). The most important feature of the MS medium is its high concentration of nitrate, potassium and ammonia. The B5 medium established by Gamborg *et al.* (1968) has a lower concentration of inorganic nutrients compared to MS medium (Misawa, 1994). The formation rate of callus and coumarin derivatives in suspension cultures of *Petroselinum crispum* (parsley) depended mainly on media composition (Deans & Svoboda, 1993).

Sucrose promotes a better growth compared to any other carbohydrate like glucose, fructose, galactose, lactose and maltose in citrus tissue cultures (Bohm, 1980). However, it does not enhance embryogenesis (Vu *et al.*, 1995). Sucrose or glucose at 2 to 4% is a suitable carbon source. In the case of *Catharanthus roseus* cell cultures, maximum biomass yields were achieved in shorter periods of time with glucose rather than sucrose (Fowler & Stepan-Sarkissian, 1985).

Fructose, maltose and other sugars also support the growth of various plant cells. The efficient production process of useful metabolites should be chosen based on the most suitable carbon source and its optimal concentration. These factors depend on plant species and products to be metabolized. Therefore, it is vital to establish an optimized medium composition together with the carbon sources for each plant species to be extracted for their respective secondary metabolites. The use of cheaper carbon sources is necessary in industry. Thus, crude sugars such as molasses have been examined as alternate carbon sources (Misawa, 1994). A high salt nutrient medium containing NO_3^- as the main nitrogen source gave best growth response in callus strains of *Betula* (Simola, 1985). MS media also contains vitamins such as myo-inositol, nicotinic acid, pyridoxine HC*l* and thiamine HC*l*. Thiamine is the most essential vitamin for many plant cells and other vitamins induce the growth of the cells (Misawa, 1994). Callus induction is very effective from *C. grandis* explants originating from fruits of 4cm in diameter and cultured on MS media modified with 510 mg/L phosphate. This concentration is three times higher than the original basal media (Jenimar, 2001).

2.3.1.1. EFFECTS OF PLANT GROWTH REGULATORS/ PHYTOHORMONES

Effects of plant growth regulators on the cell culture media vary with the type and concentration of growth regulator. Auxin (IAA, NAA, 2, 4-D and IBA) is a plant growth regulator which is known to be distributed universally in higher plants. This compound is secreted by the apical meristems of both shoots and roots and controls the expansion of the tissue cells. Auxin was also proved to be responsible for apical dominance (the inhibition of lateral bud and lateral root development by the active apical meristem), in the retention or falling of leaves and flower buds, in flower development and in the initiation and continuance of fruit development. Auxin could inhibit or promote both cell expansion and cell division. The effect of auxin on plants differs with its concentration and the sensitivity of the plant tissue. This sensitivity of tissues to auxin depends upon the kinds and development stage of the plants concerned. Tissue differentiation studies were carried out in tissue culture. For example, vascularisation (the development of xylem strands) in tissue culture was observed to take place only when auxin was present in a sufficient concentration (Street & Cockburn, 1972).

Phytohormones are necessary to induce callus tissues and to support the growth of many cell lines. Each plant species requires different types and concentrations of phytohormones for callus induction, growth and metabolites production. So, it is essential to select the most suitable growth regulators and to establish their optimal concentrations (Misawa, 1994).