SYNERGISTIC RESPONSE OF *Pogostemon cablin* (Blanco) Benth. CELL SUSPENSION CULTURES TO PRECURSOR FEEDING AND ELICITATION TOWARDS THE PRODUCTION OF PATCHOULOL

FATIN IZZATIE BINTI MAZLAN

UNIVERSITI SAINS MALAYSIA

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by

FATIN IZZATIE BINTI MAZLAN

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LIST OF ABBREVIATIONS

2-4, D	2-4, Dichlorophenoxy acetic acid
BA	Benzyladenine
BAP	6-Benzylaminopurine
CHI	Chitosan
DCM	Dichloromethane
DCW	Dry cell weight
EOs	Essential oils
FDA	Food and drug administration
FDA	Fluorescein diacetate
FPP	Farnesyl diphosphate
FRIM	Forest Research Institute Malaysia
FW	Fresh weight
GC-FID	Gas chromatography flame ionization detector
GC-MS	Gas chromatography mass spectrometer
GES	Gastric epithelial cell lines
GLU	Glucan
GMP	Genetically modified products
HCl	Hydrochloric acid
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPP	Isopentenyl diphosphate
JA	Jasmonic acid
LPS	Lipopolysaccharide
LS	Linsmaier and Skoog
MeJa	Methyl Jasmonate
MVA	Mevalonate pathway
NAA	α-Naphtaleneacetic acid
NaOH	Sodium hydroxide
PaMMV	Patchouli mild mosaic virus
PaMoV	Patchouli mottle virus

PGRs	Plant growth regulators
pH	Potential of hydrogen
ROS	Reactive oxygen species
SH	Schenk and Hilderbrandt
SMs	Secondary metabolites
USD	United states dollar
UV	Ultra violet
YE	Yeast extract

LIST OF SYMBOLS

α	Alpha
β	Beta
δ	Delta
$	Dollar
γ	Gamma
%	Percentage
±	Plus and minus
˚C	Temperature in Celsius

RESPON SINERGISTIK KULTUR AMPAIAN SEL *Pogostemon cablin* (Blanco) Benth. KEPADA SUAPAN PELOPOR DAN ELISITASI TERHADAP PENGHASILAN PATCHOULOL

ABSTRAK

Metabolit sekunder dalam *Pogostemon cablin* (Blanco) Benth, terutamanya patchoulol sangat terkenal dengan keupayaannya dalam aktiviti neuroprotektif, peningkatan kognisi dan juga mengurangkan risiko gangguan kecacatan pembelajaran. Patchoulol mampu menghalang replikasi virus influenza B secara *in vitro* dan juga merupakan salah satu komponen utama yang diperlukan dalam industri minyak wangi. Walau bagaimanapun, kandungan patchoulol dalam pokok yang ditanam di ladang adalah sangat rendah. Oleh itu, pokok *P. cablin* perlu dituai dengan lebih banyak untuk memenuhi permintaan patchoulol yang semakin tinggi. Matlamat utama penyelidikan ini dijalankan adalah untuk meningkatkan pengeluaran patchoulol melalui kaedah *in vitro* menggunakan teknik kultur sel tumbuhan dengan kaedah suapan pelopor, elisitasi serta menggabungkan kedua-dua kaedah tersebut. Sebagai permulaan, anak pokok *P. cablin* perlu dihasilkan semula melalui kaedah kultur tisu. Kultur kalus berumur 4 tahun yang diperolehi dari eksperimen sebelum ini perlu di sub-kultur dan digunakan untuk menghasilkan ampaian sel. Ampain sel *P. cablin* ini harus dikekalkan di dalam MS media yang mengandungi 1 mg/L picloram dan diinkubasi di dalam bilik gelap. Proses ini amat penting bagi memastikan bekalan bahan tumbuhan untuk eksperimen seterusnya sentiasa mencukupi. Pelbagai jenis elisitor abiotik (*methyl jasmonate* dan *chitosan*) dan biotik elisitor (ekstrak yis dan *Pycnoporus sanguineus*) dengan kadar kepekatan yang

berbeza telah dikaji untuk meningkatkan kandungan patchoulol di dalam kultur ampaian sel *P. cablin*. Elisitasi dengan menggunakan elisitor abiotik iaitu *methyl jasmonate* (MeJa; 50 µM) pada hari ke 10 iaitu pada fasa pegun pertumbuhan sel mencatatkan kandungan patchoulol yang paling tinggi iaitu 55.87 ± 2.03 µg/g apabila ia dituai pada hari ke-3 selepas penambahan MeJa. Manakala, di dalam kes elisitor biotik pula ekstrak yis (YE) dengan kepekatan 2 g/L telah dipilih sebagai elisitor biotik terbaik yang bertanggungjawab untuk meningkatkan kandungan patchoulol dalam kultur ampaian sel *P. cablin*. Kesan peringkat kitaran pertumbuhan serta tempoh pendedahan elisitor terhadap kandungan patchoulol menunjukkan bahawa rawatan menggunakan 50 µM MeJa pada fasa awal eksponen iaitu pada hari ke-4 menghasilkan pengeluaran patchoulol yang tertinggi apabila dituai pada hari ke-5 (7.60 ± 0.04 µg/g). Sementara itu, elisitasi menggunakan YE pada fasa pertumbuhan eksponen dan dituai pada hari yang sama seperti MeJa hanya menghasilkan 0.15 ± 0.06 µg/g patchoulol. Kesan sinergistik antara *trans*, *trans*-farnesol (100 mg/L) sebagai pelopor dengan 50 µM MeJa pula menunjukkan kesan negatif di mana gabungan kedua-duanya menunjukkan penurunan pengeluaran patchoulol sebanyak 0.88 kali ganda berbanding kumpulan yang di elisitkan secara individu. Kesan yang sama juga dapat dilihat apabila *trans*, *trans*-farnesol ditambah bersama YE (2 g/L). Kesimpulannya, hanya sel yang di elisitkan secara individu mampu meningkatkan pengeluaran patchoulol di dalam kultur ampaian sel *P. cablin.*

SYNERGISTIC RESPONSE OF *Pogostemon cablin* (Blanco) Benth. CELL SUSPENSION CULTURES TO PRECURSOR FEEDING AND ELICITATION TOWARDS THE PRODUCTION OF PATCHOULOL

ABSTRACT

Secondary metabolites in *Pogostemon cablin* (Blanco) Benth, especially patchoulol is known to possess strong neuroprotective activities, abilities for cognition enhancement as well as learning impairment attenuation. It was also proven to weakly inhibit replication of influenza B *in vitro* and provide the major constituents for perfumery industry. However, patchoulol content in a field planted *P. cablin* is very low and therefore higher volume of *P. cablin* plant supply is needed to fulfill the increasing demand for this active compound. The main aim of this research done was to effectively increase the production of patchoulol *in vitro* using plant cell culture technique via precursor feeding and elicitation using biotic and abiotic elicitors as well as by combining the best from each of the abiotic and biotic elicitor with precursor. The initial work involved the plantlet regeneration of *P. cablin* as well as sub-culturing the 4 years old callus obtained from previous studies. The cell suspension cultures were established using the four years old callus and was maintained in MS media containing 1 mg/L picloram and incubated in total darkness. This process is necessary in order to produce a continuous supply of plant materials for further manipulation. Various types of abiotic (methyl jasmonate and chitosan) and biotic (yeast extract and *Pycnoporus sanguineus*) elicitors with different concentrations were studied in order to produce a higher yield of patchoulol in *P. cablin* cell suspension cultures. Elicitation with abiotic elicitors namely methyl

jasmonate (MeJa; 50 µM) on day 10 corresponding to the stationary phase of cell growth yield the highest content of patchoulol (55.87 ± 2.03 µg/g) when the cultures were harvested on day 3 after MeJa addition. In the case of biotic elicitors, yeast extract (YE) with the concentration of 2 g/L was chosen as the best biotic elicitors responsible to increase patchoulol content in *P. cablin* cell suspension cultures. The effect of the stage growth cycle and duration of elicitor exposure suggest that MeJa (50 µM) treatment on the early exponential phase (day 4) yield the highest production of patchoulol when harvested on the 5^{th} day (7.60 ± 0.04 µg/g). Meanwhile, YE that was elicited on the same growth phase and harvested on the same day as MeJa was capable to yield only 0.15 ± 0.06 µg/g patchoulol. A synergistic effect between *trans, trans*-farnesol (100 mg/L) as a precursor with 50 µM MeJa yield a negative effect in which combination of them shows a decrement of 0.88 folds of patchoulol production compared to the individually elicited groups. The same effect can also be seen when *trans, trans*-farnesol were added with YE (2 g/L). In conclusion, only the individually elicited cell suspension cultures of *P. cablin* is capable in improving the yield of patchoulol.

CHAPTER ONE

INTRODUCTION

1.1 Essential Oils

In the new global economy, the use of essential oils (EOs) as complementary therapies to treat several diseases has gained momentum. EOs or also known as volatile oils are usually very concentrated and is commonly extracted from fruits, stalks, leaves, flowers and also roots of plants. Some of them can also be extracted by distillation of resins (Ali et al., 2015). These oils are frequently used as flavoring agents not only in food products but also in drinks, cosmetics, and perfumeries as well as pharmaceuticals goods. Approximately, 3000 EOs have been produced from 2000 plant species in which only 300 of them is enough to yield about 40 000 – 60 000 tonnes per annum of oils production. Surprisingly, the market value for that amount of oil production shoot up to 700 million USD and this definitely proves an increment of EOs consumption all over the world (Raut and Karuppayil, 2014).

Due to its ability to protect the plants from fungal, microbial, insects as well as viral infections, researchers around the world started to screen its chemical compositions and in-depth study on their mechanisms of action has been conducted. It is believed that EOs consists of about 20 – 60 compounds of various concentrations with some compounds were highly concentrated and the others came in trace amounts (Perricone et al., 2015). Generally, those compounds are known as secondary metabolites (SMs). SMs that are usually present in aromatic and medicinal plants are aldehydes, phenols, terpenoids, ketonic bodies, alcoholic compounds and also acidic compounds. Among those compounds, terpenes (e.g., limonene,

terpinene, myrcene), aromatic phenols (e.g., thymol, eugenol, carvacrol) and also terpenoids are known to have the most important roles in the composition of EOs (Pandey et al., 2017). However, identification of the most active compound in EOs is quite troublesome. This is because the composition of the EOs varied depending on the time of harvest, plant materials and the preferred method used to extract the oil (Hyldgaard et al., 2012; Wang et al., 2017).

Manycommon Mediterranean aromatic plants comes from the family of Lamiaceae, Rutaceae, Apiaceae, and Verbenaceae. One of the most famous EOs in Lamiaceae family is lavender oil that can be extracted from *Lavandula officinalis*. It is well believed that the active compounds called linalool and linalyl acetate in lavender oil exhibit a sedative effect and are able to act as markers for narcotic actions (Elshafie and Camele, 2017). Another example of a well-known essential oil used widely in the cosmetic industry is tea tree oil derived from *Melaleuca alternifolia* plant. It is rich in terpinen-4-ol that is believed to be advantageous in treating abrasion, acne, carbuncles, and wounds (Orchard and Vuuren, 2017). By being the center of origin as well as domestication, Mexico is famous for its production of vanilla oil (Calvo-Irabien, 2018). Extracted from *Vanilla planifolia* orchids, vanilla oil contain approximately 85-87% vanillin that can be considered as important to inhibit peroxynitrite-mediated reactions in Parkinson and Alzheimer patients (Bilcu et al., 2014; Dhanalakshmi et al., 2015).

1.2 *Pogostemon cablin* and patchouli oil production

Pogostemon cablin (Blanco) Benth can also be considered as one of the most important essential oil producing plants. It is a tropical medicinal and aromatic crop that usually grows in the south of China (He-ping et al., 2011). The fragrant plant was introduced to China during the 9th century as spices and the plant was actually native from South and South-east Asia such as Indonesia, Malaysia, Philipines and India (Liu et al., 2015). The plant patchouli belongs to the genus *Pogostemon* in the family of Lamiaceae (mint family). It has been traditionally considered as being related to Verbenaceae and plants from this family usually being used as culinary herbs such as basil, oregano, thyme and lavender (Chakrapani et al., 2013).

Another benefit of *P. cablin* is that according to Miyazawa et al. (2000), the aerial part of *P. cablin* has been extensively used to treat the common cold and it also acts as an antifungal agent in traditional medicine. Besides being used in traditional medicine, *P cablin* is also famous for its ability in aromatherapy in which it is used to calm nerves, relieve stress as well as depression (Swamy et al., 2010). Due to its perfumery uses, demand for *P. cablin* is increasing dramatically worldwide (Paul et al., 2010) however, the plant never flowers and hence vegetative propagation through stem cuttings is in practice. Despite the technique of stem cutting, this conventional method has been limited due to recurrence of mosaic virus, root-knot nematodes and insect pests (Swamy et al., 2014).

Extraction of *Pogostemon cablin* leaves yields one of the eighteen major essential oils that have a worldwide commercial importance and have been used globally in perfumery industry called patchouli oil (Santos et al., 2011). Patchouli oil

can be obtained by steam distillation and it is widely appreciated for its pleasant and long lasting woody, earthy, camphoraceous odor. Like many essential oils, patchouli oil composition is complex but distinct due to the fact that it majorly comprises of sesquiterpenes and the major constituent responsible for patchouli note is (-)-patchoulol (Deguerry et al., 2006).

The composition of patchouli oil is unique and complex because it consists of more than 24 different sesquiterpenes and this oil can be characterized by a huge number of other sesquiterpene hydrocarbons such as α-/β-/γ- patchoulenes, α-guaiene, seychellene and α-himachalene (Donelian et al., 2009). Research conducted by Kongkathip et al. (2009) stated that patchouli oil has suitable properties for anti-inflammatory activity, aromatherapy as well as a stress reliever.

Traditionally, patchouli oil has been used in India, China, and Japan for various medicinal purpose due to its antiseptic properties (Chakrapani et al., 2013). Besides that, patchouli oil is also well-known for its antibacterial effects against a broad range of bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Enterobacter aerogenes*. Till now there are more than 140 compounds including terpenoids, phytosterols, flavonoids, alkaloids as well as the organic acids can be isolated and identified from patchouli oil (Swamy and Sinniah, 2015). The major phytochemical compound obtained from the essential oil of patchouli is patchoulol and therefore, it has been extensively used as a marker for quality control of patchouli oils and the extracts (Kongkathip et al., 2009).

1.3 Plant cell culture technology

To date, there is a growing body of literature that recognizes the importance of plant secondary metabolites to chemical and pharmaceutical industries. Therefore, *in vitro* cultures of plant cells provide the most reliable approach for a sustainable yield of desired secondary metabolites of commercial interests (Ramirez-Estrada et al., 2016). The history of plant tissue culture went back to the 40s in which plant tissue culture and organ culture technology is a very complex task and factors such as mineral, nutrients, and plant growth regulators (PGRs) are not well understood (Gago et al., 2011).

According to Smetanska (2008), plant tissue culture offers a potential source of valuable secondary metabolites that can be used as food additives (flavors, fragrance, and colorants), pharmaceuticals as well as nutraceuticals. Secondary metabolites can be broadly defined as any product which is synthesized by plant and that does not necessarily carry the basic function in life such as growth or replication. It is a mysterious group of molecules that are large, varied and some of them are extraneous byproducts of metabolic pathway due to the promiscuous enzyme activity. Many of them serve as an important function in defense and protection of plants and therefore considered as biologically active thus making these molecules useful for human ailments (Kolewe et al., 2008).

Increasing demand for therapeutic and preventative healthcare products worldwide makes plant especially its cells and tissues are seen as “green factories” and due to the fact that plant tissue culture can offer an attractive and sustainable alternatives for some plants’ biosynthetic potential, it is believed that plant cell/tissue

culture is the best method to meet these demands (Mavituna et al., 2016). Other than that, tissue culture systems also offer a superior environmental containment compared to the whole plant grown in the field. Therefore, the production time can be shortened, downstream processing, as well as product purification, are always simpler and cheaper (Shih and Doran, 2009).

Increasing global demand for valuable plant biologically active compound and the increment knowledge in plant mineral nutrition has paved the way for the setup of plant cell cultures. One of the most common plant cell culture system is cell suspension culture, in which the cells are grown in liquid culture media under continuous shaking (Maathuis, 2013). Cell suspension cultures from different plant species appeared to be a very important research tool for studying cellular processes in plant biology as well as the production of priceless secondary metabolites (Vijlder et al., 2015).

A variety of research has been conducted using plant cell suspension culture and one of the latest research work is a study conducted by Mavituna et al. (2016). Mavituna and colleagues developed a theoretical model and computer simulation for the prediction of plant cell aggregate size distribution using batch suspension cultures of *Capsicum frutescens*. This proves that a lot of research work can be conducted using cell suspension culture due to the fact that suspension cells are the most amenable to genetically modified product (GMP) procedures and they can be cultivated easily in large-scale bioreactors (Hellwig et al., 2004).

In most cases, secondary metabolite production can be optimized by treating the undifferentiated cells with elicitors (Riedel et al., 2012). Elicitors can be defined as a substance in which will initiate or improves the biosynthetic pathways of specific compounds and the term elicitation is generally understood as a process of inducing or enhancing the synthesis of secondary metabolites to ensure the plants' survival, persistence as well as competitiveness (Namdeo, 2007). Another effective strategy used to influence the biosynthetic pathways to enhance the production of secondary metabolite is by feeding plant cell cultures with precursors. The addition of exogenous precursors such as amino acids is proven to increase the production of secondary metabolites in *in vitro* cultures of specific plants (Riedel et al., 2012).

1.4 Problem statement

Despite an increment on the usage of patchouli oil worldwide, production of patchouli oil around the world still vary significantly. This is because of various factors such as tissues or organs used, harvesting time, plant varieties and poorly controlled conditions during extraction and growth may affect the quality of oil produced (Chen et al., 2014). Therefore, *in vitro* cultures of patchouli plant has served as one of the major contributing factors that can successfully control the production of secondary metabolites in patchouli plants (Elhaak at al., 2016).

By being around for more than 400 million years, plants have developed thousands of structurally different secondary metabolites as a means of protection against plant-eating animals, bacteria, fungi and viruses (Wink, 2015). Due to that reason, it can be said that secondary metabolites do not have direct roles in plant growth and development as well as structure (Schmidt and Cheng, 2017). Therefore,

it is important to force the production of secondary metabolites in plants via precursor feeding or elicitation. Bunrathep and his colleagues have demonstrated the effect of precursor feeding using cis-farnesol to the production of patchoulol (major compound in patchouli oil) in cell suspension cultures of the plants. Their results revealed that only 25.5 mg/L of patchoulol can be detected after the addition of cis-farnesol into the cell suspension cultures (Bunrathep et al., 2006).

Therefore, another type of enhancement strategies such as elicitation can be attempted to improve the production of patchoulol. Elicitors have been widely used in numerousresearch to increase the production of desired secondary metabolites in plants. One of the success stories can be seen from a research conducted by Zlotek et al. (2016) in which they are finally able to increase the yield of basil oil in *Ocimum basilicum* plant using jasmonic acid as elicitor. Therefore, in this study, incorporation of biotic elicitors (yeast extract; *Pycnoporus sanguineus*) and abiotic elicitors (methyl jasmonate; chitosan) into cell suspension cultures of *P. cablin* were attempted. This is important to deeply investigate its effect on the yield of patchoulol produced by cell suspension cultures of *P. cablin*. Until now, there are no enhancement strategies using elicitors either abiotic or biotic to increase the production of patchoulol in patchouli plants being investigated.

Researchers have also suggested that combination of two different elicitors can successfully enhance the accumulation of desired secondary metabolites. This is exemplified in the work undertaken by Perassolo et al. (2017) in which they proved that combination of two different elicitors (methyl jasmonate and cyclodextrin) into *Rubia tinctarum* cell suspension cultures can synergistically induce the production of

anthraquinone. In addition, the latest finding on the combination of elicitors was done by Krzyzaniak et al. (2018) in which the combination of oligosaccharide and β-glucan laminarin successfully increase the production of secondary metabolites in grapevine plants. However, it can be seen that the study on synergistic technique between precursor and elicitors in essential oil producing plants is still scarce and limited. Due to that context, it is aimed to determine the synergistic effect of precursor and elicitors in cell suspension cultures of *P. cablin*. This study can be a breakthrough in finding whether or not a combination of both (precursor and elicitors) will increase the production of patchoulol in *P. cablin* plants and thus, providing new platforms to researchers worldwide.

1.5 Research objectives

The specific aim of this study was to increase the production of patchoulol by precursor feeding as well as elicitation using various concentration of abiotic as well as biotic elicitors. In order to accomplish this studies, three main objectives were determined and can be stated as follows:

1. To conduct a screening studies on the effect of different cell-lines, drying techniques and *in vitro* cultures of *P. cablin* as well as to select the best biotic and abiotic elicitors to enhance the production of patchoulol in cell suspension cultures of *P. cablin*.
2. To study the effects of the stage of cell growth cycle and duration of elicitor exposures on the enhancement of patchoulol production.
3. To correlate the synergistic response of *trans, trans*-farnesol feeding and elicitation on patchoulol production.

1.6 Scope of studies

This research work attempts to increase the production of patchoulol from *P. cablin* (Blanco) Benth cell suspension cultures by elicitation as well as precursor feeding. Cell suspension culture of *P. cablin* was successfully established and grown from a healthy as well as friable callus cultures. A complete growth profile of *P. cablin* cell suspension culture with cell dry weight, pH, cell viability as well as patchoulol production were investigated.

In order to increase the yield of patchoulol, precursor (*trans, trans*-farnesol) and various concentrations of abiotic as well as biotic elicitors were added into the cell suspension culture during the early stationary phase of the growth profile. In this study, only two types of biotic elicitor (yeast extract and *Pycnoporus sanguineus*) and abiotic elicitor (methyl jasmonate and chitosan) were used. The cell suspension culture was then harvested after the first, third and fifth days of treatment to analyze the cell dry weight, pH and the production of patchoulol.

The second objective of the study was to investigate the effect of the stage of cell growth cycle and duration of elicitor exposure to the patchoulol production. The best concentration of elicitors obtained from the first objective was applied into the cell suspension culture of *P. cablin* during the early exponential phase, mid-exponential phase and the stationary phase of *P. cablin* growth profile. Treated cell suspension culture was then harvested on the first, third and the fifth day after the treatment.

Lastly, the combined effects of precursor and elicitors on patchoulol production were examined in *P. cablin* cell suspension cultures. Each of the elicitors (MeJa and YE) from different concentrations (50 μM and 2 g/L) used in this experiment was coupled with *trans, trans*-farnesol to analyze and correlated their synergistic response on the production of patchoulol.

1.7 Organization of thesis

The overall structure of the study takes the form of five chapter. Chapter One introduces about the overview of *Pogostemon cablin* (Blanco) Benth, its essential oil as well as the major secondary metabolites found in the plant. This chapter also summarizes the advantages of using cell suspension culture instead of organ cultures in producing desired secondary metabolites as well as the addition of precursor and elicitors to enhance the production of the desired compound. The importance, objectives, problem statement and scope of studies are also presented in this chapter.

Chapter Two begins by laying down the literature review on *P. cablin*, plant cell culture technique used as well as the production of patchoulol and its uses in industries. The enhancement strategies using precursor and elicitors to increase the yield of patchoulol in cell suspension culture of *P. cablin* were also had been elaborated in depth throughout this chapter. In the last section, review on the synergistic response of *P. cablin* cell suspension culture towards precursor coupled with elicitors was also presented.

The third chapter is concerned with the materials and methodology used for this study. In this chapter, plant cell culture techniques, subcultures of callus as well as cell suspension culture for maintenance were described and explained in detailed. Apart from that, proper procedures and techniques required prior to addition of precursor and elicitors as well as a combination of both precursor and elicitors were also elucidated in this chapter. Lastly, the extraction methods for *P. cablin* cell suspension cultures using gas chromatography equipped with flame ionization detector was also clarified in detail in the third chapter.

The fourth chapter presents the findings of the research. This chapter was divided into two parts which are the results and discussion. Overall, this chapter shows the result for the screening of various concentration of biotic as well as abiotic elicitors towards the pH, cell dry weight, patchoulol production and cell viability of *P. cablin*. Besides that, a complete growth profile of *P. cablin* cell suspension culture, the effect of the stage growth cycle and duration of elicitor exposure, as well as the synergistic response of both precursor and elicitor feeding towards the production of patchoulol, were also discussed in detail throughout this chapter.

Chapter Five concludes all the major findings found throughout the thesis. Other than that, suggestions, as well as recommendations for better improvement on this topic, was also presented in this chapter. It will serve as a platform for future studies and extend the knowledge gain from this field of studies for future practice.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Pogostemon cablin* (Blanco) Benth

Originating from Malaysia and India, *Pogostemon cablin* is a herbaceous perennial plant from Lamiaceae family that has been extensively cultivated in southern of China (Wu et al., 2010). *P. cablin* or also known by the name of patchouli is an aromatic shrub that has a great commercial value and it has been cultivated in various parts of the world due to its economic importance (Santos et al., 2011). According to Paul et al. (2010), the main area for commercial cultivation of *P. cablin* is located in Indonesia, which accounts for approximately 80% of world patchouli production.

P. cablin is a tropical crop that can be grown under subtropical conditions and evidence suggests that it grows successfully up to an altitude of 800 – 1000 m above the mean sea level. Besides that, patchouli prefers a deep, well-drained, slightly acidic, fertile and loamy soils. It flourishes best in the soil with a pH value range from 5.5 to 7.5 and it thrives well in the coastal region with 80-90% humidity at a temperature between 20 – 35 ˚C (Ramya et al., 2013). However, research suggests that there is a rapid decrement in biomass as well as essential oil yield in patchouli plant and research done by Paul et al. (2010) claimed that it was mainly due to pathogens such as Patchouli Mild Mosaic Virus (PaMMV), Patchouli Mottle Virus (PaMoV), root-knot nematodes and insect pests.

A variety of phytochemical constituents has been isolated from *P. cablin*. Some of them possess activities such as cytotoxic activities, anti-microbial, anti-inflammatory, analgesic as well as anti-mutagenic activity (Chakrapani et al., 2013). The aerial part of *P. cablin* is commonly used to treat fever, common cold, vomiting and it also acts as an antifungal agent in Chinese medicinal materials (Wu et al., 2010). They also possess anti-insecticidal, bacteriostatic properties, act as ROS scavenger in oxidant-induced cell death of human neuroglioma cells and it was proven to inhibit the neurotoxic activity of the β-amyloid peptide (He-Ping et al., 2011). These characteristic features of *P. cablin* make them a commercial crop with a great industrial importance (Swamy et al., 2016). However, *P. cablin* can only be propagated by stem cutting and this method of cultivation was relatively slow for large-scale production of this medicinal plant (He-Ping et al., 2011).

Figure 2.1: *Pogostemon cablin* plants

2.2 Patchoulol as an essential oil

In the light of recent events in scientific development, the medicinal properties of aromatic plants have reached the greatest interest due to their pharmacological activities, low toxicity as well as economic viability. Among natural compounds enclosed in aromatic and medicinal plants, essential oils have received much attention due to its radical scavenging activities (Said et al., 2016). Essential oil also offers a potential novel template molecule as well as mixtures of bioactive aromatic substances that can be used industrially. This is due to the fact that bio-products are a promising agent for wellness, pharmaceutical, and serve as an important source to the food industries. They also demonstrated a good inhibitory effect on melanogenic activities and can be used in cosmetic industry as whitening agent and protection against skin darkening (Aumeeruddy-Elafi et al., 2016). Some of the plants that produce crucial essential oils are shown in Table 2.1.

Table 2.1: Plants producing essential oils (adapted from Ali et al., 2015; Miller et al., 2015; Spyridopoulou et al., 2017)

Essential oils	**Part of the plants**
Bergamot, lemon, lime, sweet orange, tangerine, mandarin	Fruit Peel
Cinnamon	Bark
Citronella, lemongrass, petitgrain, palmarosa, **patchouli**	**Leaves**
Geranium, lavender, rosemary, spike lavender	Entire plant
Vetiver	Roots
Jasmine, neroli (orange blossom), rose, ylang-ylang	Flowers
Ginger	Rhizome
Mastic	Resins

Recent developments in the field of essential oils have heightened the need for the production of essential oil from *P. cablin*. This plant possesses oil gland and produces a crucial essential oil in perfume industry called patchouli oil. Patchouli oil is a dark orange or brownish colored viscous liquid and the odor of patchouli is proven to be coming from a compound named norpatchoulenol. Besides its long-lasting woody, earthy and pleasant odor, patchouli oil is suitable to be used in soaps and cosmetic industries due to its fixative properties. It is also a substance that had been approved by Food and Drug Administration (FDA) for human consumption as food flavoring (Bure and Sellier, 2004; Donelian et al., 2009).

Research conducted by Harunsyah (2012) stated that patchouli oil was traded in the international market and it is one of the most important essential oil existed in Indonesia with an excellent commodity. Beek and his colleague (2017) approximated that 90% (1200-1300 metric tonnes per annum) of patchouli oil production globally comes from Indonesia. They also confirmed that patchouli oil is one of the main materials needed to the perfumer and their current review also shows that the price for a good quality of patchouli oil can shoot up to the US $ 63/kg and the annual sales were estimated to be around $ 75 million (Beek and Joulain, 2017). Deguerry et al. (2006) also stated in his studies that the composition of patchouli oil is quite unique and has identified 25 different chemical compounds from patchouli oil. The summary of the chemical compounds was tabulated in Table 2.2.

Table 2.2: Chemical compounds in patchouli oil (Deguerry et al., 2006)

Chemical Compounds		
(-)-patchoulol	α-patchoulene	β-patchoulene
α-bulnesene	α-guaiene	seychellene
trans-β-caryophyllene	(-)-pogostol	(+)-germacrene A
4,5-di-epi-aristolochene	γ-curcumene	α-selinene
farnesyl pyrophosphate	(-)-eremophilene	β-ylangene
guai-4, 11-diene	norpatchoulenol	(E)-β-farnesene
(-)-germacrene D	α-humulene	germacrene C
trans-trans-farnesol	γ-patchoulene	(-)-nerolidol
	(E, E)-α-farnesene	

Recently, investigators have isolated and identified more than 140 compounds including phytosterols, organic acids, lignin, alkaloids, glycosides, aldehydes, alcohols, and terpenoids in patchouli oil (Swamy and Sinniah, 2015). As shown in Figure 2.2, one of the major active constituents that has been isolated from *P. cablin* is patchoulol. Patchoulol (synonym to patchouli alcohol) was first isolated in a crystalline form by Gal in 1869 and it was then formulated as $C_{15}H_{26}O$ (adjusted from $C_{30}H_{26}O_2$) by Montgolfier. On the early stage of the investigation, it was believed that patchoulol is a saturated tricyclic tertiary alcohol but then later was proven wrong and the structure was finally established by single crystal X-ray diffraction (Srikrishna and Satyanarayana, 2015).

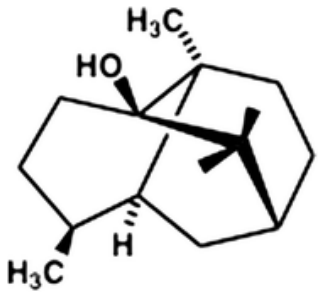

Figure 2.2: Chemical structure of patchoulol (Kiyohara et al., 2012).

Patchoulol is a tricyclic sesquiterpene that is thought to inhibit inflammatory response in various inflammatory disease models. Previous research also reported that patchoulol could successfully inhibit the overexpression of pro-inflammatory iNOS and IL-6 in lipopolysaccharide (LPS)-stimulated macrophages (Wang et al., 2016). It was also reported by Xie et al. (2016) that patchoulol has a defensive effect against apoptosis in GES-1 cells as well as preserving the mitochondrial function. Today, patchoulol that have been purified via fractional distillation is commercially available for fragrance industries under the name of "Healingwood" (Beek and Joulain, 2017).

2.3 *Pogostemon cablin* cell and tissue culture technique

A study conducted by Vakil and Mendhulkar (2013) stated that plant cell and tissue culture technique (PCT) is a very useful method for large-scale production of plant secondary metabolites. Due to the fact that many plants with high medicinal products are difficult to cultivate, biotechnological production of valuable phenolic compounds in plant cells can be considered as one of the most promising tools to enhance the production of secondary metabolites.

Not only that, PCT allows only the selected genotypes with the best multiplication rate in sterile conditions with the temperature controlled surrounding to propagate. PCT also proved to diminish the space requirement as well as labor costs to maintain the collection of the germplasm (Sundhersan et al., 2003). According to Ochoa-Villarreal et al. (2016), plant cell culture technique has gained tremendous attention not only due to the fact that it can diminish the requirement for precious water resources that usually associated with temporary agricultural production but it has also attained the consumers' acceptance because they are classified as a non-genetically modified organisms.

It is also known that PCT has several advantages compared to the whole-plant culture. This is because the desired natural products or phytochemicals can be harvested all over the world if the scientist maintains the strict quality control and production. Other than that, problems related to the climate or ecological changes can be avoided and the most important aspect is that the growth cycles of the plants were measured by weeks instead of years in an intact plant (Cusido et al., 2014).

In the same vein, some authors stated that each plant cell cultures is considered totipotent and therefore have the abilities to regenerate the whole plant body through somatic embryogenesis (Ikeuchi et al., 2013; Varhanikova et al., 2014). It was also believed that after renewing themselves, each cell is able to remarkably self-activate more than one programs of cellular differentiation and give rise to cells producing chemicals identical to the parent plants in favorable conditions (Almeida et al., 2015; Yue et al., 2016).

2.3.1 Propagation of *P. cablin* cell cultures

Researchers over the past decade have shown us that propagation of *P. cablin* via seeds is impossible due to the fact that this plant rarely flowers hence harvesting seeds is unfeasible. Therefore, the only way to propagate *P. cablin* is by stem cutting. However, mass production of *P. cablin* via this conventional method has a limitation in which the plants have become more feasible to the attack of root-knot nematodes, mosaic virus as well as insect pests (Swamy and Sinniah, 2016). According to research conducted by Kumaraswamy and Anuradha (2010), breakneck in PCT techniques worldwide can be beneficial for the continuity of *P. cablin* plantlet for field cultivation.

Several studies done by previous researchers have reported various method to mass propagate *P. cablin* via PCT technique. One of them is the regeneration via nodal callus, leaf, as well as stem tip and another important example, is the regeneration of *P. cablin* using alginate beads. These methods have proven to produce very high-quality patchouli plants due to the fact that the plants were resistant to pathogens as well as diseases (Swamy et al., 2010). Based on studies done by Hart et al. (1970), cell culture of *P. cablin* can be done in both solid (callus culture) as well as liquid medium (cell suspension culture).

2.3.1(a) Callus culture

Plant cell and tissue culture technique is a well-known method to produce high-yielding bioactive natural product instead of whole plant culture and one of the basic methods is by callus culture. A study done by Lindain et al. (2008) confirmed that callus culture of Ylang-Ylang (*Canaga odorata*) was able to yield a higher yield of benzyl acetate (one of the major compounds found in the essential oil of Ylang-Ylang) compared to the yield of benzyl acetate found in the fresh flowers of the plant (Tan et al., 2015).

According to Ikeuchi et al. (2013), the first callus formation was found over 200 years ago on a debarked tree. They were called *callum* in the Latin word which means hard and in the field of plant biology, callus was identified and referred to the enormous growth of cells. This formation of cells usually accumulates callose that usually occurred due to wounding or injury to the plant cells. Previous research on one of the essential oil producing plants (*Achyranthes aspera*) has confirmed that callus can be induced from a variety of vegetative organs such as roots, leaf, and internodes (Sen et al., 2014; Srivastav et al., 2011).

However, in the event of callus induction from patchouli plants, an earlier experimental work using MS medium added with sucrose (3%), bacto-agar (0.8%), NAA (2 mg/L) and BAP (0.5 mg/L) confirmed that the maximum frequency of callus formation can only be procured from its leaf segment (Misra, 1996). In addition to that, Inampudi and his colleagues also suggested the use of leaf explants for micropropagation due to its higher content of oil (Inampudi et al., 2017).

2.3.1(b) Cell suspension culture

Another impressive method in the world of PCT is via cell suspension cultures. Compared to the callus culture (clusters of undifferentiated cells grown on solid culture medium), cell suspension cultures which is grown in the liquid medium under vigorous consistent shaking is the most promising tool in PCT due to the fact that cell suspension cultures grow at a faster rate compared to callus cultures (Maathuis, 2013).

Apart from that, studies conducted by Bourgaud et al. (2001) also stated that cell suspension culture has the ability to recover a large number of cells and therefore ensure the isolation of enzyme which enable the scientist to observe the limiting enzyme activities for the production of beneficial metabolites. A considerable amount of literature has been published on the use of cell suspension cultures in a variety of essential oil producing plants worldwide. Some of the findings were summarized in Table 2.3.

Table 2.3: Summary of the use of plant cell suspension cultures to identify its major secondary metabolites

Plants producing essential oil	**Major secondary metabolites**	**References**
Panax vietnamensis	Ginsenosides	Trong et al., 2017
Helianthus annus	α-Tocopherol	Haas et al., 2008
Rosa damascena	Catechin	Kovatcheva-Apostolova et al., 2008
Lavandula vera	Rosmarinic acid	Kovatcheva-Apostolova et al., 2008
Salvia miltiorrhiza	Cryptotanshinone	Zhao et al., 2010
Curcuma amada	Isosorbide	Raju et al., 2015
Mentha piperita	Menthol	Samaneh et al., 2015

Although *P. cablin* is considered as one of the most important essential oil producing plants, there is a relatively little amount of literature that is concerned with the initiation of *P. cablin* via cell suspension culture. One of the earliest publication was in 1970, in which Hart et al. described that they have cultured *P. cablin* in a liquid medium comprised of 0.2 mg/L naphthaleneacetic acid (NAA), 10 % coconut milk as well as inorganic salts. However, free volatile sesquiterpene similar to the parent plant was unable to be detected. In recent years, researchers have investigated a variety of approaches to successfully initiate cell suspension culture of *P. cablin* with the secondary metabolites similar to the parent plant. Saad et al. (2016) did a study in which they manipulated several culture conditions such as carbon source, inoculum size and light conditions to initiate a healthy and friable *P. cablin* cell suspension culture. Meanwhile, Bunrathep et al. (2006) had successfully initiated the cell suspension cultures of *P. cablin* and fed them with precursor to enhance the production of the major constituent in the plant which is patchoulol.

2.3.1(c) Cell viability

The term senescence in plants biology has a very special meaning. Although senescence in human aging refers to the process of going old, plants senescence was acknowledged to be a part of the developmental phase. An example for the developmental phase in plants can be an episode of transdifferentiating that may complete the growth of the plants and this was definitely dependent on the expression of specific genes along with the plants' cell viability (Thomas, 2013).

Due to that reason, study on cell viability in plant cell cultures can be considered essential to not only identify the growth kinetics of the plant cells but also to enhance the rules and regulation for regeneration along with the induction of plant somatic embryos (Cadena-Herrera et al., 2015; Padua et al., 2014). In the field of plant cell cultures, it is rather necessary to develop a method that is both cheap and effective. Therefore, past researchers have successfully separated two methods in evaluating non-viable as well as viable cells. The first group was to stain only the dead cells and the other was to color the living cells (Fernandez-Da Silva and Menendez-Yuffa, 2006; Padua et al., 2014).

According to Truernit and Haseloff (2008), trypan blue is a good example of dyes used to stain non-viable cells. They stated in their research that plasma membrane of a dying cell refuses to function properly as a selective barrier thus allowing dyes to penetrate into the cells and colored the non-viable cells into blue color. The blue stained (non-viable) cells can be visualized using a simple light microscopy. A study was then done by Qin and colleagues in which they use trypan blue staining to investigate the effect of ultrasound pulses in tobacco cell lysis. Their results revealed that 23.4% of cells instantly lyse and undergo programmed cell death after interacted with ultrasound pulses and therefore proves the efficiency of trypan blue in detecting non-viable cells (Qin et al., 2012).

A few years later, Saruyama and his colleagues did a study on compounds that were suitable for staining living cells in plants. They agreed that fluorescein diacetate (FDA) was the best and was capable of staining viable plant cells. When incorporated into plant cells, this nonpolar compound will be hydrolyzed via esterase