METABOLIC ENGINEERING OF ESSENTIAL OIL COMPOSITION IN *LAVENDULA LATIFOLIA* BY ALTERING EXPRESSION OF BORNEOL DIPHOSPHATE SYNTHASE

By

Elaheh Najafianashrafi

B.Sc., University of Tehran, 2008

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE COLLEGE OF GRADUATE STUDIES

(Biology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Okanagan)

April 2021

The following individuals certify that they have read, and recommend to the College of Graduate Studies for acceptance, a thesis/dissertation entitled:

Metabolic engineering of essential oil composition in *Lavendula latifolia* by altering expression of borneol diphosphate synthase

submitted by Elaheh Najafianashrafi in partial fulfillment of the requirements of

the degree of Master of Science.

Dr. Soheil Mahmoud, Biology, Irving K. Barber School of Science

---

Supervisor

Dr. Michel Deyholos, Biology, Irving K. Barber School of Science

---

Supervisory Committee Member

Dr. Thuy Dang, Chemistry, Irving K. Barber School of Science

Supervisory Committee Member

Dr. Hadi Mohammadi, Faculty of Applied Science, School of Engineering

---

University Examiner

Abstract

The volatile monoterpene metabolites camphor and borneol are considered undesired constituents of high-end lavender essential oils (EO) produced for the cosmetic industry. In plants, these metabolites are derived from borneol diphosphate (BPP), which itself results from the rearrangement of geranyl diphosphate (GPP) via a reaction catalyzed by the enzyme borneol diphosphate synthase (BPPS). Mahmoud group has recently cloned a unique BPPS gene from *Lavandula x intermedia* (the LiBPPS). However, the *in planta* role of this gene has not been evaluated. In this study, we aimed to confirm the *in planta* function of *LiBPPS* through its constitutive expression in sense and antisense in spike lavender (*L. latifolia*). To achieve this goal, the coding sequence of *LiBPPS* was placed under the control of the Cauliflower Mosaic Virus 35S (CaMV35S) promoter in sense and antisense orientations, and stably expressed in transformed *L. latifolia* plants via *Agrobacterium*-mediated transformation using hygromycin as a selectable marker.

We evaluated the effect of LiBPPS overexpression (in sense and antisense) on abundance of BPPS transcript in transformed plants. As anticipated, overexpression of *LiBPPS* in sense resulted in an increase in *BPPS* mRNA levels, while overexpression of the gene in antisense led to a decrease in *BPPS* mRNA levels in transgenic plants.

To determine whether altering the expression of BPPS influenced EO yield, and abundances of camphor, borneol and other monoterpenes, we analyzed the EO of the transgenic plants by gas chromatography mass spectrometry (GC-MS). Manipulating BPPS expression did not have a significant effect on oil yield. However, we observed a significant reduction in borneol and camphor, and a significant increase in the production of other EO constituents such as 1,8 cineole and limonene in 90.9 % of the BPPS-antisense plants relative to wild-type plants. Furthermore, meeting our expectations, 60% of the BPPS-sense plants produced higher levels of camphor and borneol, and lower amounts of other oil constituents including limonene and 1,8 cineole compared with wild-type plants. Results obtained in this

study provide insight into the regulation of camphor production in lavenders, and confirm that monoterpene metabolism can be modified through genetic manipulation of terpene synthase expression.

Lay Summary

This thesis aimed to investigate the function of the enzyme borneol diphosphate synthase from Lavandin (*Lavandula x intermedia*) (LiBPPS) through the expression of the LiBPPS gene in spike lavender. To achieve this goal, the *LiBPPS* gene was expressed in sense and antisense orientations in stably transformed spike lavender plants. The effects of *LiBPPS* overexpression on the abundance of BPPS transcript in transformed plants were then evaluated by Polymerase Chain Reaction (PCR). As well, the effects of *LiBPPS* overexpression on the production of EO, and EO constituents borneol and camphor were evaluated by gas chromatography / mass spectrometry. The results confirmed the function of LiBPPS, and demonstrated that altering the expression of the *LiBPPS* gene can affect production of borneol and camphor in transformed plants.

Preface

I conducted this project in Dr. Soheil Mahmoud's lab under the supervision of Dr. Soheil Mahmoud. All experiments and analysis of research data in this work were completed by Elaheh Najafianashrafi with the assistance of Dr. Ayelign Adal. Identification, cloning and *in vitro* functional characterization of *LiBPPS* were conducted by Dr. Ayelign Adal. I cloned this gene in another vector with hygromycin selectable marker and transformed spike lavender with *LiBPPS* via *Agrobacterium*-mediated transformation. All the tissue culture regeneration and molecular analysis of transgenic plants via PCR and qPCR were completed by Elaheh Najafianashrafi. I also conducted EO screening of transgenic plants and wild-type plants by GC-MS. Results of this study are being prepared for publication in a journal. This thesis was written with the guidance of Dr. Soheil Mahmoud and was reviewed and approved by my supervisory committee: Dr. Soheil Mahmoud, Dr. Michael Deyholos, and Dr. Thuy Dang. While completing my thesis, I also was co-author of the following publication: *Planta* 251(41): 1-11, Cloning and functional characterization of a floral repressor gene from *Lavandula angustifolia*, Rebecca S. Wells, Ayelign M. Adal, Lina Bauer, Elaheh Najafianashrafi and Soheil S. Mahmoud, © 2020, license 4900371164986.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ABA	Abscisic acid
ADH	Alcohol dehydrogenase
BA	6-benzylaminopurine
BDH	Borneol dehydrogenase
BM	Basal medium
BPPS	Bornyldiphosphate synthase
BRM	Basal regeneration medium
2,4-D	2,4-dichlorophenoxyacetic acid
DMAPP	Dimethylallyl diphosphate
EO	EO
ER	Endoplasmic reticulum
FPP	Farnesyl diphosphate
GC-MS	Gas chromatography-mass spectrometry
GPP	Geranyl diphosphate
GUS	β- glucuronidase
IAA	Indole-3-acetic acid

IBA	Indole-3-butyric acid
2iP	N6 -(2-isopentenyl) adenine
IPP	Isopentenyl diphosphate
KIN	kinetin
LB	Luria-Bertani
LiBPPS	*Lavendula x intermedia* borneol diphosphate synthase
MEP	2-C-methyl-d-erythritol 4-phosphate
mTPS	Monoterpene synthases
MVA	Mevalonate
MS	Murashige and Skoog
NAA	Naphthylacetic acid
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PGRs	Plant growth regulators
qPCR	Quantitative reverse transcriptase PCR
RE	Restriction enzyme
SDR	Short chain alcohol dehydrogenase

SEM	Shoot elongation medium
TDZ	Thidiazuron
RM	Rooting media

Acknowledgements

First, I would like to express my sincere gratitude to my research supervisor, Dr. Soheil Mahmoud for providing me with a chance to conduct my research. His devoted guidance and patience helped me to flourish and to get invaluable experiences throughout my studies. I would also appreciate my committee members, Drs. Michael Deyholos and Dr. Thu-Thuy T. Dang for their technical advice and feedback. I would like to express my heartfelt gratitude to my co-workers in Dr. Mahmoud's laboratory, Dr. Ayelign Adal for his continuous assistance and mentorship, Ms. Rebecca Wells for her nice attitude and guidance, Ms. Savanna Maddock, Dr. Lukman Sarker, Ms. Lina Bauer, Ms. Lisa Remedios and Ms. Sakshi Khanna. I would like to thank Ms. Barb Lucente for her technical support and assistance during my studies. I also would like to acknowledge UBC Okanagan, Mitacs, Natural Sciences and Engineering Research Council of Canada, and the Canadian Foundation for Innovation for supporting my project through grants to Dr. Soheil Mahmoud.

I am extremely grateful to all my family for their love, caring and continuous support. My special thanks go to Dr. Ali Akbarisehat, my caring and loving husband for his constant support and encouragement.

Dedication

This thesis is dedicated to:

My husband and my family

For all your support and love

My mom,

For your strong and gentle soul, I always keep you in my heart, rest in peace!!!

Chapter 1: Introduction

1.1 Lavenders

Lavenders are perennial shrubs in the genus Lavandula, in the mint family (*Lamiaceae*). The genus Lavandula comprises of over 30 species, about 400 cultivars, and hybrid varieties, which differ by growth habit, morphological characters, and chemical composition (Lesage-Meessen et al., 2015). Among the existing species, only three are commercially cultivated worldwide for the production of their essential oil (EO): *Lavandula angustifolia*, commonly known as English Lavender, the most common lavender with many pretty cultivars, and blossom colour; *Lavandula latifolia* or spike lavender, a Mediterranean grass-like lavender; and *Lavandula x intermedia* or lavandin, which is a sterile cross between *L. latifolia* and *L. angustifolia* (Koulivand et al., 2013; Sarker et al., 2012; Woronuk et al., 2011).

Although Lavenders originate from the Mediterranean region, they are now cultivated largely in Europe, Canary Islands, Madeira, North Africa, South West Asia, Arabian Peninsula, India, North and South America (Aprotosoaie et al., 2017). While France, Bulgaria, UK, China, Ukraine, Spain, and Morocco are the world's top producers of lavender EO, lavandin and spike lavender are largely cultivated in France and Spain (Lesage-Meessen et al., 2015).

Lavender EO (EO) is a complex mixture of volatile compounds, including monoterpenes (C_{10}) and sesquiterpenes (C_{15}), which are produced mainly in the glandular trichome secretary cells localizing the surfaces of leaves and floral tissues (Guo et al., 2020). Lavenders are famous for their monoterpenes as the main constituent in their EO, which is widely used in pharmaceutical preparations, the perfumery industry, and cosmetics (Falk et al., 2009; Juan Segura et al., 2019).

The major monoterpenes found in EO of lavenders include linalool, linalyl acetate, borneol, camphor, and 1,8-Cineole. These constituents are varied based on the lavender species. The major components of the cultivated Lavandula species are the following: linalyl acetate, linalool, β-Ocimene, terpinen-4-ol, lavandulyl acetate and borneol for lavender oil; linalool, 1,8-Cineole, camphor, borneol for

spike lavender oil; and linalool, linalyl acetate, camphor, 1,8-Cineole and borneol for lavandin oil (Aprotosoaie et al., 2017; Lauren Alexandra Elizabeth Erland, 2015; Lesage-Meessen et al., 2015; Upson et al., 2004) (Table 1).

Table 1. Chemical composition of major monoterpenes in lavender (*L. angustifolia)*, spike lavender (*L. latifolia)* and lavandin (*L. x intermedia)* as a percentage of total oil composition.

Compound	***L. angustifolia***	***L. latifolia***	***L. x intermedia***
1,8-Cineole	Trace	22–27	4–7
Camphor	Trace	12–16	6–8
Linalool	25–38	27–41	25–47
Linalyl acetate	25–45	Trace	26–48
Terpinen-4-ol	4–5	Trace	Trace
β-Ocimene	3–4	Trace	Trace
Borneol	0-24	0.16-5.9	1.71-26
Lavandulyl acetate	2-3.5	Trace	0-3.1
Limonene	0-1	0.5–3	0.5–1.5

The typical olfactory characteristics of oil quality depend on the ratio of undesirable to desirable monoterpenes (Despinasse et al., 2017). The finest and most desired lavender oils contain high percentages of linalool and linalyl acetate, and are used in the cosmetic and flavour industries, while oil

quality and the prize of the essence drops with increasing camphor and 1,8 cineole ratios (Aprotosoaie et al., 2017; Dušková et al., 2016; Juan Segura et al., 2019; Woronuk et al., 2011).

L. angustifolia (English lavender) has the most favourable lavender oils with high linalool/linalyl acetate and low camphor, and is used for the perfumery industry. Spike lavender yields more EO, but with a higher level of unpleasant camphor and borneol, making it unpleasant for cosmetics and perfumes. Lavendin, crossed lavender, produces much more oils than English lavender (120 kg/ ha compared to 40 kg/ha) but has lower application in perfumery and therapy due to the high levels of unpleasant camphor and borneol (Aprotosoaie et al., 2017; Juan Segura et al., 2019). 1, 8-cineole has an aromatic camphor-like odour while camphor presents musty, penetrating, slightly minty notes. Also, borneol is found in the EO from leaves and aerial parts than in lavender flowers EO (Aprotosoaie et al., 2017; Salido et al., 2004). Limonene has been used as an insecticide to control ectoparasites of pet animals and might be employed for pest and weed control in agriculture. Also, linalool content is a trace monoterpenes found in leaves and but with higher content (more than 15% of the total oil) in flowers of spike lavender (Juan Segura et al., 2019).

1.2 Lavender EO

EO are volatile compounds plants produce for their protection rather than nutrition. Essentials oils are accumulated in secretory cells of glandular trichomes present on stem, leaf, fruit, and main flower in plants. Trichomes are globules with secretary cells, stalk, and storage cavity (Chamorro, 2012). These structures (especially in the *Lamiaceae* family) are involved in plant defense against herbivores and pathogens and deterring insects (Sharifi-Rad et al., 2017). They also have non-defensive roles, such as temperature regulation, light reflectance, protection against UV, decreased water loss and photosynthesis (via light reflection), the attraction of pollinators, mediate allopathy and seed dispersal (Glas et al., 2012; Santos Tozin et al., 2016).

Lavender has a long history of medical usage. The lavender EO has been known for its antidepressive, anxiolytic, sedative, anticonvulsant, analgesic activity. It also has shown antioxidant, antifungal, antimicrobial, anti-inflammatory, spasmolytic, carminative properties (Baker et al., 2012; Sharifi-Rad et al., 2017) It also has applications in insect & pest control, cosmetics, and the food industries, aromatherapy massage, and bathing (Koulivand et al., 2013; Wells et al., 2018).

Several studies have reported the mechanism of action of lavender EO in neuronal tissues. Lavender EO has neuroprotective activity against cerebral ischemia and alleviated neurological function, which might be associated with the augmentation in endogenous antioxidant defense and inhibiting oxidative stress in the brain (Vakili et al., 2014; D. Wang et al., 2012). Exposure to lavender is reported to reverse spatial memory deficits induced by dysfunction of the cholinergic system. Linalool and linalyl acetate act as anxiolytic and antidepressive agents and improve spatial memory deficits (Hritcu et al., 2012).

Sedative, anesthetic, and antispasmodic actions have been reported due to linalool and linalyl acetate activity. Also, linalool, 1, 8-cineole, camphor, terpineol, and α-and β-pinene have shown antibacterial and antifungal activity (Blažeković et al., 2010; Lesage-Meessen et al., 2015; Moon et al., 2007). Insecticidal and insect repellent properties have been attributed to limonene, borneol, linalool, linalyl acetate, and 1,8-cineole (Rozman et al., 2007; Attia et al., 2016; Germinara et al., 2017). Also, monoterpenes such as linalool, camphene, and 1,8-cineole and camphor, are involved in pollinator attraction, mediating plant to plant interactions, and allelopathy (Mahmoud & Croteau, 2002; Okamoto et al., 2011). Oils obtained for the alternative medicine sector usually come from spike lavender with high levels of linalool, camphor, and 1, 8-cineole, but trace linalool acetate (Sarker et al., 2012).

Although short-term treatment of lavender for some neurological disorders is recommended, long-term trials are needed as a precaution (Koulivand et al., 2013). It is reported that lavender and lavandin oils may also have toxic effects at certain doses and these effects are especially due to linalool,

camphor, and linalyl acetate. Also, camphor at higher concentrations might have serious consequences, including convulsions, coma, heart failure, circulatory collapse, abortion, and even death (W. Chen et al., 2013; Lesage-Meessen et al., 2015).

Also, the antibacterial effect of lavender EO against *Escherichia coli* O157: H7, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus* and *Salmonella typhimurium* have been researched. Some studies have reported the antifungal activity of lavender EO against *Candida albicans*, *Aspergilus niger*, and plant pathogenic fungi like *Botrytis cinerea* and *Phytophthora infestans.* Camphor, α-terpineol, and terpenen-4-ol have antibacterial properties while limonene, pinene, 1, 8-cineole, and myrcene have antifungal activity (Sharifi-Rad et al., 2017; Wells et al., 2018). The lavender EO has also shown anti-inflammatory, free- radical scavenging, and antioxidant properties due to the activity of linalool, linalyl acetate, α- pinene, and caryophyllene oxide (Peana et al., 2002).

EO has been widely used in the cosmetic and food industry for its pleasant fragrance and flavour. They have shown promise as natural preservatives in food and cosmetics due to their antimicrobial and antioxidant properties (Djenane et al., 2012; Sharafati Chaleshtori et al., 2015).

1.3 Biosynthesis of EO

Lavender EO is mainly comprised of monoterpenes (C10), and small amounts of sesquiterpenes (C15). Monoterpene biosynthesis can be divided into four phases (A. Lane et al., 2010; Mahmoud & Croteau, 2002; Muñoz-Bertomeu et al., 2006): (1) construction of the basic C5 units isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP); (2) condensation of IPP and DMAPP by prenyltransferase to form linear geranyl diphosphate (GPP; C10), the respective precursor for regular monoterpene biosynthesis; (3) conversion of GPP to the parent skeleton of the various monoterpene subfamilies, through the catalytic action of specific terpene synthases; and (4) enzyme-mediated transformation of the parent structures to various metabolites (Figure 1 & 2).

The biosynthesis of monoterpenes begins with the synthesis of two five-carbon units, IPP and

DMAPP. IPP and DMAPP are derived from two different precursor routes, namely the mevalonate (MVA) pathway localized in the cytosol and the plastid-localized non-MVA pathway, also termed as 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway. Both MVA and MEP biosynthetic routes produce IPP and DMAPP as the common precursors of terpenes (Gershenzon et al., 2000; Laule et al., 2003; Liu et al., 2005; G. Wang, 2014). The MEP pathway gives rise to the mono-, di-, and polyterpenes, while the MVA pathway brings about sesqui- and triterpene (Hampel et al., 2005; Vranová et al., 2012). Although studies on the biosynthesis of monoterpenes in Lamiaceae are scarce, investigations in peppermint (*Mentha piperita*) and *L. angustifolia* suggest that the MEP pathway might be the main source of precursors (IPP and DMAPP) for monoterpenes in this family (Guo et al., 2020; W. A. Lane & Mahmoud, 2008; B Markus Lange & Ahkami, 2013; Mahmoud & Croteau, 2003). The MVA pathway begins with the condensation of three units of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by acetoacetyl-CoA thiolase (AACT) and continues to form 3-hydroxy- methylglutaryl CoA (HMG) by 3-hydroxy- methylglutaryl CoA synthase (HMGS) and then reduced to mevalonate (MVA) by HMGR (HMG-CoA reductase). Mevalonic acid is then phosphorylated and carboxylated to generate IPP through the action of mevalonate kinase (MVK), phosphomevalonate kinase (PMK), and mevalonate diphosphate decarboxylase (MVD) (Croteau et al., 2000; B Markus Lange & Ahkami, 2013). The abundance of monoterpenes can be affected by substrate flux through the MEP pathway (Muñoz-Bertomeu et al., 2006). MEP pathway, referred to as a newly discovered route for biosynthesis of isoprene, starts with the condensation of pyruvate and glyceraldehyde-3-phosphate (GAP) to make 1-deoxy-D-xylulose 5-phosphate (DOXP), catalyzed by DOXP synthase (DXPS). DOXP is then reduced by DX reductoisomerase (DXR) to form 2-C-Methyl-Derythritol 4-phosphate (MEP). MEP synthesis is followed by the formation of the cytidine 5-diphosphate derivative, phosphorylation, and cyclization to 2- C-methylerythritol-2,4cyclodiphosphate (MECP). MECP is then oxidized to 1-hydroxy-2 methyl-2-(E) -butenyl 4diphosphate (HMB-PP) by HMBPP synthase (HDS). Isopentenyl diphosphate and Dimethylallyl pyrophosphate are produced as final products by HMB-PP reductase (HDR) (Liu et al., 2005; Vranová et al., 2012) (Figure 1).

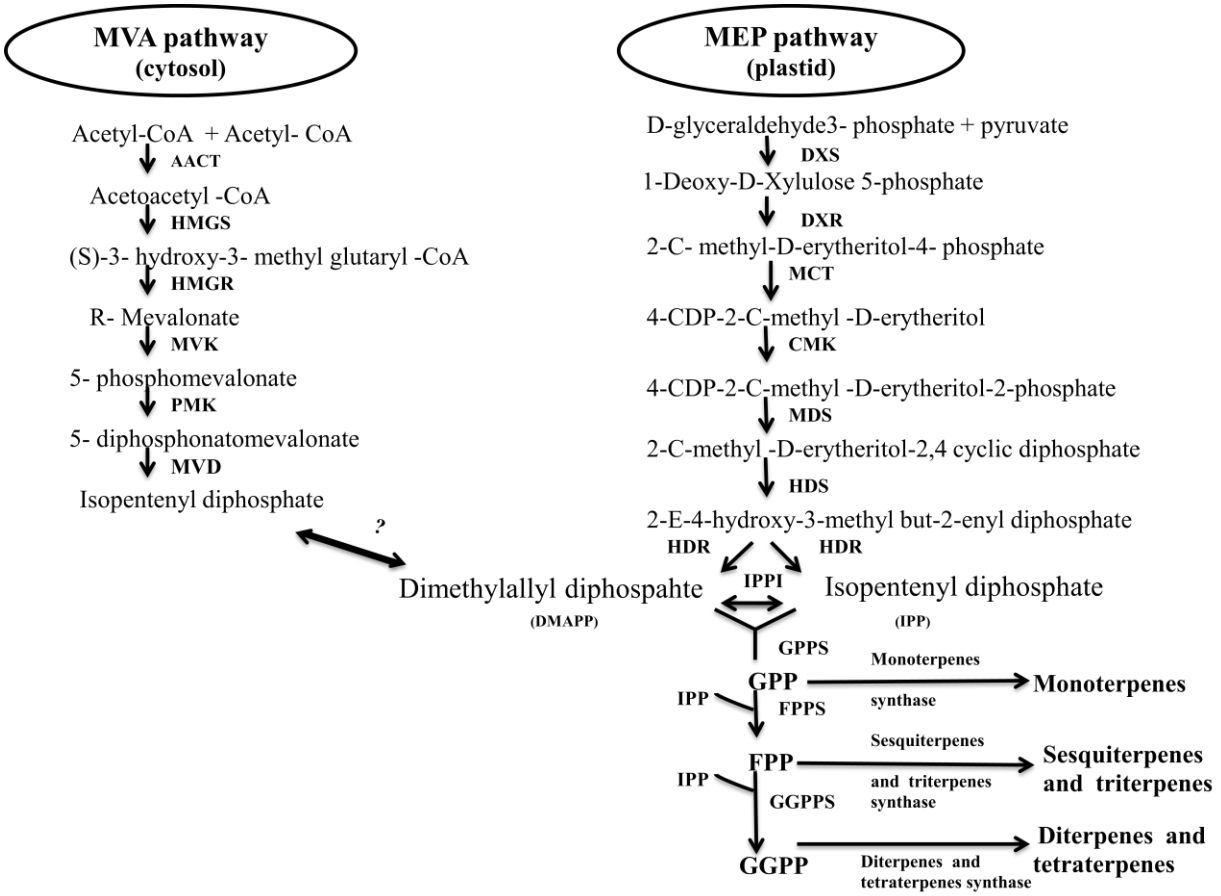


Figure 1. Terpenoid biosynthesis pathways in plants.

Biosynthesis of IPP and DMAPP through the plastidial-localized MEP pathway (right) and the MVA pathway (left) in the cytosol. Enzymes in the MEP pathway are 1-deoxy-D-xylulose 6-phosphate (DOXP) synthase (DXS), DOXP reductoisomerase (DXR), 2-C-methyl-Derythritol cytidyltransferase (MCT), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEC-PP) synthase (MDS); 1-hydroxy-2 methyl-2-(E)- butenyl 4diphosphate (HMB-PP) synthase (HDS), and HMBPP reductase (HDR). Enzymes included in the MEP pathway analysis are acetoacetyl-CoA thiolase (AACT), 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase (HMGS), HMG-CoA reductase (HMGR), mevalonate kinase (MVK), phospho-mevalonate kinase (PMK), 5-phosphate-mevalonate decarboxylase (MVD). IPP isomerase (IPPI); geranyl diphosphate (GPP); GPP synthase (GPPS); farnesyl diphosphate (FPP); FPP synthase (FPPS); geranylgeranyl diphosphate (GGPP); GGPP synthase (GGPPS).

In the following stage of terpene biosynthesis, one molecule of IPP with one molecule of DMAPP is condensed by prenyltransferase to yield geranyl diphosphate (GPP), the precursor of most monoterpenes as regular monoterpenes. Terpenes are classified by the number of isoprene units that they contain, and monoterpenes contain two isoprene units with a molecular formula of $C_{10}H_{16}$. Further

condensation of one GPP with one IPP produces farnesyl diphosphate (FPP), the precursor of sesquiterpenes (Boeckelmann, 2008; Demissie et al., 2012; Glas et al., 2012).

Based on how isoprene units are condensed, regular or irregular monoterpenes are biosynthesized. The precursor of regular terpenes, geranyl diphosphate synthase (GPP), is produced by the sequential head-to-tail addition of DMAPP to IPP, whereas the non-head-to-tail joining of the two isoprene units led to the precursor of less common 'irregular' terpenes, lavendulyl diphosphate (LPP) (Boeckelmann, 2008; Mahmoud & Croteau, 2002).

These precursors are subsequently modified into various monoterpenes by specific enzymes known as monoterpene synthases (mTPS) (Figure 2). Based on their chemical structures, monoterpenes are classified into cyclic monoterpenes such as pinene, borneol, camphor, 1,8 cineole and limonene and acyclic or linear monoterpenes, including geraniol and linalool, and ocimene. Relatively few monoterpenes are acyclic (Degenhardt et al., 2009; Zhang et al., 2017).

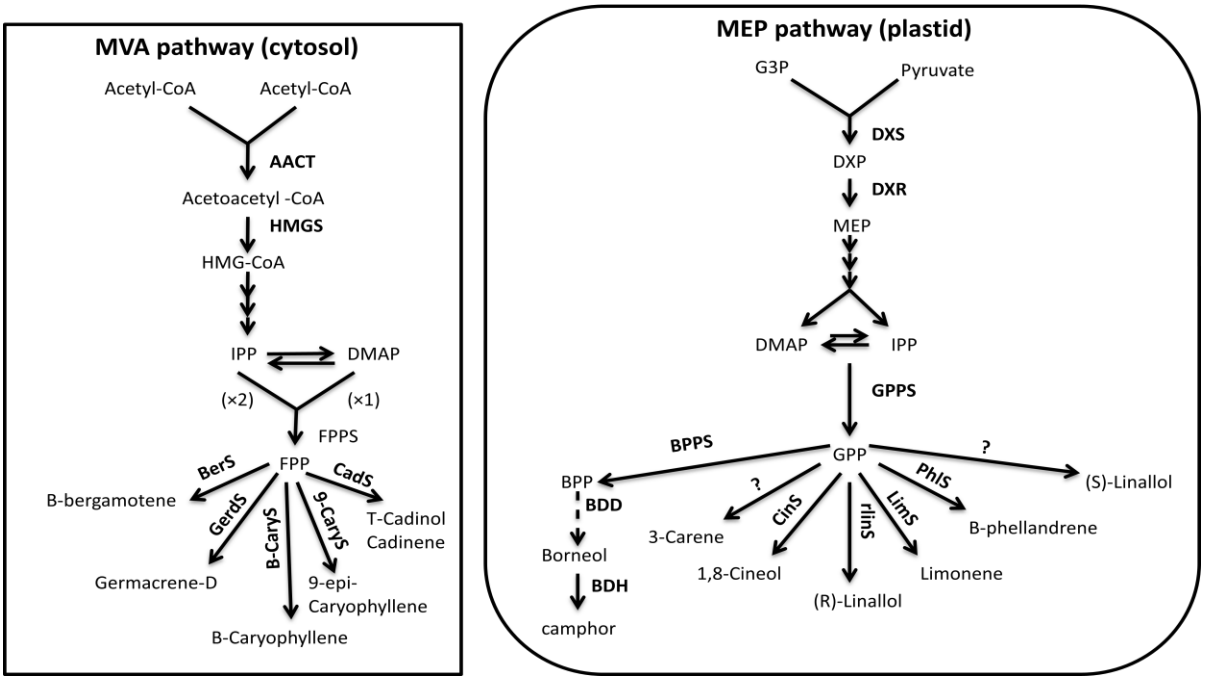


Figure 2. Biosynthesis pathway of mono and sesquiterpenes.

MEP- 2-C-methyl-D-erythritol 4 phosphate; DXS- 1-deoxy-D-xylulose 6-phosphate (DOXP) synthase; DOXP reductoisomerase (DXR); isopentenyl diphosphate (IPP); methyl-D-erytritol-4 phosphate (MEP); Dimethylallyl pyrophosphate (DMAPP); geranyl diphosphate (GPP); β-phellandrene synthase (PhlS); (R)-linalool synthase (rLinS); limonene synthase (LimS); 1,8-Cineole synthase (CinS); borneol diphosphate synthase (BPPS); bornyl diphosphate (BPP); borneol diphosphate diphosphatase (BDD); borneol dehydrogenase (BDH); Mevalonate (MVA); acetoacetyl-CoA thiolase (AACT); 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase (HMGS); farnesyl diphosphate (FPP); FPP synthase (FPPS); β -bergamotene synthase (BerS); germacrene D synthase (GerS); T-cadinol synthase (CadS); β-caryophyllene synthase (β –CaryS), and 9-epi-caryophyllene synthase (9-CaryS).

The molecular weight of plant-derived monoterpene synthases are in general between 50 kDa and 100 kDa; a predicted isoelectric point (pI) near 5.0 and a pH optimum in the neutrality area. They also need a divalent metal ion (usually Mg^{2+} or Mn^{2+} for angiosperms, K^{+}, Mn^{2+}, Fe^{2+}) as a cofactor for catalysis (Bohlmann et al., 1998). Monoterpene synthases contain three conserved motives among which a tandem arginine motif (RR(X8) W-motif) is located at the very beginning of the N-terminus of the protein. This motif plays a role in the isomerization of the monoterpene cyclization of the GPP substrate and enzymatic activity of mTPS (Bohlmann et al., 1998; Williams et al., 1998) and protein stability (Boyle et al., 2007). The RR(x8) W motif is involved in producing cyclic monoterpenes and is absent in mTPS that produce acyclic products (F. Chen et al., 2011). The second and third motifs presented at the

C-terminus are DDxxD and (N, D)D(L, I, V)x(S, T)xxxE, have a function in substrate binding and coordination of divalent metal ion cofactors (Christianson, 2006; Degenhardt et al., 2009). All mTPS also bear transit peptides, which are N-terminal extensions rich in serine and threonine, low in acidic and basic amino acids (aa), and they are about 45-70 aa long. They facilitate the targeting and translocation of cytosolically synthesized precursors into plastids via a post-translational mechanism (Bohlmann et al., 1998; Degenhardt et al., 2009).

1.3.1 Camphor biosynthesis in lavenders

Spike lavender is known as a high oil-yielding lavender variety producing low-grade oils with more camphor (Falk et al., 2009). Among monoterpenes, linalool, 1,8-cineole, and camphor are found mostly in flowers, while leaves have 1,8-cineole and camphor as main undesirable flavour components in spike lavender, leading to a depreciated EO (Mendoza-Poudereux et al., 2017).

In the monoterpenes biosynthesis route, camphor is derived from the rearrangement of GPP by specific enzymes (Despinasse et al., 2017). Also, according to the results achieved with NMR and GC-MS, the biosynthesis of camphor and 1,8-cineole in spike lavender happens through the MEP pathway (Mendoza-Poudereux et al., 2017). Studies in sage (*Salvia officinalis*) showed that camphor and borneol are biosynthetically related (Wise et al., 1998). Bornyl diphosphate synthase (BPPS), the first enzymatic step of camphor biosynthesis, gives bornyl diphosphate (BPP) as a major prenyl diphosphate, and several monoterpenes as minor compounds. BPP is then dephosphorylated by bornyl diphosphate diphosphatase (BDD), leading to the borneol, and then oxidized to camphor by a borneol dehydrogenase (BDH) (Figure 3) (Croteau et al., 1978; Mendoza-Poudereux et al., 2017; Sarker et al., 2012; Wise et al., 1998; Woronuk et al., 2011). A monoterpenoid alcohol dehydrogenase (ADH) has been previously purified from *Artemisia annua* (Polichuk et al., 2010). This enzyme as non-specific short-chain alcohol dehydrogenase (SDR) had the lowest specific activity for borneol, indicating that borneol is not a primary substrate for *Artemisia annua* ADH2 (Sarker et al., 2012). Later, a homology-based cloning strategy was conducted to

clone an SDR from glandular trichomes of *L. x Intermedia* cDNA library (LiBDH) in *E.coli*, which converts borneol into camphor (Sarker et al., 2012). This LiBDH is the first borneol specific dehydrogenase reported from plants. Recently, a new derived *BPPS* from floral-based glandular trichomes of *L. angustifolia* (Despinasse et al., 2017) showed that LaBPPS produced BPP (in low amounts) and some other terpenes such as pinenes and camphene. Based on these results, they concluded the possible role of their new identified *LaBPPS* gene in the biosynthesis of BPP and derived monoterpenes such as borneol and camphor.

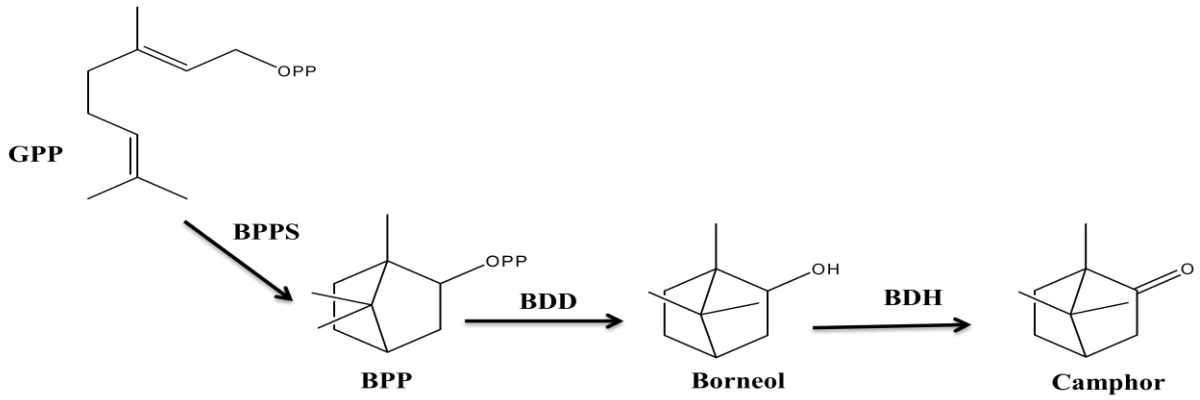


Figure 3. Putative camphor synthesis in *Lavandula latifolia.*

Bornyl diphosphate synthase (BPPS) gives bornyl diphosphate (BPP). BPP is then dephosphorylated by bornyl diphosphate diphosphatase (BDD), leading to the borneol, and borneol then oxidized to camphor by a borneol dehydrogenase (BDH).

1.4 Intracellular compartmentalization of monoterpene biosynthesis and secretion

The intracellular compartmentalization of terpene biosynthesis is still unclear. It has been reported that monoterpene synthesis is compartmentalized to specialized leucoplasts within glandular trichomes in the *Lamiaceae* family (Markus Lange & Turner, 2013; Turner & Croteau, 2004). Leucoplast is the first site for the biosynthesis of monoterpene through MEP pathway. Then, the monoterpenes are transported to the cytosol for downstream transformation and/or secretion (Turner et al., 2000; Markus Lange & Turner, 2013; Sharifi-Rad et al., 2017).

1.5 Regulation of monoterpene biosynthesis

1.5.1 Environmental regulation of monoterpenes

Monoterpene metabolites play several ecological and physiological functions, and therefore their production by differential expression profiles of monoterpene synthase genes are undergoing environmental factors, physiological, biochemical, metabolic, and genetic regulation (Sangwan et al., 2001; Tholl, 2006). Regarding the implication of monoterpene metabolites for the interaction of plants with the environment, volatile and non-volatile monoterpenes are suggested to serve as both pollinators and predators of herbivores attractants, especially at the flowering stage (Boeckelmann, 2008). Monoterpenes also act as pathogen deterrents. For example, expression of (S)-limonene synthase in rice is induced in response to defence against its pest (Magnaporthe oryzae) infection (X. Chen et al., 2018). In lavender, both non-oxygenated (ocimene, limonene) and oxygenated monoterpenes (linalool and terpinen-4-ol) could act as repellents to protect immature flowers and seeds against damaging insects (Guitton et al., 2010).

Monoterpenes have also been involved in mediating thermotolerance, and especially isoprene emission may have played an important role in surviving plants under rapid temperature changes (Sharkey & Yeh, 2001). High temperature causes water loss and decreased photosynthesis but does not prevent increased rates of isoprene emission because of the very low heat capacity of isoprene relative to water (Sharkey & Yeh, 2001). Also, isoprene emission showed a typical daily variation with a light- and temperature-dependent increase in the morning and a decline in the emission rate during the night (Mayrhofer et al., 2005). With higher light, isoprene increased thermotolerance of kudzu (*Pueraria lobata*) leaves, and when photosynthesis declined to zero, thermotolerance increased with added isoprene (Singsaas et al., 1997). It was reported that, long days improve plant growth and induce larger leaves and flowers and consequently produce higher EO yield in peppermint (Clark & Menary, 1980). Also, it is showed that low photon flux density and high temperatures led to the accumulation of pulegone and

menthofuran, both of which contribute to an 'off' odour and taste (Burbott & Loomis, 1967; Clark & Menary, 1980).

1.5.2 Developmental regulation and spatiotemporal of monoterpenes

The developmental stage of the plant also triggers the monoterpene profile of plants. For instance, in *Salvia officinalis*, camphor, and borneol levels are higher in expanding leaves compared to mature leaves (Croteau et al., 1981). Studies showed that β-ocimene and myrcene levels increased on the second day after anthesis in snapdragon flowers (Natalia Dudareva et al., 2003). In *Menta piperita*, monoterpene profiles like menthone and menthol increase with leaf age advancement (Maffei & Codignola, 1990; Turner et al., 2000). A separate study conducted on *L. latifolia* showed a decrease in camphor and 1, 8 cineole contents and an increase in linalool level from flowering to fruiting. Also, plants during the full flowering period showed higher EO yield than those in the fruiting period (Salido et al., 2004). Also, monitoring monoterpene abundance in lavender showed the highest level of linalool at the stage in which 30-70 % of flowers were in bloom (Boeckelmann, 2008).

The biosynthesis of monoterpenes is regulated spatiotemporally as well. In some plants, flowers are the major sources of monoterpenes (Boeckelmann, 2008). In others, monoterpenes are abundantly produced in leaf tissues, for example, 1, 8-cineole and camphor in *L. x intermedia*. In Arabidopsis, monoterpene biosynthesis is limited to the flower stigma, anthers, nectaries, and sepals instead of flower petals (Tholl et al., 2005).

It was reported that there is a higher level of camphor and 1, 8-cineole in spike lavender leaf oil but a high percentage of linalool and borneol in oils from the flower. Linalool is considered a major constituent in spike lavender oils from flowers, not the leaves. Linalyl acetate has a trace amount in both flowers and leaves of spike lavender. Furthermore, EO is mostly made of monoterpenes (99.4%-99.9% and 95.6%-99.5% in leaves and flowers, respectively) and the oxygenated monoterpenes such as borneol, camphor and 1,8 cineole are more abundant than the hydrocarbon monoterpenes including pinenes,

limonene and myrecene in *L. latifolia* (Muñoz-Bertomeu et al., 2007). A study showed that the leaves and bracts of *L. angustifolia* have about ten times less volatile compounds compared with the blooming heads (Guitton et al., 2010). Another study revealed that the amount of borneol/camphor was higher in flowers, especially in the first two stages of inflorescent, than leaves of *L. latifolia* in different developmental stages (Despinasse et al., 2017).

1.5.3 Genetic regulation of monoterpenes

Monoterpene production also is regulated through gene expression. Since monoterpenes have specific functions in plant life, their biosynthesis and emission need to be regulated strictly (Boeckelmann, 2008; McConkey et al., 2000; Tholl, 2006). For example, it was showed that there is a correlation between menthofuran content and the level of the menthofuran synthase (*mfs*) transcript, implying that menthofuran biosynthesis is controlled primarily by transcriptional regulation of menthofuran synthase (Mahmoud & Croteau, 2003). Using relative expression assay of borneol dehydrogenase (BDH), it was revealed that the level of camphor reached its maximum in glandular trichomes of mature flowers due to higher expression of its biosynthetic enzyme (BDH) in *L. x intermedia* (Sarker et al., 2012). Also, the abundance of mRNA, protein, and enzymatic activity of β-ocimene synthase in snapdragon flowers (Natalia Dudareva et al., 2003), S-linalool synthase expression in *Clarkia breweri* flowers (N Dudareva et al., 1996), and menthofuran synthase expression in peppermint leaves (Mahmoud & Croteau, 2003; Turner et al., 2000) correlate with the emission of corresponding monoterpenes. Higher amounts of transcripts for R-linalool synthase and 1,8-cineole synthase were found in flowers and leaves of *L. x intermedia*, respectively (Demissie et al., 2012).

Metabolic engineering for *de novo* monoterpene biosynthesis and monoterpene emission suggests that monoterpene production is regulated at the level of gene transcription (N Dudareva et al., 1996; McConkey et al., 2000). There is a growing interest in improving the quality and yield of lavender EO through metabolic engineering. Metabolic engineering is a quick and direct breeding tool to alter plant

oil profile and can be used in the development of oil with the desired monoterpene profile (Gonçalves & Romano, 2013; Sharif et al., 2019). One of the strategies is identifying any of the genes involved in controlling the biosynthesis of monoterpenes at the enzyme level (Gonçalves & Romano, 2013; Muñoz-Bertomeu et al., 2008). Using various molecular tools, numerous genes that encode mTPS and contribute to EO production have been identified from different lavender species (Adal et al., 2017, 2019; Benabdelkader et al., 2015; Demissie et al., 2012; Jullien et al., 2014; Landmann et al., 2007; Sarker et al., 2013; Wise et al., 1998). For the understanding of the regulation of monoterpene synthesis in lavenders, some studies have dealt with the *in vitro* cloning and functional characterization of some monoterpenes like limonene synthase, linalool synthase, trans-α-bergamotene synthase, and Bornyl-diphosphate synthase in *L. angustifolia* (Demissie et al., 2012; Despinasse et al., 2017; Landmann et al., 2007) 1,8-cineole synthase and borneol dehydrogenase in *L. x. intermedia* (Demissie et al., 2012; Sarker et al., 2012), 3-carene synthase, (S)-linalool synthase and (R)-linalool synthase in *L. x intermedia* (Adal et al., 2017, 2019). For example, cloning, heterologous protein expression in *E.coli*, and functional characterization of 1,8-cineole synthase (LiCINS) from leaves and flowers of *L. x. intermedia* were reported (Demissie et al., 2012). By showing a correlation between LiCINS mRNA and 1, 8-cineole content in mature flowers of lavandin, they concluded that the production of this monoterpene is likely controlled through transcriptional regulation of LiCINS. Furthermore, *in vitro* cloning and functional characterization of borneol dehydrogenase (BDH) from glandular trichomes of *L. x. intermedia*, showed that BDH has a function for *in vitro* converting of borneol to camphor (Sarker et al., 2012). They also reported that the LiBDH transcripts were significantly expressed in glandular trichomes of mature flowers. Also, in another study, the *in vitro* cloning and functional characterization of BPPS from *L. angustifolia* showed production of less BPP and more terpenes such as pinenes and camphene (Despinasse et al., 2017).

1.5.4 Genetic transformation of lavenders

The successful application of genetic engineering depends on the transgene being expressed and inherited stably and predictably (Muñoz-Bertomeu et al., 2006). Thus, genetic engineering, along with sufficient information about the genes involved in the biosynthetic pathways, could increase or improve EO in lavenders (Nebauer et al., 2000).

There are numerous methods to deliver a foreign gene into host plant genomes classified into two groups: indirect gene transfer - where exogenous DNA is introduced by a biological vector and direct gene transfer - where physical and chemical processes are responsible for DNA introduction (Alves et al., 1999). Among the methods proposed for achieving the gene transformation approach, indirect transformation mediated by *Agrobacterium* (Sheng & Citovsky, 1996; Zupan & Zambryski, 1995) or direct systems using particle bombardment and microinjection is mostly utilized (Crossway et al., 1986; Zhanji et al., 2001). Particle bombardment is a technique that inserts the exogenous DNA directly into the tissue cells using high-pressure helium gas (Altpeter et al., 2005; Zhanji et al., 2001). In microinjection, a solution of DNA is injected into either cytoplasm or nucleolus of cells using a fine needle or pipette (Chou et al., 2004; Crossway et al., 1986). An *Agrobacterium*-mediated transformation is a tool for introducing a foreign gene in the form of T-DNA into the plant genome by using different *Agrobacterium* strains. This method is the most common tool for plant transformation due to several merits, including its simplicity, high reproducibility, and low experimental cost. Although only a small proportion of target plant cells receive the DNA, this method can introduce a large fragment of the foreign gene but with a small copy number into the host plant genome (Birch, 1997; Gelvin, 2003; Hwang et al., 2017; S. Li et al., 2017).

Also, for *Agrobacterium*-mediated transformation, establishing an efficient and reliable transformation system and the optimum conditions (e.g., tissue materials, *Agrobacterium* strains) for each plant species is usually necessary (Mishiba et al., 2000; Nebauer et al., 2000). For example, for the first

time, the transient expression of the neomycin phosphotransferase II (nptII) marker gene, driven by the nopaline synthase (nos) promoter, for plant selection of lavandin was obtained (Dronne et al., 1998). They also showed that *Agrobacterium* susceptibility was cultivar dependent. Also for the first time, a stable transformation of spike lavender by *Agrobacterium tumefaciens* (EHA105 strain) was reported (Nebauer et al., 2000). In a similar work, it was reported an efficient transformation of spike lavender mediated by two strains of *Agrobacterium* (LBA4404 and EHA101) with the *gusA* gene (Mishiba et al., 2000).

One of the approaches for metabolic engineering of plants is implementing sense and antisense technology as useful tools to modulate the expression of the gene of interest. The antisense RNA strategy as a potential tool provides the opportunity to reduce the expression of specific genes and thereby produce plants with modified phenotypes or unwanted metabolites. Reducing the expression of a specific gene by this technique also provides a way to study either the role of a gene whose function was previously unknown or the role of a protein whose gene has been identified (Bird & Ray, 1991). For years, antisense RNA technology has been applying to reduce the expression of targeted genes in plants (Tiwari et al., 2014). The antisense technology is based on blocking the informational flow from DNA to protein via introducing a homologous antisense version of the cDNA encoding antisense RNA (Bird & Ray, 1991; van der Krol et al., 1988). A few parameters determine the potential of antisense RNA in gene regulation. These include the region and length of target mRNA covered by the antisense transcript, the organization and potential of antisense transcript to form secondary structure, and some signals controlling the synthesis and treating of antisense transcript *in vivo* (Green et al., 1986). In plants, antisense RNAs are mainly used as a potential tool in the inhibition of fruit maturation, an increase of fruit shelf-life, virus resistance, flower coloration, starch synthesis, male sterility, and fertility (Xu et al., 2018). Some studies reported the application of antisense RNA technology for modification of EO in plants. For example, in a study, the flux into the native limonene pathway was reduced in spearmint (Mentha spicata) by knocking down the expression of limonene synthase through using RNAi method (C. Li et al., 2020). It was also showed that transgenic down-regulation of menthofuran synthase, by the antisense approach, led to the

anticipated decrease in oil content of (1)-menthofuran without a change in EO yield and transgenic up-regulation of menthofuran synthase improved flux of precursors for monoterpene biosynthesis and increase in EO in peppermint (*Mentha piperita*) (Mahmoud & Croteau, 2003). Gene overexpression is defined as a process that a fragment of the desired gene is obtained by artificial gene synthesis or is directly grafted from the plant genome and subcloned into a plasmid to clone the gene. The constitutive gene overexpression via sense technology in plants has been used widely to determine gene functions, metabolic engineering of plants, and to improve useful phenotypes (Endo et al., 2018; Xu et al., 2018). Until now, overexpression of a particular terpene synthase gene has been studied in a few *Lamiacea*e such as peppermint (Krasnyanski et al., 1999; Bernd Markus Lange et al., 2011; Mahmoud et al., 2004), spearmint (Q. Wang et al., 2016), cornmint (*Mentha arvensis*) (Diemer et al., 2001), lavendin (Desautels et al., 2009), spike lavender (Mendoza-Poudereux et al., 2014, 2017; Muñoz-Bertomeu et al., 2006, 2008) but with varying success rates. For example, it was reported that overexpression of spearmint limonene synthase in spike lavender did not affect the EO profile of spike lavenders from flowers and leaves (Muñoz-Bertomeu et al., 2008). They showed that EO accumulation was higher in developing than the mature leaves. In another study, it was reported that the overexpression of the spearmint limonene synthase (*MsLS*) gene did not lead to changing EO composition in mint but led to an increase in the accumulation of limonene in spike lavender (Mendoza-Poudereux et al., 2014). Besides, it was reported in another study that overexpression of *Clarkia breweri* linalool synthase gene in spike lavender resulted in an increase in linalool content in leaves without modifying the EO yield (Mendoza-Poudereux et al., 2014).

Arguably increasing the availability of biosynthetic precursors would be the most obvious approach for enhancing essential oil yield in the transgenic plants (B Markus Lange & Ahkami, 2013; Juan Segura et al., 2019). Thus, in addition to monoterpene synthase level regulation of monoterpene biosynthesis, changes in the expression of enzymes involved in the MEP pathway affected the precursor supply (IPP and DMAPP) for monoterpene biosynthesis (Juan Segura et al., 2019). The first documented

success at increasing EO yield was reported in mint by overexpressing the endogenous gene coding for DXR (Mahmoud & Croteau, 2001). They obtained two types of transgenic lines: the first lines contained low oil yields due to co-suppression of DXR transcript levels; the second lines with overexpressed DXR showed up to 44% increased EO yield compared with wild-type controls. In lavender, the overexpression of the DXS gene led to EO increment for up to 359% in leaves and up to 74% in flowers of transgenic spike lavender with essentially no negative effects on their EO composition (Muñoz-Bertomeu et al., 2006) which might highlight the importance of the MEP pathway for precursor supply. Interestingly, the consecutive expression of DXR in spike lavender also resulted in significant increases in oil yields and other terpenoid end products derived from the MVA pathway (sterols) but had no effects on the accumulation of carotenoids and chlorophylls, which are primarily derived from the MEP pathway (Muñoz-Bertomeu et al., 2007). Also, it was reported that the generation of double transgenic spike lavender plants overexpressing both DXS and LIS genes improved neither linalool content nor EO yield (Mendoza-Poudereux et al., 2014).

1.6 Plant tissue culture

Plant tissue culture is *in vitro* maintenance of cells, tissue, organs, or whole plant under aseptic nutritional and environmental conditions (Thorpe, 2007). Apart from its use as a tool of research, plant tissue culture has different applications in the production of pathogen-free plants (P.-J. Wang & Charles, 1991), genetic manipulation (Pierik, 1988), organogenesis (Fransz & Schel, 1994), regeneration of haploid, sterile, and seedless plants through the application of pollen or microspore cultures (Reed, 1996), isolation of variants, clones, and mutant plants with enhanced resistance to biotic and abiotic stresses (Nabors et al., 1975), overcoming seed dormancy and embryo sterility (Hu & Zanettini, 1995) and production of secondary metabolites (Verpoorte et al., 1994).

Micropropagation as an effective technique of *in vitro* plant breeding provides fast propagation, high multiplication rates, independence from the environmental factors, and protection from diseases

under controlled conditions (Debnath et al., 2006). Plant micropropagation can be classified into two principal methods: propagation from axillary or terminal buds (meristem proliferation), and organogenesis via the formation of adventitious shoots or somatic embryos (George et al., 2008a; Oseni et al., 2018). In propagation by meristem, pre-existing shoot buds or primordial buds (meristems) are placed on media or soil to promote root formation and proliferation. Organogenesis involves the formation of shoot or embryo directly from an explant tissue without callus formation (direct organogenesis or direct somatic embryogenesis), or indirectly when shoots or embryos regenerate on the previously-formed callus or in cell culture (indirect organogenesis or indirect somatic embryogenesis). In somatic embryogenesis, somatic embryos resemble the seed embryos that are formed but without seed coat and can be regenerated into shoot and roots simultaneously (George et al., 2008a; Gonçalves & Romano, 2013; Oseni et al., 2018).

Plant growth regulators (PGRs) play an essential role in determining the development pathway of plant cells and tissues in the culture medium. Auxins and cytokinins are the most important hormones for regulating growth and morphogenesis in plant tissue and organ cultures. The type and the concentration of hormones used depend mainly on the species of the plant, the tissue or organ cultured, and the objective of the experiment. Auxins and cytokinins are the most widely used PGRs in plant tissue culture, and their amount determines the type of culture established or regenerated. Cytokinins are purine compounds applied into tissue culture medium mainly to stimulate cell division and morphogenesis control by differentiation of adventitious shoots from callus and by releasing lateral buds from dormancy in shoot tissue culture (George et al., 2008a; Skoog & Armstrong, 1970). Some natural cytokinenes used in tissue culture are as follow: trans-zeatin, 4-hydroxy-3-methyltrans-2-butenylaminopurine (iP), dihydrozeatin (N6 - Δ2 isopentenyl adenine), and (6-(4- hydroxy-3-methyl-trans-2- butenyl) aminopurine). Despite the presence of endogenous cytokinins in whole plants, many tissues and small organs are unable to synthesize sufficient amount of these substances in *in-vitro* culture. Thus, using exogenous cytokinin in the media seems necessary (George et al., 2008a; Jameson & Song, 2016; Oseni

et al., 2018; Skoog & Armstrong, 1970). Also, some commercially synthetic cytokinins such as kinetin (KIN) and 6-benzyl amino purine (BA) are commonly used in micropropagation works (Skoog & Armstrong, 1970).

Auxins are another widely used growth regulators in plant tissue culture. Chemically, they have either an indole or an aromatic ring in their structure. Auxins are involved in the maintenance of polarity of tissues and maintenance of apical dominance in whole plants. In tissue culture, they promote callus growth, cell suspensions, and also organogenesis (such as inducing roots and somatic embryogenesis) by initiating cell division and elongation (George et al., 2008a; Trigiano & Gray, 2016). The most common natural auxins are indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), while naphthyl acetic acid (NAA) 2,4-dichlorophenoxyacetic acid (2,4-D) are used as synthetic auxins in tissue culture (Skoog & Miller, 1957; Trigiano & Gray, 2016). Moreover, Thidiazuron (TDZ) has been used, with considerable success, to promote plant regeneration (Jones et al., 2007).

The ratio of auxin and cytokinin included in the media is an essential factor determining the type of organ induced differentiation of the cultured tissue (Skoog & Miller, 1957). Either a higher value of auxin/cytokinin or the use of auxin alone tends to produce roots, while the inclusion of cytokinin alone in the medium most often induces shoot formation. A balance of both auxin and cytokinin leads to the development of the mass of undifferentiated cells known as callus (George et al., 2008a, 2008b; Oseni et al., 2018).

1.6.1 Tissue culture studies in spike lavender

There are some germination constraints in lavender (poor germination of spike lavender and lavender and not producing seeds in lavandin), which are mainly reproduced vegetatively as cutting (Kara & Baydar, 2012; Slavova et al., 2004). Thus, effective protocols for producing these plants while keeping their valuable metabolites and avoiding exploitation of wild populations are required (Gonçalves & Romano, 2013).

In vitro propagation of lavenders under controlled environmental conditions allow for facilitating the rapid multiplication of selected clones, extraction of valuable metabolites without limitation factors (climate, water availability, and susceptibility to diseases), application of biotechnology to improve the quality and quantity of EO and to increase the vigour of Lavandula spp. (Maria Carmen Calvo & Segura, 1989; Lauren A E Erland & Mahmoud, 2014; Gonçalves & Romano, 2013).

Only a few studies on tissue culture of spike lavender, especially on regeneration of this plant are reported. For example, in a study regeneration via somatic embryogenesis from leaf bud-derived callus of spike lavender was reported (Quazi, 1980). In another study, the hypocotyl sections from spike lavender was used as preliminary explants for callus formation and cell culture (M C Calvo et al., 1988). Also, an indirect adventitious bud induction from both hypocotyls and cotyledons of spike lavender with callus formation intervention was achieved in a separate study (M C Calvo & Segura, 1988). In another effort, adventitious buds were regenerated from hypocotyl sections of spike lavender (Maria Carmen Calvo & Segura, 1989). Also, high auxin concentration and darkness inhibited regeneration from the cultured leaves. The light might modify the endogenous level of hormones by reducing free endogenous auxins like IAA (J Segura & Calvo, 1991; Stirk et al., 2014).

A method for bud regeneration from leaf explants using MS with 8.8 μM BA and 0.6 μM IAA and MS with 0.06 μM IAA plus 8.9 μM BA for shoot elongation was reported for *Agrobacterium* transformation of spike lavender (Nebauer et al., 2000). Also, a new indirect organogenesis protocol was used for *Agrobacterium*-mediated spike lavender transformation (Mishiba et al., 2000). In their indirect organogenesis, lavender leaves first were cultured in MS with 4.5 μM 2, 4-D for callus induction under light conditions. After the inoculation of induced callus with *Agrobacterium*, they were transferred to MS supplemented with 4.4 μM BA for shoot regeneration.

1.7 Functional analysis of LiBPPS in transformed *L. latifolia* plants

Monoterpene synthases are attractive targets for monoterpene metabolism engineering to improve essential oil yield or alter monoterpene composition in plants (Mahmoud & Croteau, 2002). The monoterpene profile of spike lavender EO differs from that of the most valuable common lavender (*Lavandula angustifolia*) by higher relative amounts of camphor and 1, 8-cineole with a trace amount of linalyl acetate (Aprotosoaie et al., 2017; Mendoza-Poudereux et al., 2014). Many genes associated with the EO pathway in this plant have been reported (Adal et al., 2017, 2019; Benabdelkader et al., 2015; Demissie et al., 2012; Jullien et al., 2014; Landmann et al., 2007; Sarker et al., 2013). Our group has recently cloned a novel *BPPS* gene (LiBPPS) from *L. intermedia* plants (unpublished). The identity of this gene was confirmed through *in vitro* functional characterization of the encoded LiBPPS enzyme. The results showed a high *in vitro* activity of LiBPPS in the conversion of GPP to BPP. The catalytic function of this gene has not been confirmed *in planta*. This thesis aimed to examine the *in planta* function of LiBPPS through constitutive expression of *LiBPPS* in sense and antisense orientations under the control of CaMV35s promoter.

1.8 Research Objectives and Hypotheses

The main goal of this thesis was to examine the *in planta* role of *LiBPPS* gene in camphor biosynthesis through altering its expression in transformed *L. latifolia* plants. Specific objectives were to:

1) Overexpress *LiBPPS* in antisense in stably transformed *L. latifolia* plants
2) Overexpress *LiBPPS* in sense in stably transformed *L. latifolia* plants

We hypothesized that:

1) The overexpression of a homologous sense version of *LiBPPS* will lead to increased expression of the gene in some transformed *L. latifolia* plants while the overexpression of a homologous antisense version of *LiBPPS* results in downregulation of *BPPS* in *L. latifolia.*

2) The overexpression of *LiBPPS* will lead to increased production of BPP, borneol, and camphor, while downregulation of the *BPPS* gene will result in reduced biosynthesis of these metabolites in *L. latifolia* plants.

To achieve these objectives, we attempted to:

1) Clone a cDNA- encoding LiBPPS in sense and antisense orientation in *L. latifolia* plants separately, using *Agrobacterium*-mediated transformation.

2) Evaluate the transcriptional expression of BPPS in transformed plants via molecular analysis.

3) Screen the essential oil of both BPPS-sense and BPPS-antisense plants via the GC-MS technique and compared them to that of wild-type plant.

Chapter 2: Materials and methods

2.1 Plant material

Spike lavender seeds were purchased from Seedneeds LLC (United states), and germinated under sterile conditions described earlier (M C Calvo et al., 1988) with some minor changes. Lavender seeds were surface-sterilized by immersion in 70% (v/v) ethanol for 1 min followed by soaking in 20% commercial bleach containing 1 drop of Triton X-100 (Fisher Scientific, Canada) for 20 minutes. Finally, seeds were rinsed with sterile distilled water three times, 5 minutes each. Seeds were kept in the dark at 25 °C for growing. The first pair of leaves from 40-50 days old seedlings was used as primary explants in transformation experiments.

2.2 Culture conditions

The basal medium (BM, Appendix D) consists of MS salts and vitamins (Murashige and Skoog, 1962, PhytoTechnology laboratories, USA). This media contains 3% (w/v) sucrose (Fisher Scientific, Canada), 0.4 % (w/v) Gellan gum (PhytoTechnology laboratories, USA) without hormones. pH was adjusted to 5.7 before autoclaving. Growth regulators were added to the media after autoclaving (20 min at 120 °C, 1×10^5 Pa). Regeneration of spike lavender was carried out according to previous study (Nebauer et al., 2000). Hence, for direct organogenesis in the form of bud regeneration, the leaf explants with their petiole segments were cut in 1 cm^2 using a surgical blade and cultured (with the abaxial surface to the medium) on 25mm× 100 mm Petri plates containing basal regeneration medium (BRM) (Appendix D). This medium consisted of MS medium supplemented with 0.6 µM IAA (Sigma Aldrich) and 8.8 µM BA (Sigma Aldrich). Cultures were then kept in the dark at 25 °C for around 30-50 days until the callus with tiny adventitious buds were emerged. The newly emerged 0.5-1 cm buds were then excised and transferred to 250 ml magenta jars containing 30 ml shoot elongation medium (SEM, Appendix D) in light (16/8 hour photoperiod) for shoot elongation. This medium contained 8.9 µM BA but with less concentration of auxin (0.06 µM IAA). After 30 days, the individual elongated shoots were sub-cultured

on fresh SEM media for further developments. The individual 2-3 cm elongated shoots were transferred onto rooting media (RM) for root induction. Since the RM reported in the previous work (Nebauer et al., 2000) did not yield a high percentage of rooting, we followed rooting media (RM) reported by Ernald (2015) (Lauren Alexandra Elizabeth Erland, 2015). To do this, the developed shoots were cultured in magenta jars containing half-strength MS media with 2.9 μM IAA (Appendix D), and pH of 5.7 for 20-25 days in the greenhouse at 25 °C and a photoperiod of 16 hours light and 8 hours darkness. Regenerated plants were transplanted to small pots (100 ml) with transparent plastic covers to do acclimation and maintain relative humidity. Relative humidity was reduced by gradually enlarging the plastic covers. Complete removal of the covers took place after 4-5 weeks of placement.

2.3 Antibiotic resistance test

The sensitivity of spike lavender explants to antibiotic selectable markers such as kanamycin and hygromycin was studied prior to the plant transformation to determine the effective concentration for selecting transformed plants. Lavender seedlings cultured on MS media (as mentioned above) were cut 1 cm from above the roots and then transferred to rooting media containing kanamycin B (Sigma-Aldrich) with different concentrations (0, 5, 7.5, 10, 15, 25, 50, and 100 mg L^{-1}). To test the sensitivity of spike lavender to another type of antibiotic, we used hygromycin B (A.G. Scientific) as one of the common antibiotics for transformed plant selection with the same concentration used for the kanamycin resistance test. Experiments involving 2-4 explants carried out in three replicates.

2.4 Constructing appropriate vectors and bacteria transformation

The primary spike lavender transformation was accomplished using the sense and antisense constructs (BPPS-sense: pGA and BPPS-antisense: pGA). The full length of LiBPPS in both sense and antisense orientation had already cloned separately into the pGAdekG/NIb.L vector derived from pGA482 (named hereafter as pGA). The GUS reporter gene was removed by EcoRI and KpnI digestion, and replaced by *LiBPPS-sense* and *LiBPPS-antisense* gene separately. The PGA vector harboured

neomycin phosphotransferase II (NPTII; kanamycin resistance gene), cauliflower mosaic virus (CaMV 35S) promoter, and NOS (nopaline synthase) terminator (Figure 3).

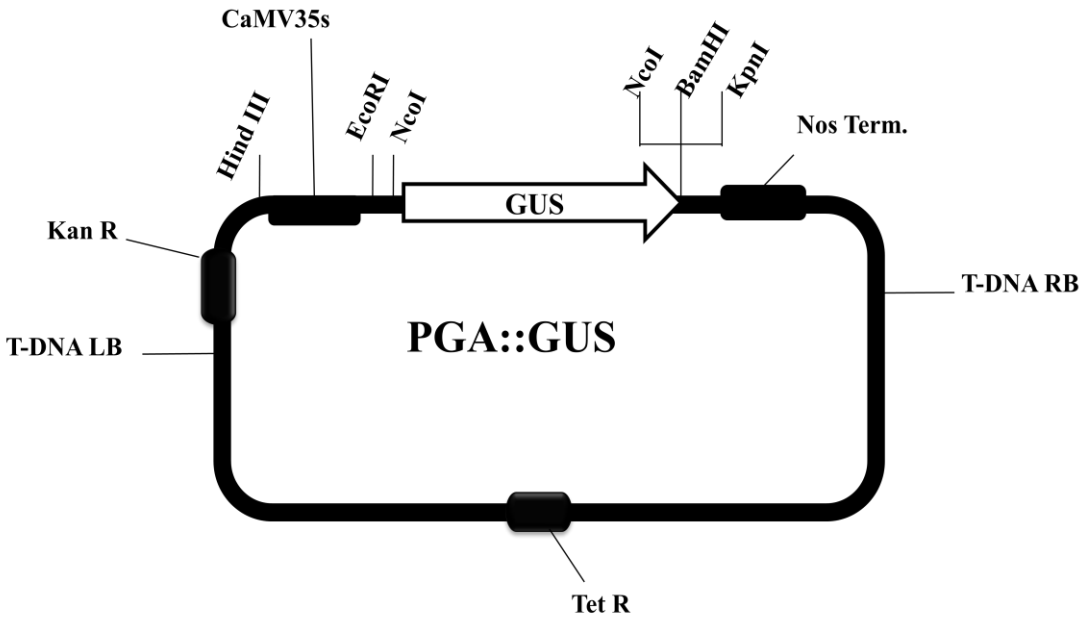


Figure 4. A schematic representation of pGA (pGA482) vector carrying GUS.

GUS gene was removed by EcoRI and KpnI digestion, and replaced by *LiBPPS-sense* or *LiBPPS-antisense*. The expression of LiBPPS in the construct is driven by the Cauliflower mosaic virus (CaMV) 35S promoter; The vector also harboured Neomycin phosphotransferase gene (which confers resistance to Kanamycin); a nopaline synthase terminator (NOS Term); upstream MCS: EcoRI and NcoI; downstream MCS: NcoI, BamHI and KpnI; left border (T-DNA LB); right border (T-DNA RB); a gene encoding a tetracycline-resistant protein for bacteria selection (Tet R) (An 1986).

Since, kanamycin neither suppresses the growth of untransformed and transformed spike lavender with the above constructs nor led to selection of transgenic plants in tissue culture medium, hygromycin as a different antibiotic was introduced for selection of transgenic plants. To do this, pCambia 1390 vector (Marker Gene Technologies, Inc., Appendix A) harbouring hygromycin resistance gene (*HygR*) was used for transformation of spike lavender. Since there is a lack of CaMV 35S-Cauliflower mosaic virus promoter for the multiple cloning site (MCS) of pCambia1390 binary vector, we amplified CaMV 35S::GUS fragment from PGA vector and inserted it in the MCS site in pCambia1390. This vector also harbours: NOS- a nopaline synthase terminator; upstream MCS - EcoRI

and NcoI; downstream MCS- EcoRI and NcoI; T-DNA LB - left border; T-DNA RB - right border; Kan R- a gene encoding a kanamycin-resistant protein for bacteria selection. To construct pCambia1390:: BPPS-sense (sense plasmid) and pCambia1390:: LiBPPS-antisense (antisense plasmid), the coding sequence for *LiBPPS-antisense* with a homologous antisense version of the borneol diphosphate synthase (BPPS) cDNA in the opposite orientation and *LiBPPS-sense* with the normal homologous version of BPPS cDNA in the correct orientation were amplified by PCR mentioned in Table C.1 using their gene-specific primers (Table 2) and cloned into the pCambia 1390 vector. Separately, the *35s-gus A* gene was also cloned in the pCambia vector with the above mentioned PCR program listed in Table C.1 and specific primers (Table 2). Thus, three different vectors were constructed: pCambia1390:: *35s-gus A* (GUS construct), pCambia1390:: *LiBPPS-sense* (sense construct), and pCambia1390:: *LiBPPS-antisense* (antisense construct) (Figure 5).

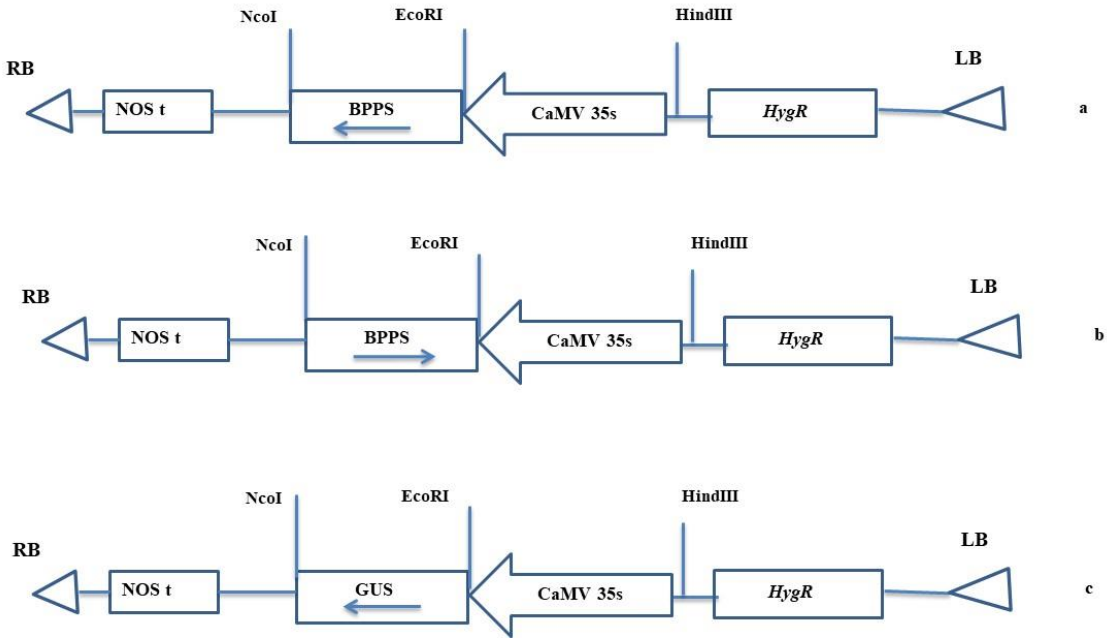


Figure 5. Schematic representation of the T-DNA from pCambia1390.

It shows three constructs: sense construct containing the normal homologous version of BPPS cDNA in the correct orientation (a), antisense construct with a homologous antisense version of BPPS cDNA in the opposite orientation (b), and GUS construct with the GUS cDNA (c) under control of CaMV35s. Restriction sites for HindIII, EcoRI and NcoI are indicated. *GUS* gene was removed by EcoRI and NcoI and replaced by *BPPS* gene. A gene encoding a hygromycin-resistant protein for plant selection (*HygR*); a nopaline synthase terminator (NOSt); Cauliflower mosaic virus 35S (CaMV35s) promoter; left border (LB); right border (RB).

Then the resulting constructs were transformed into *Escherichia coli* (*E.coli*) strain DH5α (Appendix B) via the heat shock method (Froger & Hall, 2007). The plasmid was extracted from positive colonies grown on LB media containing kanamycin antibiotic.

After extraction and validation of constructs in the transformed *E.coli* by either restriction enzyme digestion or PCR using gene-specific primers for the gene of interests, the positive plasmids containing the resulting constructs extracted from the bacteria using plasmid DNA Miniprep kit (Omega) and transformed into *Agrobacterium tumefaciens* strain GV3101 (Appendix B) via the freeze-thaw method (Jyothishwaran et al., 2007). The bacteria were plated on LB agar medium supplemented with 50 mg/l kanamycin and 25 mg/l rifampicin. Then, the positive colonies were selected. A single transformant bearing each construct was isolated and grown to log phase in test tubes containing 5 ml LB with the above antibiotics for 24 h on a horizontal shaker (200 rpm) at 28°C. Then 1ml of the overnight culture was transferred into fresh 100 ml of LB with the same antibiotics and grown overnight at 90 rpm at 28 °C. The following day, when the measured OD_{600} of the bacterial culture via UV/Visible Spectrophotometer (Ultrospec 2100 pro) was 0.2-0.3, 100 μM acetosyringone (PhytoTechnology laboratories) added to the bacterial culture and incubated for three hours more shaking at 200 rpm at 28°C. When the OD_{600} reached the range of 0.56 to 0.6, the culture was centrifuged at 4000 rpm for 10 min at room temperature (22 °C). The culture was then resuspended and diluted in 5 ml liquid BM (pH 5.5) (Appendix D) supplemented with 100 μM acetosyringone (PhytoTechnology l).

Table 2. List of primers used in this study

Genes	Primers	Sequences (5' ==> 3')	vectors
For *L. latifolia* transformation and PCR detection			
Li BPPS-sense	bpps_sense_EcoRI_F	CTAGA**GAATTC**ATGCCTGTGGGAATCCT	pCambia1390
	bpps_sense_NcoI_R	AAT**CCATGG**TTAGGCATATGGCTCGAAC	
Li BPPS-antisense	bpps_antisense_NcoI_F	ATAT**CCATGG**ATGCCTGTGGGAATCCT	
	bpps_antisense_EcoRI_R	TGACA**GAATTC**TTAGGCATATGGCTCGAAC	pCambia1390
35s:gusA	35s_ HindIII_F	ATTA**AGCTT**ATGGTGGAGCACGACACTC	pCambia1390
	gus_ BamHI_R	TATA**GGATCC**TTGTTTGCCTCCTGCT	
HygR	HygR_F	ATG AAA AAG CCT GAA CTC ACC G	
	HygR_R	TTT CTT TGC CCT CGG ACG	
β-Actin	actin_F	TGTGGATTGCCAAGGCAGAGT	
	actin_ R	AATGAGCAGGCAGCAACAGCA	
For qPCR analysis			
Li BPPS	BPPS-F	AGAATTTGGGCAAGGGTATT	
	BPPS- R	CGAGTTGAGACTCAGCATTAG	

Note: Underlined sequences indicate the respective restriction site.

2.5 *Agrobacterium*-mediated transformation of *L. latifolia*

Agrobacterium-mediated leaf transformation of *L. latifolia* was carried out based on protocol (Nebauer et al., 2000) already was set up with some modification. Between 440-450 young leaves with

their petioles from 40-50 days seedlings were excised and pre-cultured for 24 h on BRM without antibiotics. The pre-cultured explants were transformed by *A. tumefaciens* GV3101 carrying pCambia 1390::BPPS-sense, pCambia 1390::BPPS-antisense, and pCambia 1390::35s:gusA (control) by immersing in the *Agrobacterium* suspension as described in 2.4 for 20 min. After blot drying on sterile Whatman filter papers, the explants were cultured on BRM for co-cultivation of explants and *Agrobacterium* for 24 hours at 22 °C in the dark. The next day, the explants were transferred to the bacterial elimination medium, which was BRM supplemented with the demonstrated concentration of 60 mg L^{-1} TIMENTIN (Gold Biotechnology, USA), 120 mg L^{-1} cefotaxime (Gold Biotechnology, USA) (Adal, 2019) (Adal et al., 2019) in a growth chamber. After seven days of culture, the explants were transferred to the selection medium, which consists of BRM with 60 mg L^{-1} TIMENTIN, 120 mg L^{-1} cefotaxime, and 7.5 mg L^{-1} hygromycin B for plant selection. After 4 weeks of culture on the selection medium, explants with newly emerged buds were subcultured to SEM with antibiotics for shoot elongation. Elongated shoots were rooted in RM in the presence of antibiotics and then transferred to the pots for acclimatization, as mentioned earlier in 2.2. In each transformation experiment, there were 50 wild-type explants as positive controls. A schematic procedure for *L. latifolia* transformation and regeneration is shown in Figure 6.

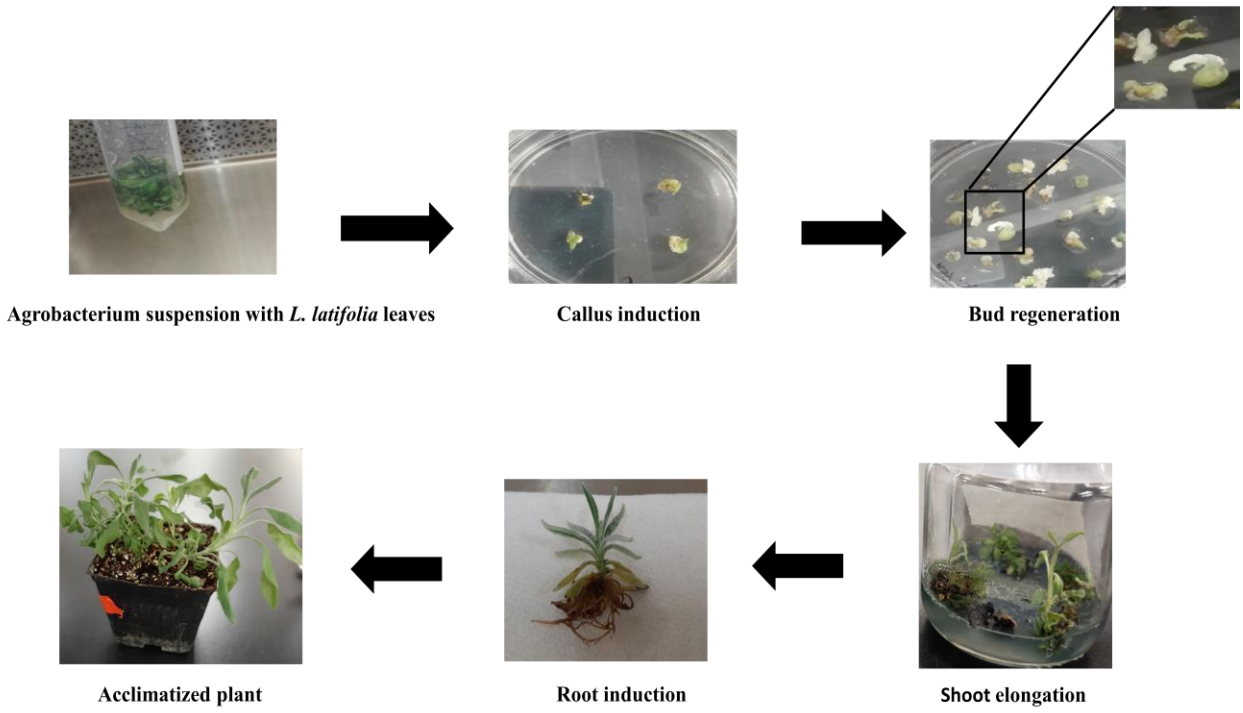


Figure 6. A schematic protocol for *Agrobacterium*-mediated transformation and regeneration of *L. latifolia.*

Photo credit by Elaheh Najafianashrafi

2.6 Evaluation of putative transgenic spike lavenders

2.6.1 Antibiotic selection of putative transgenic spike lavenders

The primary selection of putative transgenic spike lavenders was performed in the selection medium (BRM consisted of 60 mg L^{-1} TIMENTIN, 120 mg L^{-1} cefotaxime, and also 7.5 mg L^{-1} hygromycin B). The transgenic explants with emerged shoots were transferred to the shoot elongation medium for further process.

2.6.2 Gus assay

Stable expression of β- glucuronidase (GUS) by histochemical staining in transformed *Agrobacterium tumefaciens* with GUS construct- a positive control for constructed vector, and in the transgenic spike lavenders containing GUS vector- a control for transformation was assayed according to

previous reported study (de Ruijter et al., 2003). Transformed bacteria, and freshly excised leaf segments were separately immersed in GUS buffer containing 100 mM sodium phosphate buffer (pH 7.0), 0.1% Triton X-100, and the substrate, 2 mM 5-Bromo-4-chloro-3- indolyl glucuronide (X-gluc), and then incubated overnight at 37 °C. Subsequently, the plant material was rinsed in 70% ethanol to extract pigments and visualize the blue staining.

2.6.3 Detection of plant selectable marker *(HygR)* and *BPPS* genes from cDNA

RNA was extracted from the young leaves of putative transgenic plants (BPPS-sense and BPPS-antisense plants) using the RNAeasy Mini Plant Kit (Qiagen). The resulting RNA was quantified via the NanoDrop-1000 Spectrophotometer running on an agarose gel to assay the quality and quantity of RNA. Then, the total RNA (1 μg) was reverse transcribed into cDNA with 50 μM oligo d(T), 50mM random hexamer primers, 0.2 M RNAase inhibitor, and 1M-MuLV Reverse Transcriptase, ×10 MR buffer (New England Biolabs) following the manufacture's procedure. The resulting cDNAs were amplified by mi-quantitative PCR (rt-PCR) and normalized with β-*actin* using a specific primer for β-*actin*, *HygR* and *BPPS* genes (Table 2) in untransformed and the putative transgenic plants according to PCR program listed in Table C.2, Table C.3 and Table C.4. Each cDNA sample was amplified, and the PCR products were subsequently run on a 1% agarose gel. Band intensities were measured by densitometry, using the ImageJ software, used to estimate expression level.

2.6.4 Real-time quantitative PCR (qPCR)

Transcript levels for BPPS in young leaves of BPPS-sense, BPPS-antisense, and untransformed plants, were determined by quantitative real-time PCR (qPCR). The transcript levels for BPPS in young leaves of the putative transgenic plants were compared in relative to that of WT young leaf. The qPCR analysis was performed using SYBR Premix (Applied Bioscience) in the StepOne Plus Real-Time PCR system (Applied Bioscience) using Primer sets listed in Table 2. qPCR was run with a final reaction volume of 10 μL, consisting of 5 μL of SYBR premix, 5 μM of each primer, and ~150 ng of cDNA template. The

following program was set for qPCR: 50 °C for 2 min, and 95 °C for 2 min, 50 cycles at 95 °C for 3 s and 60 °C for 30 s, 95 °C for 15 s and 60 °C for 1 min as a final melting curve stage. The generated data were analyzed using the Livak method (Livak & Schmittgen, 2001), expressed as $2^{-\Delta\Delta CT}$ of a gene of interest using β-*actin* as the endogenous control gene for data normalization. All reactions were repeated three times, and two biological assays (different leaves of each transgenic plant) were performed.

2.6.5 Screening of EO for modified composition in transgenic plants

To determine monoterpenes variation in transgenic plants, their EO was extracted. To do this, approximately 0.5 g of young leaves harvested from 9- month BPPS-sense, BPPS-antisense and WT plants (Figure 7) were submerged in 25-ml test tubes containing 5 ml pentane and 50 µg of thymol as an internal standard.

a b c

Figure 7. 9- month *L. latifolia* plants. a) BPPS-sense plant, b) BPPS-antisense plant, c) WT

The samples were then sonicated in an ice bath for 30 min using an Ultrasonic water bath sonicator (VWR) to extract their oil. Qualitative EO analysis was accomplished by gas chromatography-

mass spectrometry (GC-MS) using a Varian GC 3800 Gas Chromatographer (Varian Inc, USA) coupled to a Saturn 2200 Ion Trap mass detector following the procedure used in our research group (Demissie et al., 2013). In brief, for each sample without dilution, 1 μL was injected with cold on-column mode, at which the injector temperature was set to 40 °C. The oven temperature was set at 40 °C for 3 min, ramped to 130 °C at a rate of 10 °C $min^{-1,}$ and held for 2 min. The temperature was further ramped to 230 °C at a rate of 50 °C min^{-1} and finally held for 8 min. The flow rate of the carrier gas (helium) was set to 1 mL min^{-1}. The product identity was confirmed by comparing their retention times and mass spectrum to those of the corresponding authentic standards. For EO constituents for which a standard was not available, mass spectra were compared to those in the NIST libarary (Figure 8 & 9).

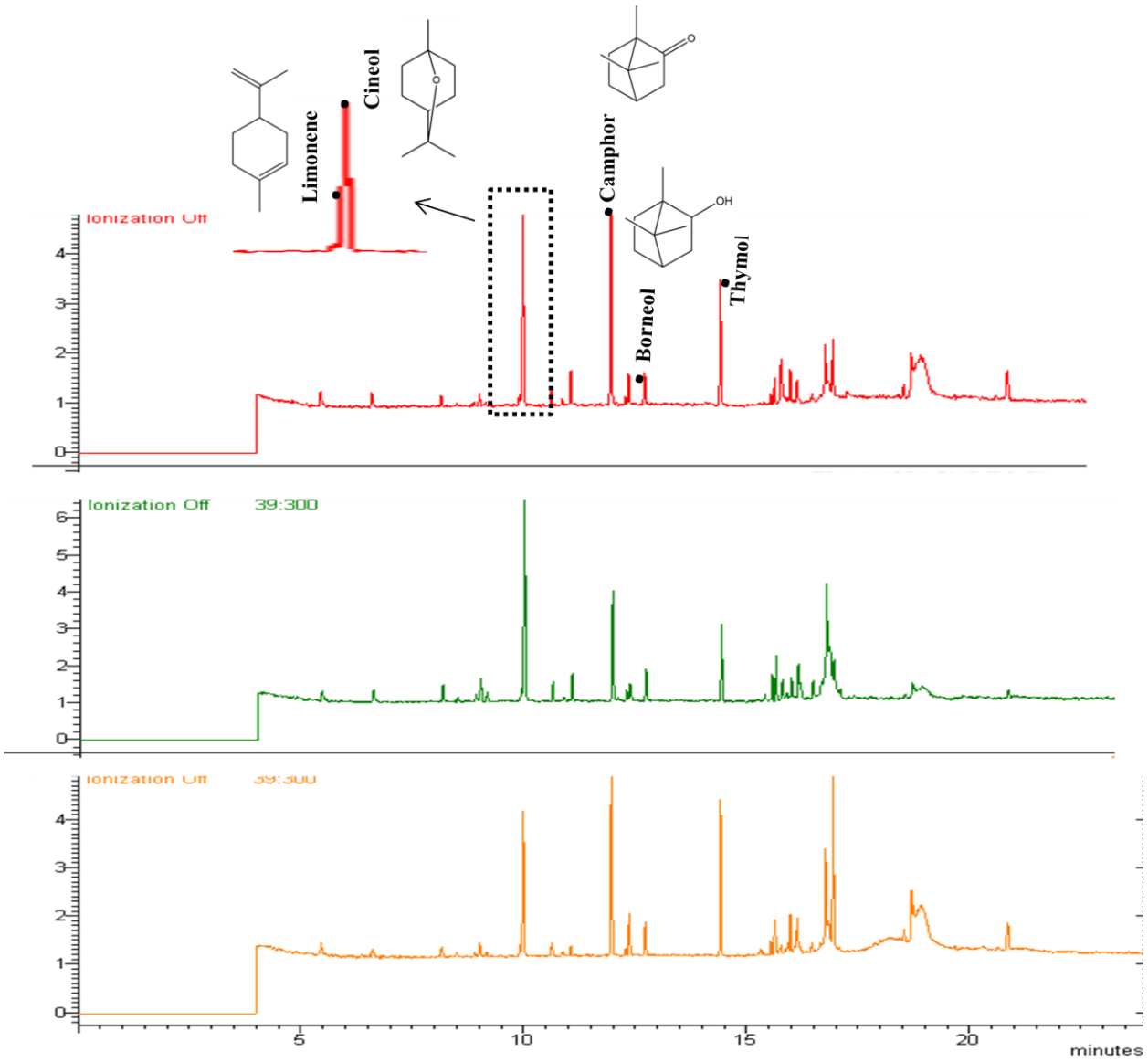


Figure 8. Sample chromatograms from EO of representative WT (top), BPPS-antisense (middle) and BPPS-sense (bottom). Thymol was used as an internal standard.

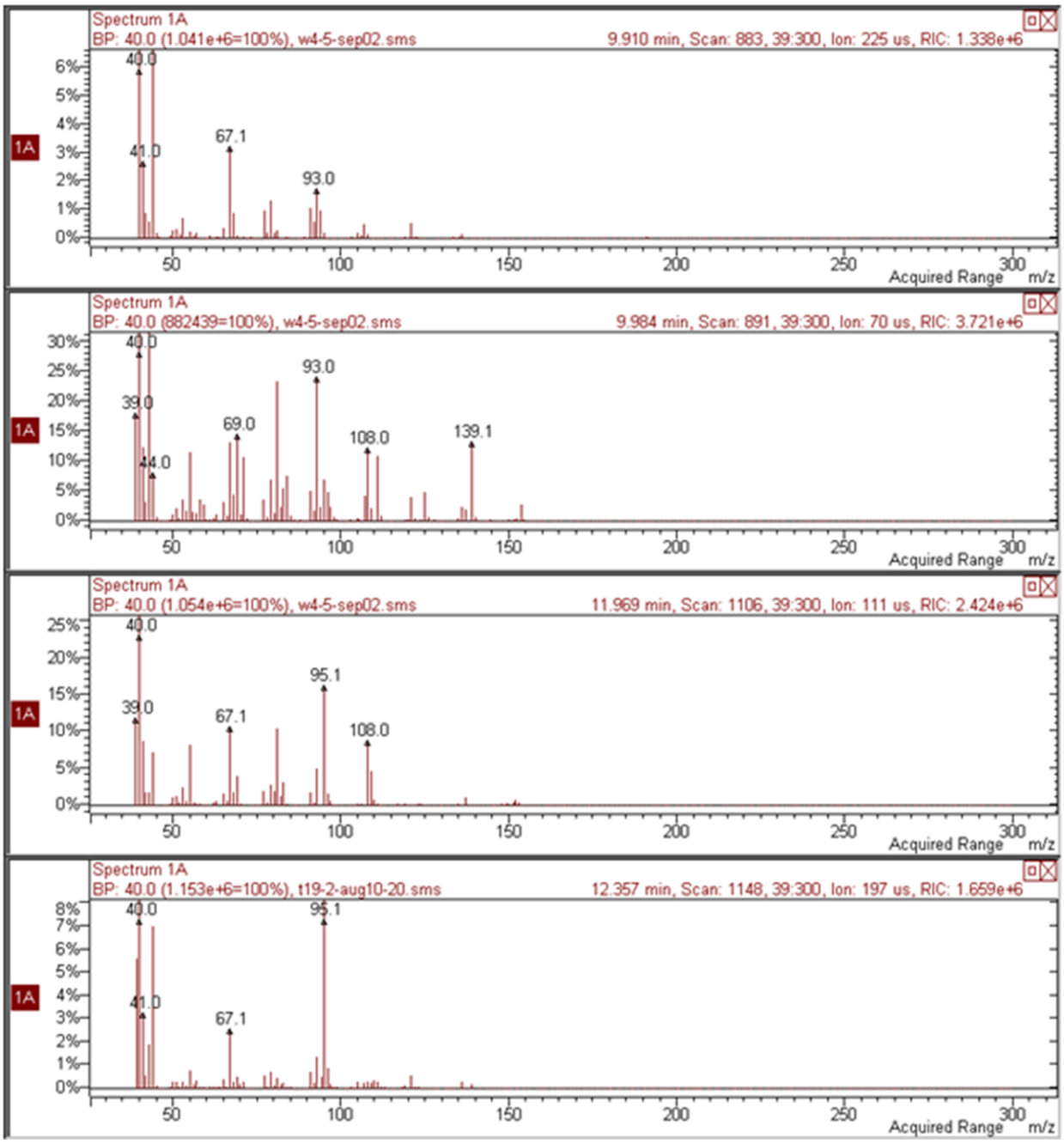


Figure 9. Mass spectrum of limonene, 1,8 cineole, camphor and borneol (from top to bottom) from EO of WT.

The concentration of each EO constituent was calculated by comparing its peak area to that of the internal standard (thymol). Peak areas of 21 major constitutes and internal standard were used to calculate relative percent composition and oil yield concentration using the formulae 1 & 2, as already has

described (Lauren Alexandra Elizabeth Erland, 2015). The oil yield concentration was normalized to the amount of tissue used in the extraction. Also, oil yield concentration and relative monoterpene composition were averaged for 3 extractions. Thymol was used as internal standard for oil analysis.

Formula 1:

$$\text{Relative percent composition} = \frac{\text{Peak area major constituent}}{\sum \text{Peak area all constituents}} \times 100$$

Formula 2:

$$\text{Oil yield} = \frac{\sum \text{Peak area all constituents}}{\text{Peak area Thymol}} \times \text{ug thymol added}$$

For WT, tissues from three biological replication were used to extract the EO. To select the appropriate tissue for screening oil composition in transgenic plants, initially, oil samples were taken from the different developmental stages (young, medium, and old) of WT *L. latifola* leaves. Leaves of three developmental stages were selected based on the position of the leaf (Figure 10). The young leaves were collected from the four nodes of apical meristem; medium leaves were taken from the 6th-8th node of the stem, while old leaves with a darker colour, sampled from the bottom of two nodes from the base of the stem. EO from young leaves of 9 BPPS-sense and 11 BPPS-antisense plants was screened by GC-MS.

a b c

Figure 10. Three developmental stage of *L. latifolia* leaves, a) old, b) medium and c) young leaf

2. 7 Statistical Analysis

Statistical analysis (Student's t-test) was performed using GraphPad Prism 8.4.3 (Graphpad Software, US) with a significant difference at $P < 0.05$ or $P < 0.01$ to determine significant difference between transgenic spike lavenders and WT.

Chapter 3: Results

3.1 Sensitivity of *L. latifolia* to selectable markers

Due to using kanamycin as a general selectable marker for lavender transformation, the kanamycin resistance test was initially implemented for the selection of transgenic plants. After culturing unrooted seedlings of WT spike lavender in ½ MS media with 0 to 100 mg L^{-1} kanamycin, the sensitivity of explants to the antibiotic was determined. Kanamycin did not inhibit growth and root induction in WT plants (Figure 11). The antibiotic resistance test was also performed to select transformed spike lavender leaves with BPPS-sense: pGA and BPPS-antisense: pGA in selection media containing the above-mentioned kanamycin concentration. Results showed that kanamycin did not inhibit callus induction and shoot regeneration from WT leaf explants in selection media (data not shown). This observation revealed that kanamycin is not a suitable selective agent for transforming spike lavender. Thus, hygromycin was used as the substitution for kanamycin to select the putative transgenic plants. Hygromycin at a concentration of 7.5 mg L^{-1} and higher annulled root induction in all plants. The concentration above 10 mg L^{-1} led to necrosis in the plants after 30 days. A concentration above 7.5 mg L^{-1} hygromycin interfered with the regeneration process, mainly shoot regeneration and shoot elongation of explants in SEM (Figure 12). Therefore, 7.5 mg L^{-1} hygromycin is the lowest concentration of the selective agent that suppressed the growth of untransformed cells was used for the selection of putative transgenic plants.

a b c

Figure 11. The sensitivity of spike lavender to kanamycin.

Concentrations of 100, 50 and 15 mg L^{-1} kanamycin did not suppress plant growth and root induction, indicating that this antibiotic is not a suitable selecting agent for selecting transgenic spike lavender.

a b c

Figure 12. The sensitivity of spike lavender to hygromycin.

Suppressed root induction and necrosis in the plants grown on hygromycin at 15 mg L^{-1} (a), 7.5 mg L^{-1} (b) and 5 mg L^{-1} hygromycin (c). While there was no effect on root induction and plant growth at 5 mg L^{-1}, hygromycin effectively inhibited root formation at 7.5 and 15 mg L^{-1}.

3. 2 Confirmation of constructed vectors in transformed bacteria

The constructs were successfully transformed into competent *E. coli* DH5 α cells and further into *Agrobacterium tumefaciens* GV3101. The colonies grew well for each candidate, and positive colonies were successfully selected via antibiotic selection media. Validation of the presence of the respective constructs (GUS, sense, and antisense constructs) in transformed *E. coli* DH5a was accomplished by restriction enzymes (EcoRI and NcoI) (Figure 13). Also, the presence of the genes of

interest (*GUS* gene or *BPPS*) in transformed *Agrobacterium tumefaciens* was confirmed by PCR using specific primers (Table 2). The PCR amplification resulted in ~ 1.6 kb band for *GUS* gene, and ~1.8 kb for both BPPS-sense and *BPPS-antisense* genes indicated that both genes of interest were introduced correctly into the constructs and therefore into the transformed *Agrobacterium* (Figure 14).

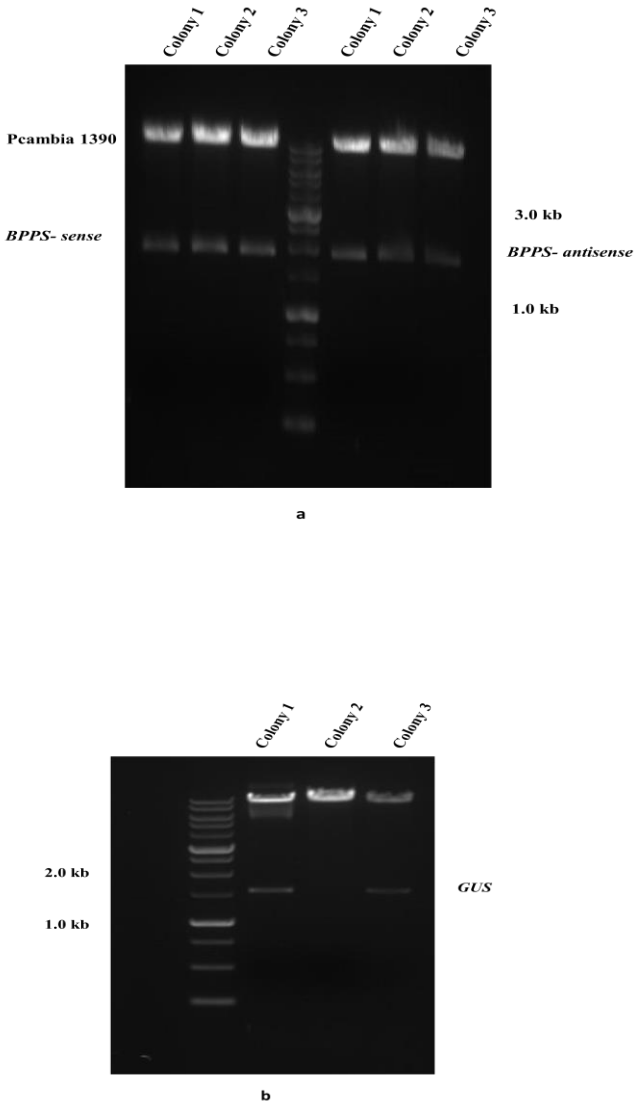


Figure 13. Agarose gel of restriction digestion of GUS, sense, and antisense constructs extracted from *E. coli* DH5a.

The digestion of the constructs by EcoRI and NcoI showed a 1.8 kb band for both *BPPS-sense* and *BPPS-antisense* in sense and antisense constructs, respectively (a) and 1.6 kb band for *GUS* in GUS construct (b) alongside the ladder on the agarose gel.

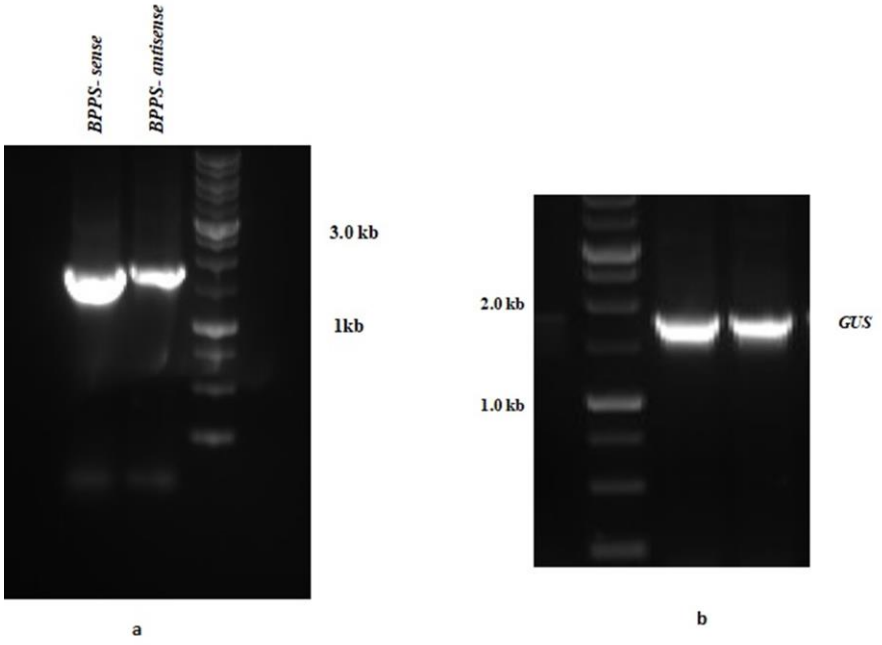


Figure 14. PCR confirmation of *LiBPPS- sense*, *LiBPPS-antisense* and *GUS* genes using gene-specific primers.

The amplification using gene-specific primers in transformed *Agrobacterium tumefaciens* showed a 1.8 kb band for both *BPPS-sense* and *BPPS-antisense* in sense and antisense constructs respectively (a) and 1.6 kb band for *GUS* in pCambia1390:: GUS (b) alongside the ladder on the agarose gel.

3.3 Regeneration of hygromycin-resistant shoots

A procedure previously developed for the regeneration of transformed *L. latifolia* (Neuber et al., 2000) was used in our study for the regeneration of transformed *L. latifolia* with *A. tumefaciens* separately harboring sense, antisense, and GUS constructs. *Agrobacterium* growth was not observed in elimination media, indicating that the implemented 60 mg L^{-1} TIMENTIN and 120 mg L^{-1} cefotaxime in elimination media were appropriate concentrations to suppress *Agrobacterium* bacteria growth (Figure 15). Callus emergence was observed on the surface of explants, particularly around the petioles, within 2-4 weeks of culturing in selection media. At this stage, all WT leaves turned dark brown and died; thus, this stage could be considered a primary stage for selecting transformed explants (Figure 16). Also, some of the transformed explants were unable to regenerate into buds and turned dark brown. Within 30-50 days,

some emerged calli were differentiated into adventitious buds on the selection media (Figure 17). Shoot developments were observed within 60-75 days after transferring the adventitious buds to the SEM (Figure 18).

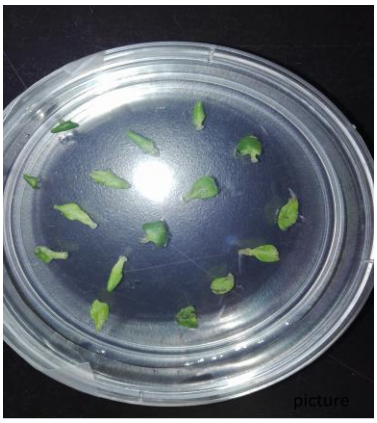
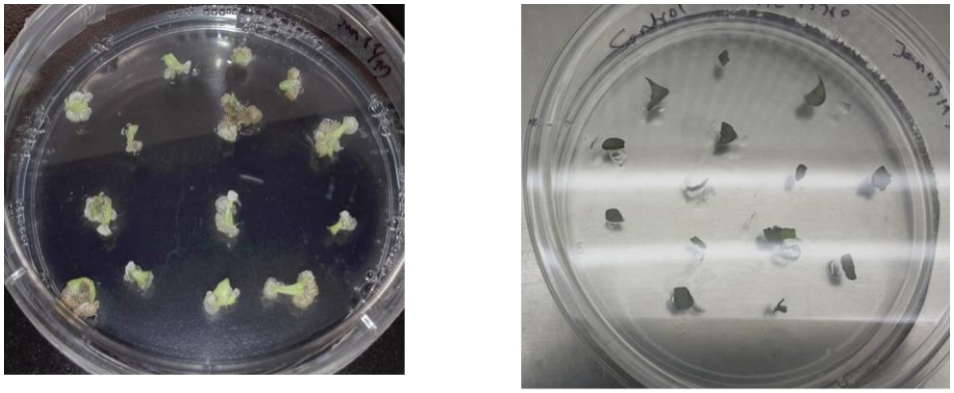


Figure 15. *L. latifolia* leaves infected with *Agrobacterium tumefaciens* in the elimination media.

No trace of *Agrobacterium* growth was observed.



a **b**

Figure 16. *In vitro* callus induction from *L. latifolia*.

Callus emergence from *L. latifolia* leaves treated with *Agrobacterium tumefaciens* on selection media containing 7.5 mg L^{-1} hygromycin within 2-4 weeks (a), and no callus induction was observed on WT leaves within 2-6 weeks (b)

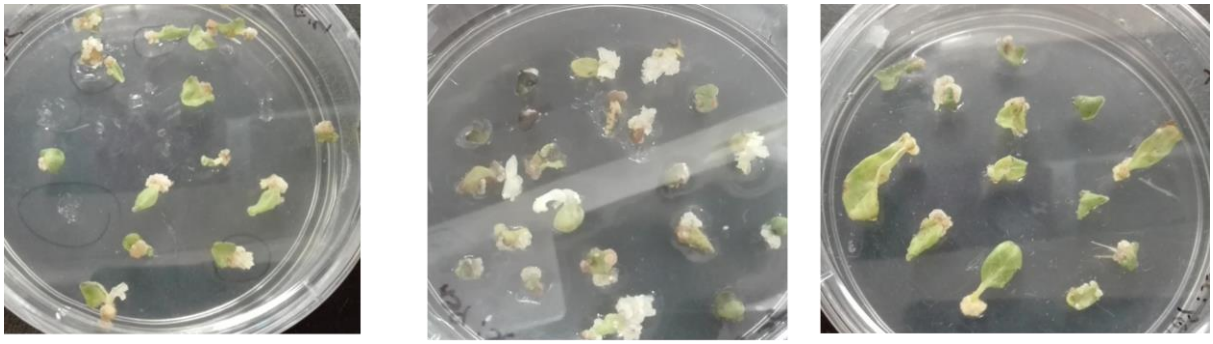


Figure 17. *In vitro* shoot induction from *L. latifolia* leaves.

Bud induction on emerged callus from *L. latifolia* transformed with *A. tumefaciens* harboring sense (a), antisense (b), and GUS (c) constructs on selection media containing 7.5 mg L^{-1} hygromycin within 30-50 days

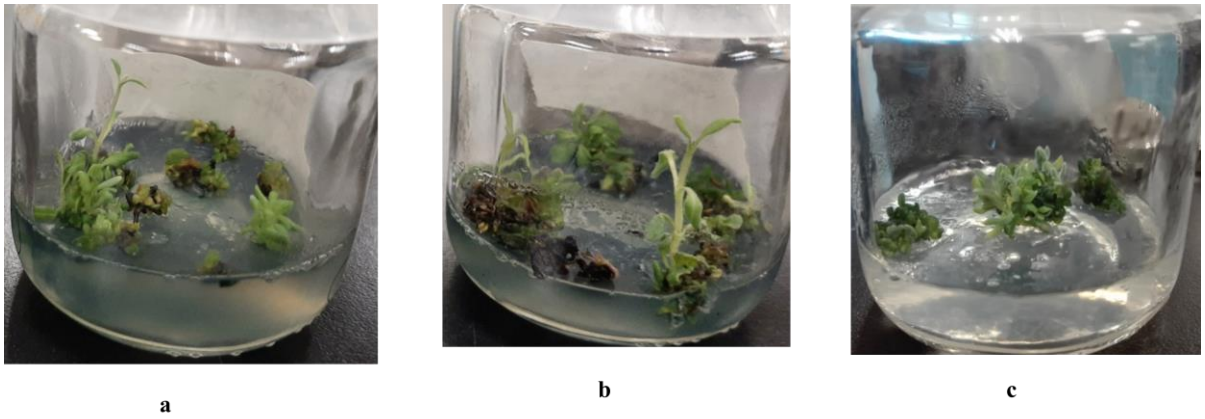


Figure 18. *In vitro* shoot development from *L. latifolia*.

Shoot development of putative transformed *L. latifolia* with *A. tumefaciens* harboring sense (a), antisense (b), and GUS (c) construct on SEM with 7.5 mg L^{-1} hygromycin within 60-75 days.

3. 4 Rooting of hygromycin-resistant shoots and plant development

The elongated shoots (~ 3 cm) obtained in SEM were excised and transferred to RM containing 7.5 mg L^{-1} hygromycin for root induction. Results showed that ~ 70 % of the shoots were rooted within 3 weeks in RM (Figure 19). To acclimatize, the rooted plants transferred to soil as explained earlier in section 2.2 (Figure 20).

After acclimatization, 38- 67% of putative transgenic plants survived. The putative transformed *L. latifolia* was defined as follows: The plants harboring sense construct (BPPS-sense), the plants harboring antisense construct (BPPS-antisense), and the plants harboring GUS construct (GUS). Transformed plants were visually similar to WT plants while grown in the greenhouse (Figure 21).

Figure 19. *In vitro* root induction of *L. latifolia*.

Rooting of putative transformed *L. latifolia* plants harbouring sense (a), antisense (b) and GUS (c) constructs.

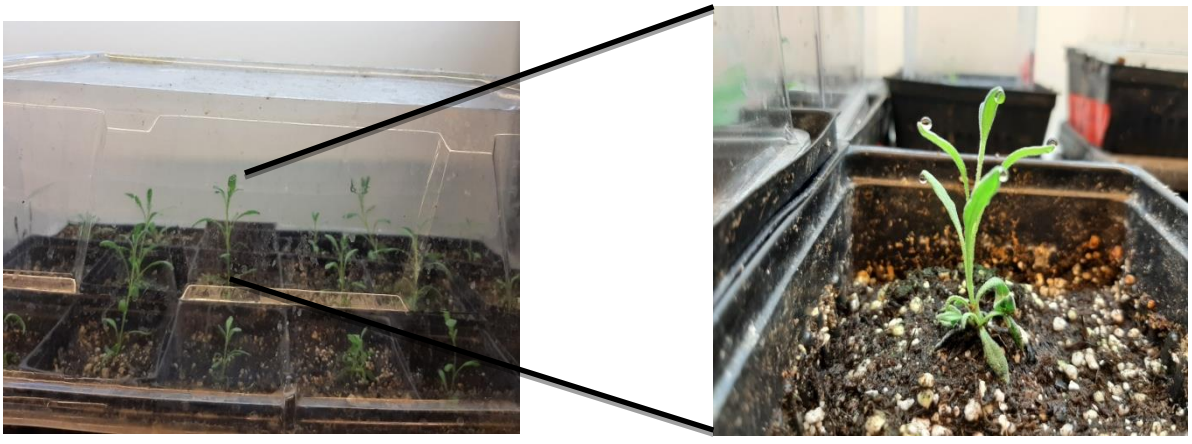


Figure 20. Acclimatization of rooted *L. latifolia* in soil in greenhouse condition

a b c

Figure 21. 7-months old putative transgenic *L. latifolia* plants growing in the greenhouse.

WT (a); BPPS-antisense plant (b); BPPS-sense plant (c).

Rate of successful organogenesis in transformed *L. latifolia* was calculated based on the number of explants initially infected with *Agrobacterium tumefaciens* in bud induction medium, and the number of explants regenerated to shoot and then developed into the whole plants. Out of the initial 150 treated explants for each construct transformation experiment, 65 calli from transformed leaves with BPPS-sense gene and 80 calli from transformed leaves with BPPS-antisense gene emerged in selection media. Of these calli, 32 BPPS-sense and 59 BPPS-antisense plants were regenerated, of which 9 (31.25%) of BPPS-sense and 40 (67%) of BPPS-antisense plants were established in the greenhouse (Table 3). Although all BPPS-antisense plants were phenotypically indistinguishable from WT, most of the BPPS-sense plants showed severe growth retardation. Of the 9 rooted plants, 3 (BPPS-S1, BPPS-S2, BPPS-S5) grew well and resembled WT plants in growth habit; 2 (BPPS-S3 and BPPS-S4) grew poorly and produced little vegetative tissue, and BPPS-S6, BPPS-S7, BPPS-S8 and BPPS-S9 did not survive in soil (Figure 22).

a **b** **c**

Figure 22. Some representative BPPS-sense plants growing in the greenhouse.

BPPS-S1 grew well and resembled WT plants in growth habit (a) BPPS-S3 grew poorly and produced little vegetative tissue (b), and BPPS-S8 did not survive in soil (c).

Table 3. Summary of *Agrobacterium tumefaciens* experiments

Transformation experiment (inserted gene)	**≠ of initially inoculated explants with *Agrobacterium tumefaciens***	**≠ of explants developed into a callus (%)**	**≠ of callus developed into shoots (%)**	**≠ of shoots developed roots (%)**	**≠ of survived transformed plants**
BPPS-sense (BBPS-S)	150	65 (43.3%)	48 (73.8%)	32 (66.6%)	9 (28.12%)
BPPS-antisense (BPPS-As)	150	80 (53.3%)	66 (82.5%)	59 (73.2%)	40 (67%)
GUS	150	59 (39.3%)	22 (54.2%)	15 (68.18%)	Not transferred to the soil

3.5 Histochemical assay of GUS plants

A histochemical assay for β-glucuronidase activity showed blue precipitate in transformed *Agrobacterium tumefaciens* with GUS construct- positive control for constructed vector and also in callus and regenerated shoots of GUS plants transformed with GUS construct. GUS expression was not detected in the WT *L. latifolia* plants (Figure 23).

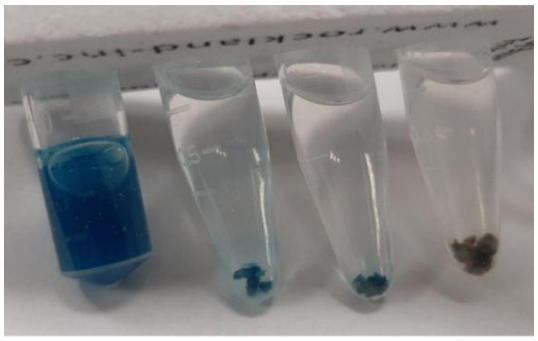


Figure 23. GUS expression of transgenic *L. latifolia.*

Transformed *Agrobacterium* harboring GUS construct- a positive control (a), regenerated callus and shoots from GUS plants, respectively (b and c), WT leaves- negative control (d).

3.6 Molecular analysis of putative transgenic plants

3.6.1 Detection of *HygR* gene

The *HygR* gene from young leaf-derived cDNA of transformed *L. latifolia* was detected with rt-PCR using specific primers. The *HygR* gene was successfully amplified from some of the putative transgenic plants, but it was not amplified from WT *L. latifolia* cDNA as was expected. Rt-PCR analysis of the *HygR* gene amplified the expected fragments of ~1.0 kb. Figure 24 shows the *HygR* amplification in some representative transformants, WT- a negative control, and sense construct harboring *HygR* gene- a positive control. All the 9 putative BPPS-sense transgenic plants were positive for *HygR.* In

comparison, 31 out of 40 putative BPPS-antisense plants were positive for this gene showing high transformation efficiency of the *HygR* gene in our transgenic plants.

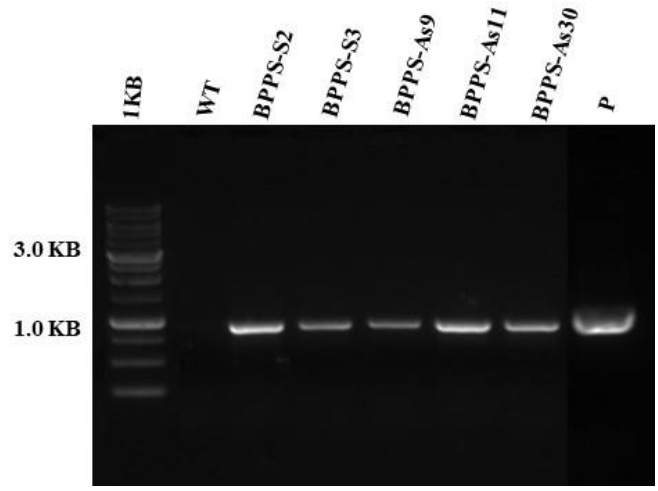


Figure 24. rt-PCR amplification of the *HygR* gene from some representative putative transgenic *L. latifolia* plants using gene-specific primers amplified the expected fragments of ~1.0 kb.

The *HygR* gene was successfully amplified from most transgenic plants, but it was not amplified from wild-type *L. latifolia* cDNA. 1 KB = 1 kb ladder, P = sense construct- positive control, WT= wild -type *L. latifolia*- negative control, BPPS-S= BPPS-sense and BPPS-As= BPPS-antisense plants.

3.6.2 Expression analysis of *BPPS* gene in generated transgenic plants

3.6.2.1 rt-PCR analysis of *BPPS* expression in generated transgenic plants

The expression of *BPPS* gene in young leaves of BPPS-sense, BPPS-antisense, and WT plants, were assessed by rt-PCR using gene-specific primers and normalized to β-actin. The cDNA derived from young leaves of WT plants was used as a negative control for the plant genetic transformation in this study. The PCR products were resolved on agarose (1%) gel. Band intensities were measured by densitometry using the ImageJ software. *BPPS* and *β-actin* genes with ~ 1.8 bp and ~ 500 bp respectively were observed within WT since *BPPS* is an indigenous gene in lavender plants, the expected fragment

was observed in WT plants. All tested BPPS-antisense plants showed significantly lower *BPPS* expression than WT except for BPPS-As12, and BPPS-As22, which showed near-average BPPS expression compared with WT. All BPPS- sense plants showed significantly stronger expression of *BPPS* in comparison with WT and BPPS- antisense plants (Figure 25).

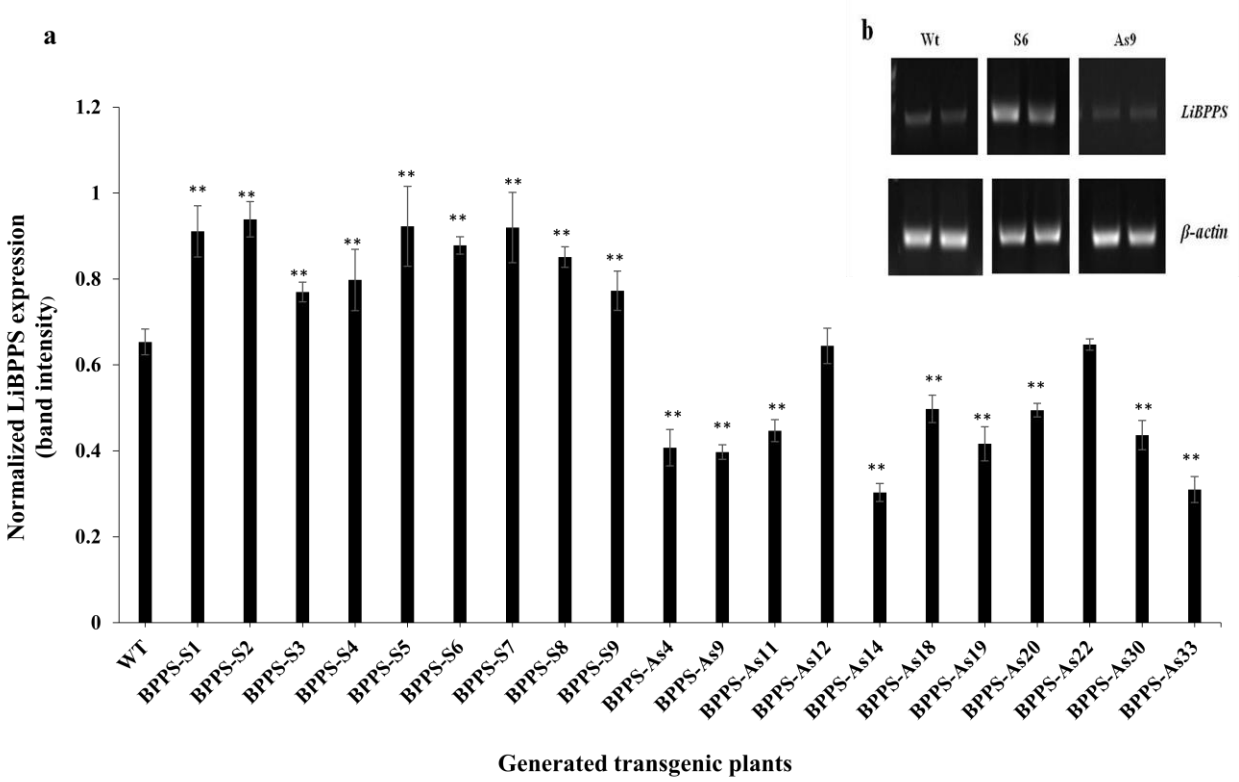


Figure 25. rt-PCR amplification of the *BPPS* gene in WT and transgenic *L. latifolia* plants using gene-specific primers.

Normalized (to *β-actin*) expression of *BPPS* in wild-type (WT) and some representative BPPS-sense (BPPS-S) and BPPS-antisense (BPPS-As) plants assessed by rt-PCR (a), and PCR products were resolved on agarose (1%) gel (b). Band intensities were measured by densitometry using ImageJ software. All the BPPS-sense plants had stronger expression than BPPS-antisense and WT. Data represented as mean ±standard error for two biological replicates and three technical repetitions, $^{**}P \leq 0.01$.

3.6.2.2 qPCR analysis of generated transgenic plants

To quantify the expression of BPPS in transgenic plants, the qPCR quantified-transcript levels for BPPS in young leaves of BPPS-sense, BPPS-antisense were compared relative to that of WT leaf. Results showed that the transcriptional expression level of BPPS in all BPPS-sense plants was significantly high ranging from 6.2 – 10.5 fold. 8 out of 10 tested BPPS-antisense plants showed an opposite trend toward significantly low BPPS expression ranging from 0.20 - 0.69 fold. Lines BPPS-As12 and BPPS-As22 showed a near-average BPPS expression compared with WT. For BPPS-sense plants, lines BPPS-S5, BPPS-S7, BPPS-S8 and BPPS-S9 presented the highest level of transcripts, and among BPPS-antisense lines, BPPS-As4, BPPS-As11, BPPS-As19, and BPPS-As30 presented the lowest levels of BPPS transcript. When we compared the highest transcript levels of BPPS line (BPPS-S8) with the lowest transcript level of BPPS-antisense plants (BPPS-As 19), we calculated differences of up to 10.6 times for BPPS (Figure 26).

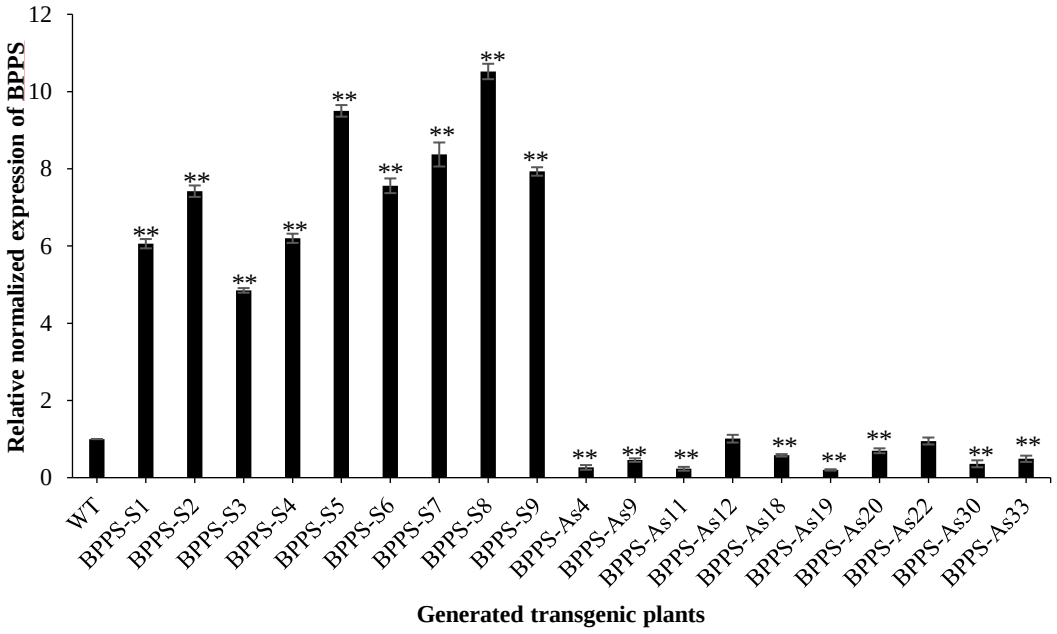


Figure 26. qPCR analysis of WT and generated transgenic *L. latifolia* plants using gene-specific primers.

The transcript levels for BPPS in young leaves of BPPS-sense (BPPS-S) and BPPS-antisense (BPPS-As) was normalized to actin. Data represented as mean ± standard error for two biological replicates and three technical repetitions, **P ≤ 0.01. The control for relative expression (WT) was assigned the arbitrary value of 1.0.

3.7 Screening of EO

3.7.1 Oil analysis of three developmental stages of wild-type *L. latifoila* leaves

The EO obtained from spike lavender leaves of three developmental stages (young, medium, and old leaves) showed that the oil yield decreased with the age of leaves from 36.3 % in young leaves to 11.2 % in old leaves (Figure 27). Since borneol diphosphate (BPP) produced by BPPS is not a volatile compound, detection of this monoterpene was not feasible by GC-MS (Despinasse et al., 2017). We detected the abundance of borneol, camphor, 1,8 cineole and limonene as the derived monoterpenes from GPP to study the manipulating BPPS expression on the abundance of these monoterpenes in the transgenic plants. EO analysis showed that camphor content decreased from 23% in the young leaf to 8.6 % in medium leaves and then increased to 12.4 % in old leaves (Figure 28). It seems likely that camphor abundance did correlate less with the developmental stage of leaves. Also, borneol, along with 1,8

cineole and limonene, decreased gradually as the leaf matured. Due to higher oil yield in young leaves of spike lavender, this type of tissue was selected to screen monoterpenes in the transgenic plants.

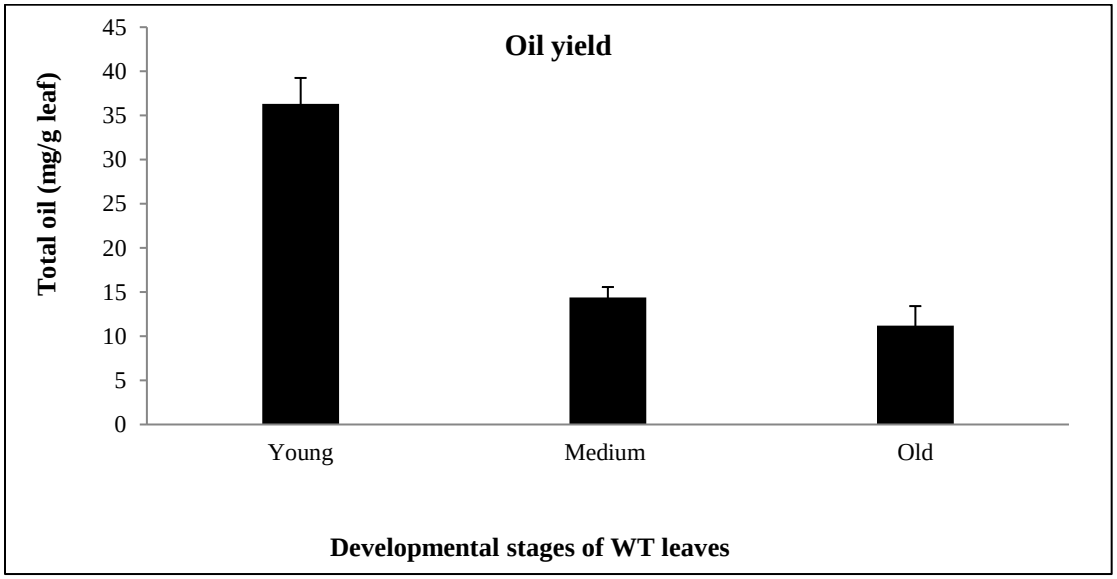


Figure 27. Oil yield (mg /g leaf) obtained from leaves of three developmental stages (young, medium, and old) of wild-type *L. latifolia.*

Data represented as mean ± standard error for three biological replicates and three technical repetitions.

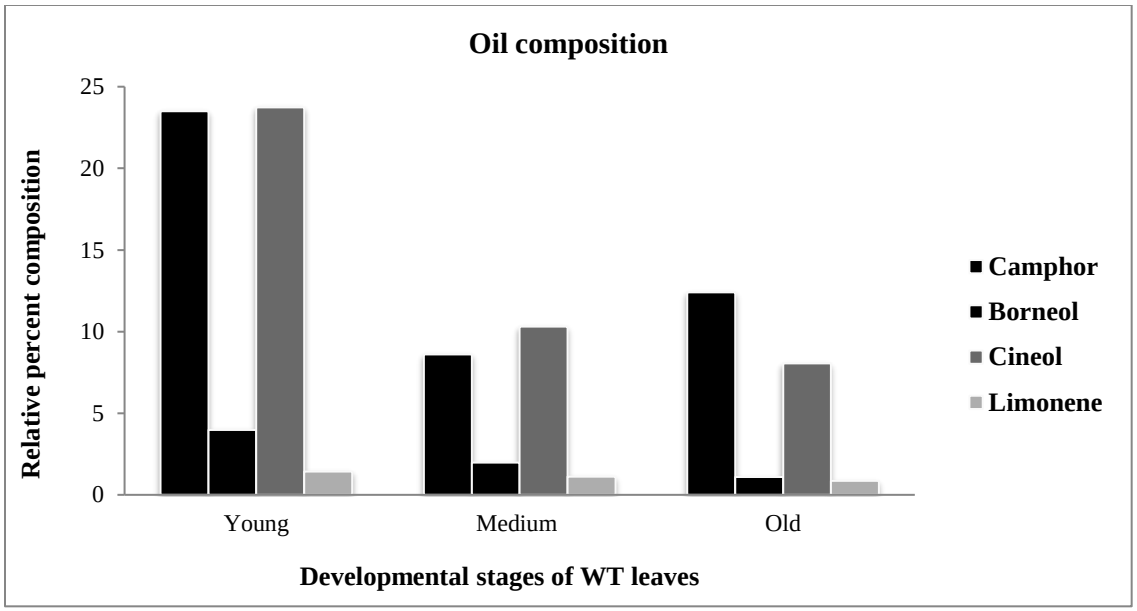


Figure 28. Relative percent abundance of four monoterpenes obtained from leaves of three developmental stages (young, medium, and old) of wild-type *L. latifolia.*

Data represented as mean ± standard error for three biological replicates and three technical repetitions.

3.7.2 Screening of EO in BPPS-antisense plants

In order to correlate camphor production and BPPS expression in transgenic plants, GC-MS analyses were performed on young leaves of each transgenic line as an *in planta* evaluation of LiBPPS function. The EO of 11 BPPS-antisense plants that their transcriptional BPPS expression has been already evaluated was screened. Twenty-one constituents were identified in both transformants and wild-type *L. latifoila* (See Appendix E). The oil yield in WT and each transgenic plant was calculated. BPPS-antisense plants show an oil of near-average yield compared with WT except for BPPS-As33, which had significantly ($p < 0.05$) less oil yield (32.21 mg oil/g leaf) compared with WT (37.3 mg oil/g leaf) (Figure 29).

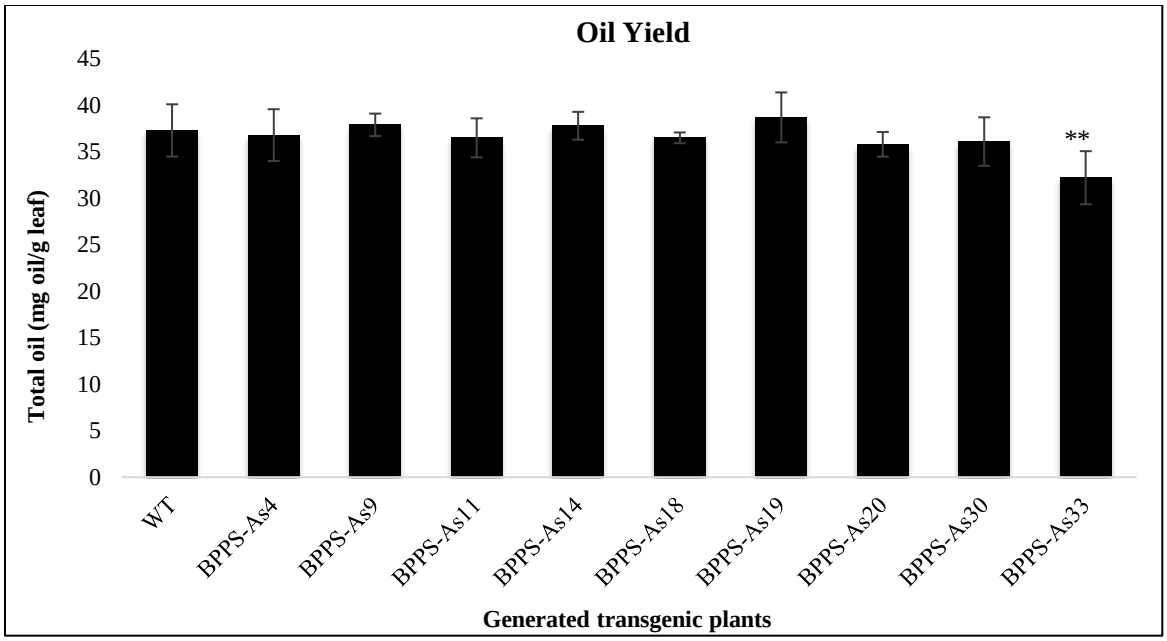


Figure 29. The total EO (mg oil/ g leaf) of 9 BPPS-antisense *L. latifolia* plants.

All the BPPS-antisense plants showed an oil of near-average yield compared with WT except for BPPS-As 33, with significantly less oil yield. WT= Wild-type and BPPS-As= BPPS-antisense plant. Data represented as mean ± standard error for three biological replicates and three technical repetitions, **P ≤ 0.01 and * P< 0.05.

The monoterpene synthase candidates tested for oil screening showed consistency with the results obtained from the transcriptional expression of BPPS. All BPPS-antisense plants demonstrated a significantly different abundance of borneol, 1,8 cineole, camphor, and limonene compared with WT except for BPPS-As12, and BPPS-As22 (See Appendix D- Table 4). Borneol content was considerably reduced in 9 of the BPPS-antisense plants with the highest and lowest reduction in BPPS-As4 (~ 80%) and BPPS-As33 (37%), respectively, relative to WT (Figure 30). Likewise, camphor abundance also decreased in 9 of tested transgenic plants, ranging in concentration from 10.8 % in BPPS-As30 to 16.06% in BPPS-As20 compared to 23.5% in WT (Figure 31). Statistical analysis showed that 1,8 cineole in 8 of BPPS-antisense plants was significantly ($p < 0.05$) higher, and ranged in concentration from 28.15% in BPPS-As20 to 36.33% in BPPS-As19 compared to 23.74% in WT. The 1,8 cineole in BPPS-As33

showed a similar percentage of abundance (25.24 %) compared to WT, which is consistent with its low total EO (Figure 32).

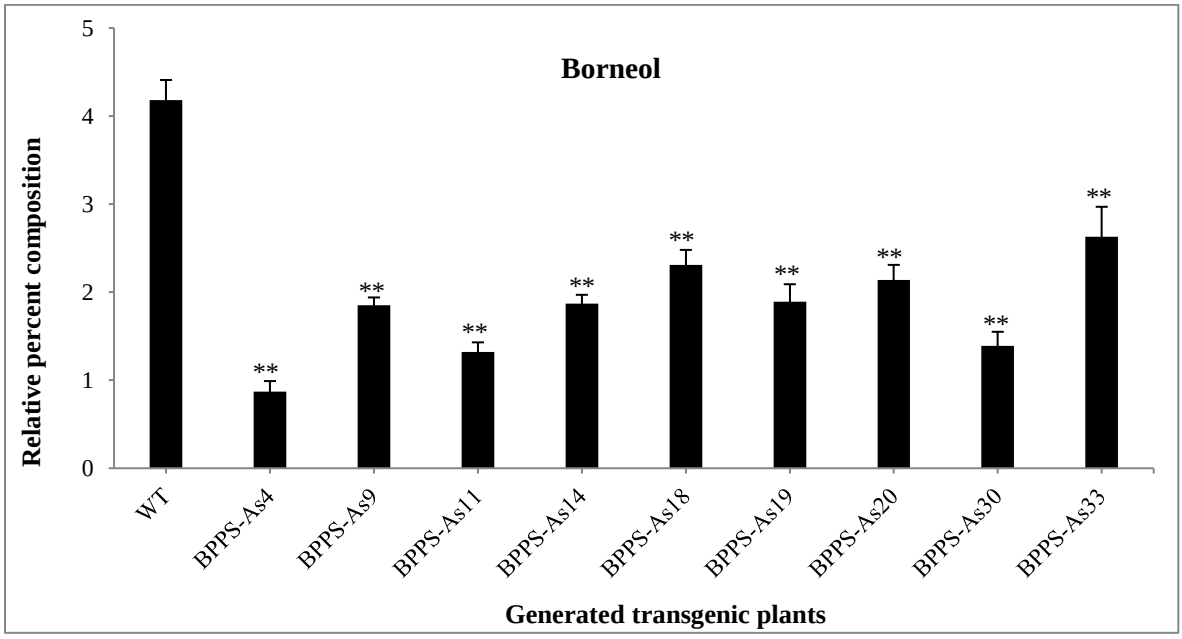


Figure 30. The borneol abundance of 9 of BPPS-antisense *L. latifolia* plants.

The borneol contents were considerably reduced in 9 of the BPPS-antisense plants with the lowest and highest reduction in BPPS-As4 (~ 80%) and BPPS-As33 (37%) respectively, relative to WT. WT= Wild-type and BPPS-As= BPPS-antisense plant. Data represented as mean ± standard error for three biological replicates and three technical repetitions, * P< 0.05.

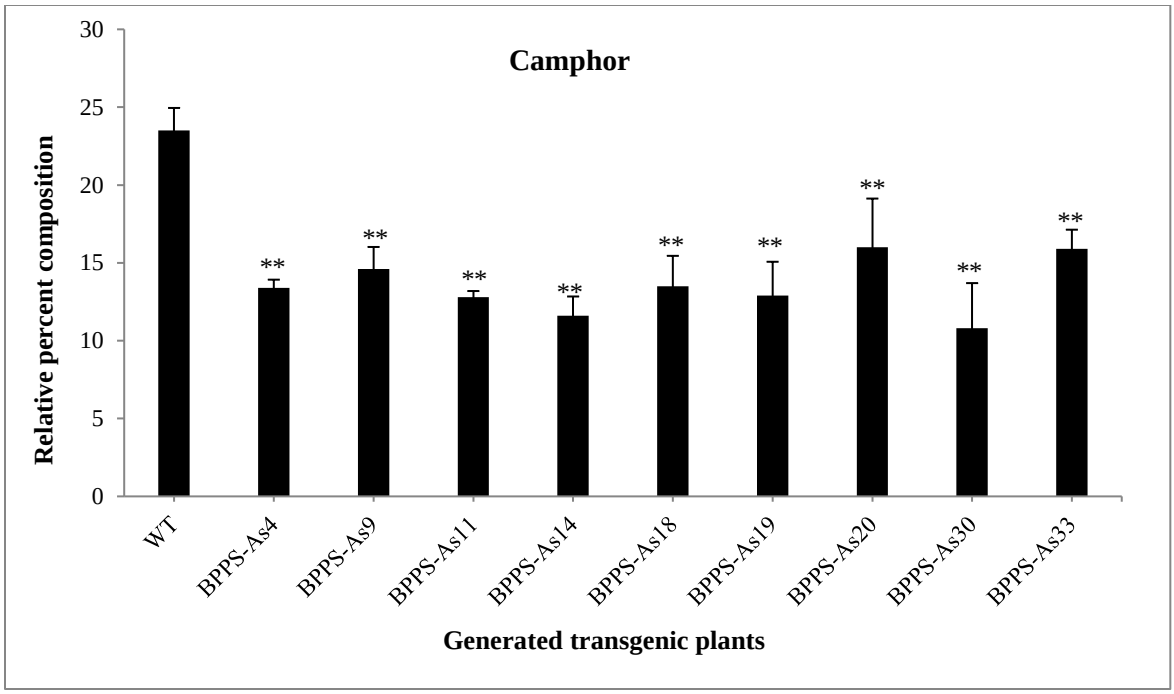


Figure 31. The camphor abundance of 9 of BPPS-antisense *L. latifolia* plants.

The camphor contents were considerably reduced in 9 of the BPPS-antisense plants with the highest and lowest reduction in BPPS-As30 (54%) and BPPS-As20 (~ 32%) respectively, relative to WT. WT= Wild-type and BPPS-As= BPPS-antisense plant. Data represented as mean ± standard error for three biological replicates and three technical repetitions, * P< 0.05.

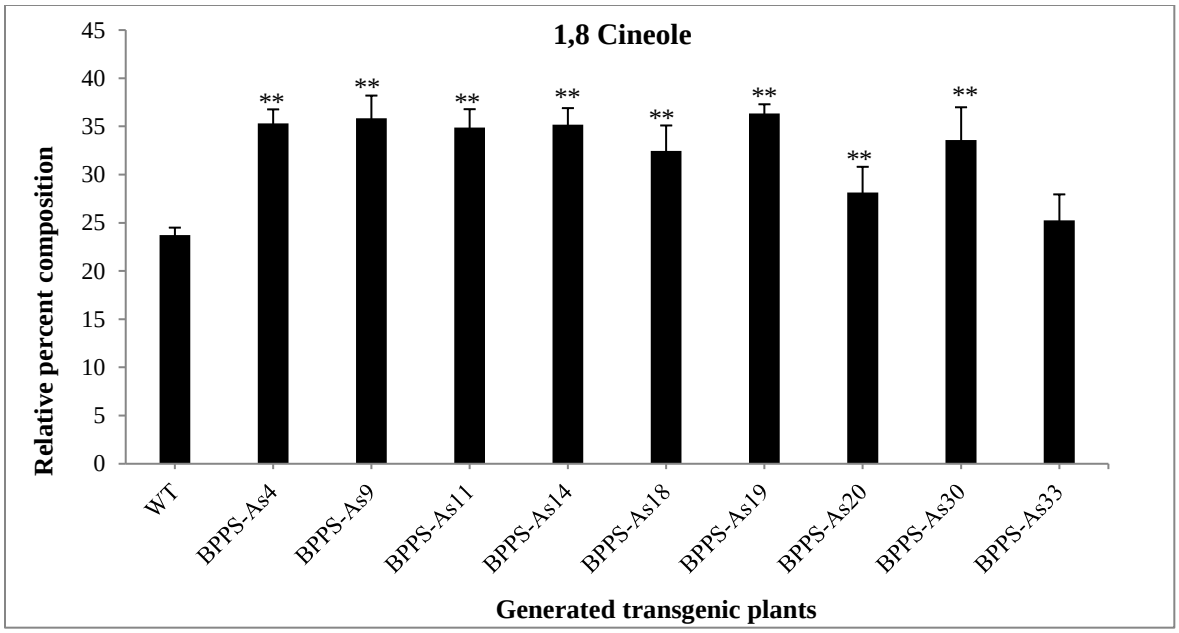


Figure 32. The 1,8 cineole abundance of 9 BPPS-antisense *L. latifolia* plants.

All the BPPS-antisense plants showed a significantly higher level of 1,8 cineole compared with wild-type except for BPPS-As33. The 1,8 cineole abundance showed a significant increment in 8 of the BPPS-antisense plants with the lowest and highest increase in BPPS-As20 (18.5 %) and BPPS-As19 (53%) respectively, relative to WT. WT= Wild-type and BPPS-As= BPPS-antisense plant. Data represented as mean ± standard error for three biological replicates and three technical repetitions, * $P< 0.05$.

A consistent trend toward a decreased abundance of borneol and camphor with an increased 1,8 cineole level was observed in the 9 of BPPS-antisense plants except for BPPS-As33. Limonene was detected in minute quantities in WT, but was significantly higher ($p< 0.05$) in 9 of BPPS-antisense plants ranging from 1.49 in BPPS-As20 to 3.74% in BPPS-As14 compared with WT (1.22%) (Figure 33).

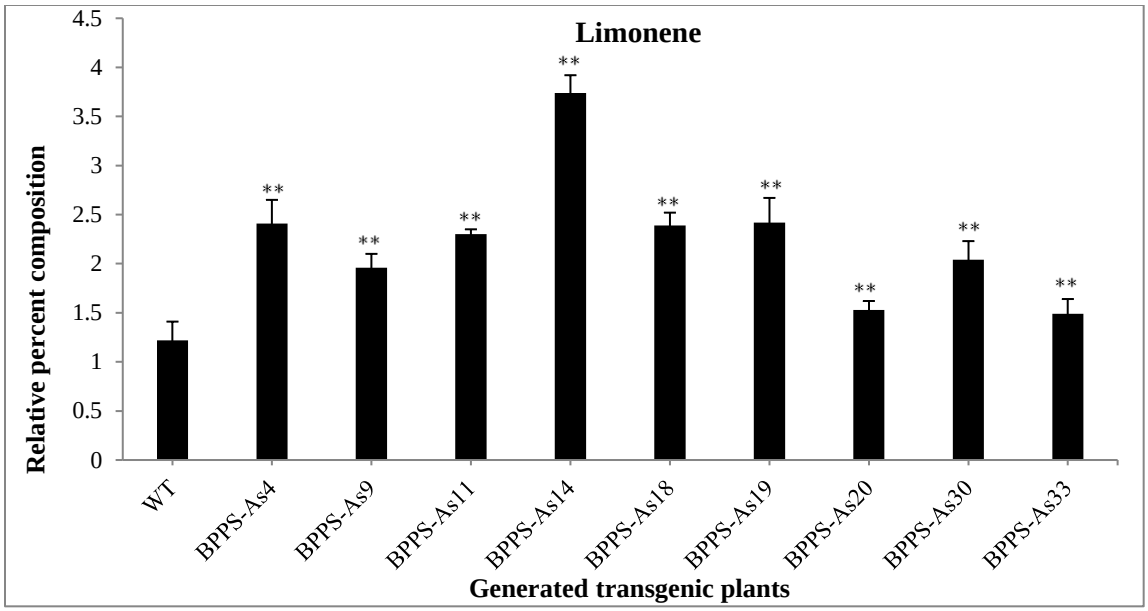


Figure 33. The limonene abundance of 9 BPPS-antisense *L. latifolia* plants.

All the BPPS- antisense plants showed a significantly higher level of limonene with the lowest and highest increase in BPPS-As20 (~22 %) and BPPS-As14 (206%) respectively, relative to WT. WT= Wild-type and BPPS-As= BPPS-antisense plant. Data represented as mean ± standard error for three biological replicates and three technical repetitions, * P< 0.05.

3.7.3 Screening of EO in BPPS-sense plants

The EO of the 5 BPPS-sense *L. latifolia* plants with a significant increase in BPPS expression were analyzed. Over a period of 9 months, only 5 of the transgenic plants survived for oil analysis. Similar to BPPS-antisense plants, twenty-one constituents were identified in BPPS-sense plants. The transgenic plants showed alteration toward oil yield as BPPS-S2 and BPPS-S5 showed a significantly higher oil yield (40.33-41.12 mg oil/g leaf), BPPS-S3 and BPPS-S4 exhibited a decrease in their oil yield compared with WT (37.3 mg oil/g leaf). BPPS-S1 showed an oil of near-average yield compared with WT (Figure 34).

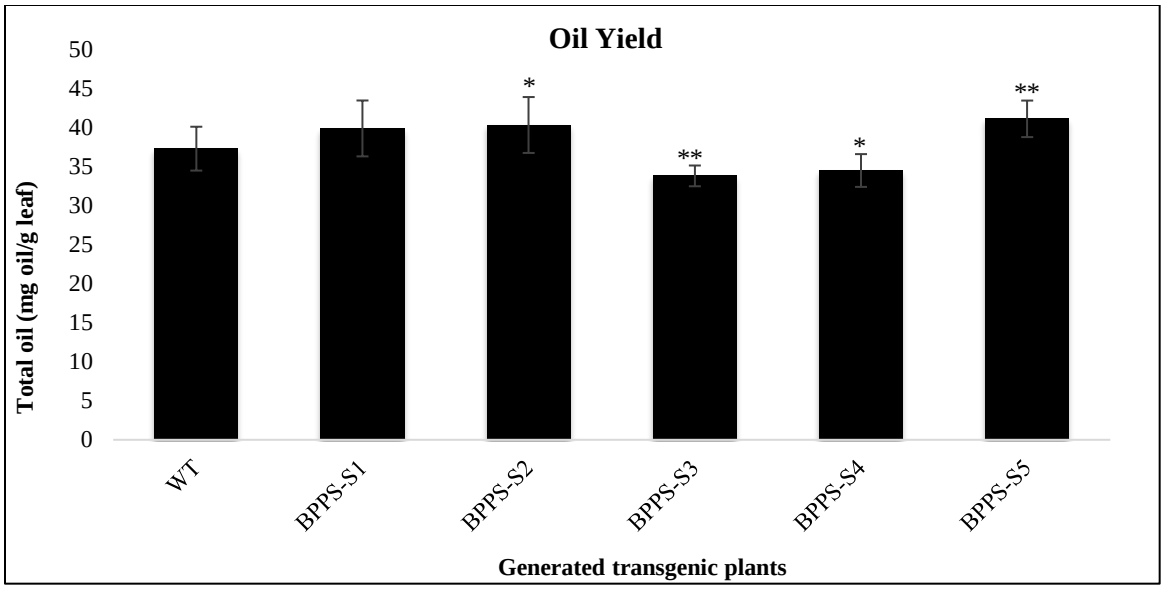


Figure 34. The total EO (mg oil/ g leaf) of 5 BPPS-sense *L. latifolia* plants.

BPPS-S2 and BPPS-S5 showed a significantly higher oil yield (8.1 and 10.2 % respectively), while S3 and S4 showed a significant decrease in their oil yield (9.3 and 7.5 %) compared with WT. BPPS-S1 showed an oil of near-average yield compared with WT; WT= Wild-type and BPPS-S= BPPS-sense plant. Data represented as mean ± standard error for three biological replicates and three technical repetitions, ** P< 0.01 and * P< 0.05.

Borneol abundance in BPPS-sense plants showed a significant (P< 0.05) increase ranged from 6.88 to 9.24% except for BPPS-S3 and BPPS-S4 compared with WT (4.18%) (Figure 35).

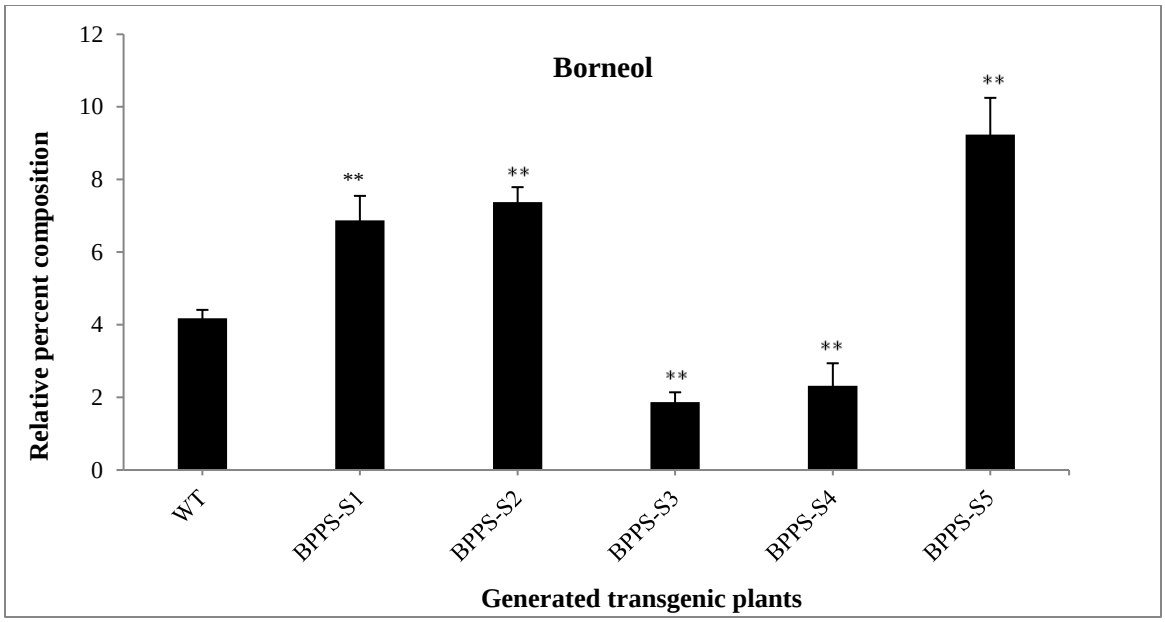


Figure 35. The borneol abundance of 5 BPPS-sense *L. latifolia* plants.

While borneol in BPPS-S1, BPPS-S2, and BPPS-S5 increased considerably (~65, ~76, and ~220% respectively), BPPS-S3 and BPPS-S4 showed a significant decrease (~55 and ~44 %) in borneol relative to WT. WT= Wild-type and BPPS-S= BPPS-sense plant. Data represented as mean ± standard error for three biological replicates and three technical repetitions, * P< 0.05.

While camphor showed a significant (P< 0.05) increase in BPPS-sense plants ranging from 30.11-36.88%, BPPS-S3 and BPPS-S4 showed a significantly lower level of camphor (18-20.8 %) compared with WT (23.5%) (Figure 36).

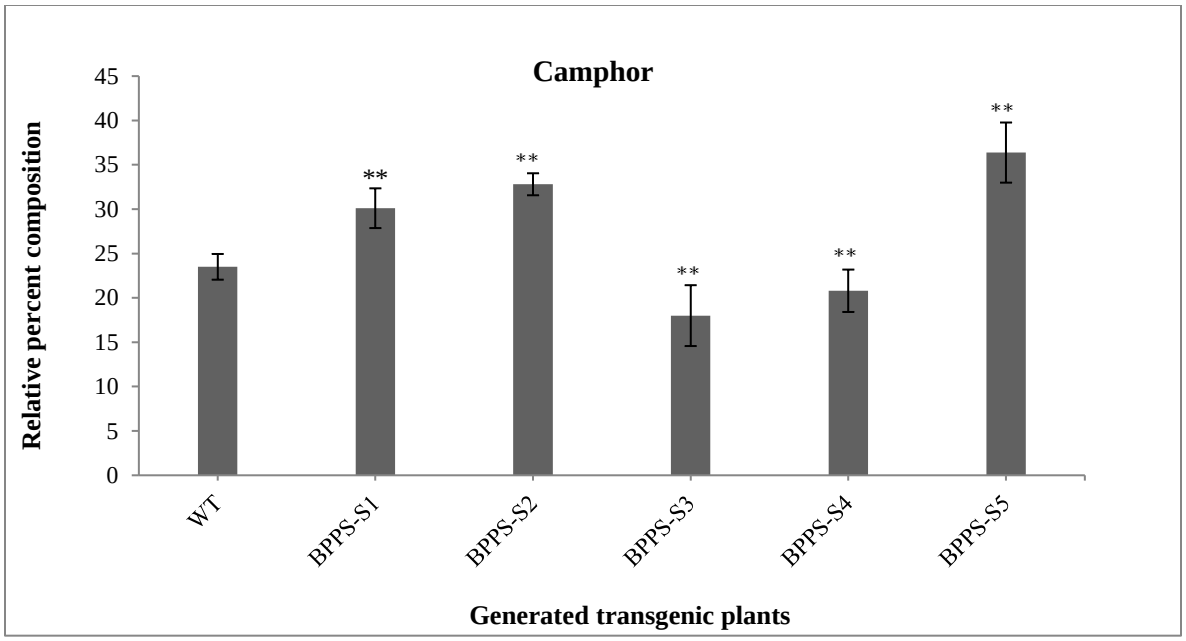


Figure 36. The camphor abundance of 5 BPPS-sense *L. latifolia* plants.

While camphor in BPPS-S1, BPPS-S2 and BPPS-S5 increased significantly (~ 28, ~ 40 and ~55 % respectively), BPPS-S3 and BPPS-S4 showed a significant decrease (~23 and ~ 11% in camphor compared with WT. WT= Wild-type and BPPS-S= BPPS-sense plant. Data represented as mean ± standard error for three biological replicates and three technical repetitions, * P< 0.05.

On the contrary, the 1,8 cineole content considerably reduced in all transgenic plants ranging from 16.79% in BPPS-S5 to 20.19% in BPPS-S3 compared with WT (23.74%) (Figure 37).

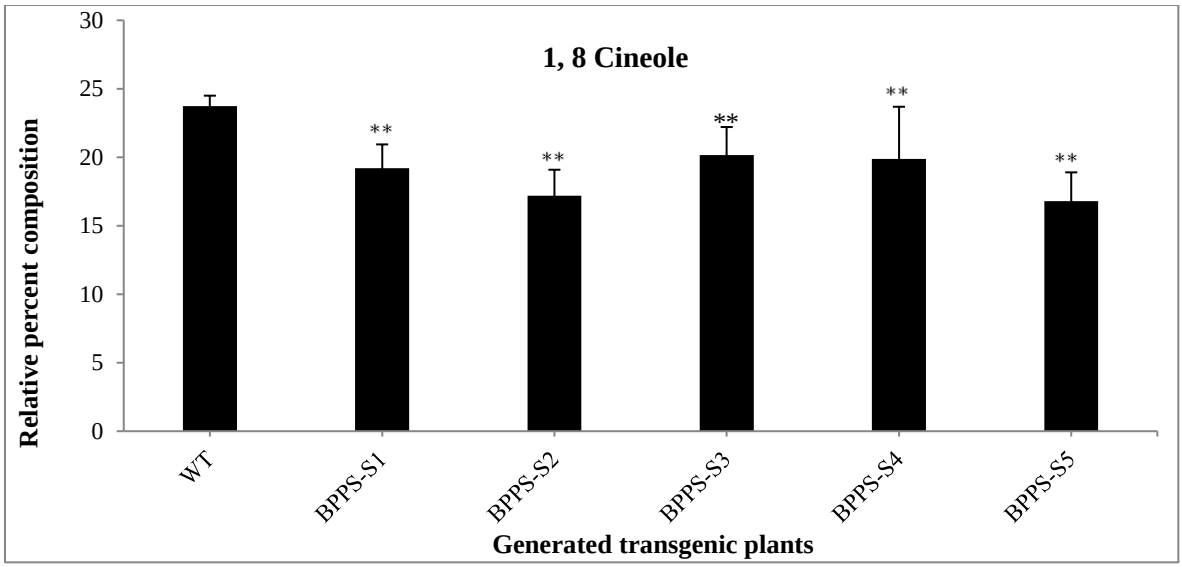


Figure 37. The 1,8 cineole abundance of 5 BPPS-sense *L. latifolia* plants.

The 1,8 cineole content was considerably reduced in all transgenic sense plants, with the lowest and the highest reduction in BPPS-S3 (~15%) and in BPPS-S5 (~30%) relative to WT. WT= Wild-type and BPPS-S= BPPS-sense plant. Data represented as mean ± standard error for three biological replicates and three technical repetitions, * P< 0.05.

While limonene showed a significantly lower abundance (p< 0.05) in 3 of BPPS-antisense plants ranging from 0.56% in BPPS-S1 to ~1.1% in BPPS-S5, BPPS-S3 and BPPS-S4 showed a significantly higher (p< 0.05) abundance of limonene (1.83-2.3 % respectively) compared with WT (1.22%) (Figure 38).

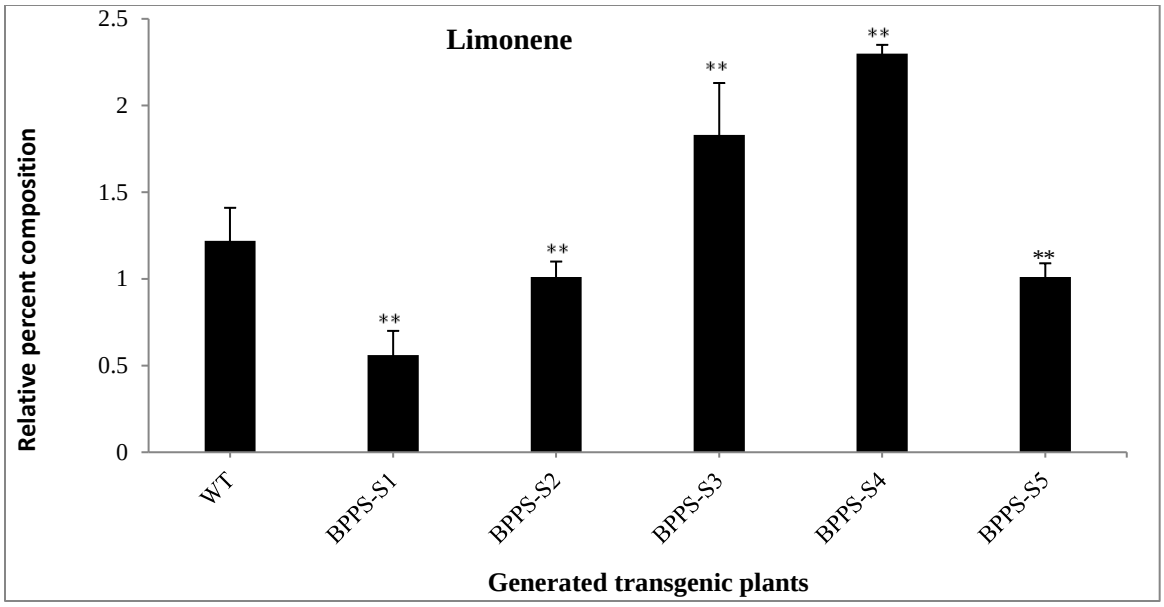


Figure 38. The limonene abundance of 5 BPPS-sense *L. latifolia* plants.

Limonene in BPPS-S1, BPPS-S2 and BPPS-S5 decreased significantly (~ 54, ~ 17 and ~17 % respectively), BPPS-S3 and BPPS-S4 showed a significant increase (~50 and ~ 88% respectively) in limonene compared with WT. WT= Wild-type and BPPS-S= BPPS-sense plant. Data represented as mean ± standard error for three biological replicates and three technical repetitions, * P< 0.05.

Chapter 4: Discussion

Among lavender cultivars, spike lavender is not considered as a desired plant for the perfumery industry due to producing high camphor. It is widely assumed that the production of monoterpenes is regulated genetically and that the difference in camphor levels in *L. latifolia, L. angustifolia and L. x intermedia* is due to the distinct genetic background of these species (Clark & Menary, 1980; Turner et al., 2000). Monoterpene production is also influenced by environmental factors and varies throughout the development of the plant (Boeckelmann, 2008; Tholl, 2006).

Although borneol has been identified as the main component in EO of leaf and aerial parts of lavender cultivars, the abundance of this monoterpene in spike lavender is low compared with other cultivars (Aprotosoaie et al., 2017). Also, the higher amount of 1,8 cineole and camphor in spike lavender EO makes it different from other valuable lavender cultivars (Mendoza-Poudereux et al., 2014). Studies showed that camphor and 1,8-cineole in lavenders are mostly found in both young leaves and flowers and greatly affected by developmental and environmental factors mainly in the Mediterranean region (Aprotosoaie et al., 2017). The 1,8-cineole abundance increases in low temperature (Muñoz-Bertomeu et al., 2007) and after rainfall while lower altitudes favor the biosynthesis of camphor (Hassiotis et al., 2014). Although monoterpene biosynthesis is undergoing environmental factors, physiological, biochemical, metabolic and genetic regulation (Clark & Menary, 1980; Sangwan et al., 2001; Tholl, 2006), the reason behind this diversity in the monoterpene profile in lavender cultivars is not well elucidated. It seems that more research in terms of knowledge in genetic and biochemical data is needed to reveal the regulation of monoterpene in the plants (Aprotosoaie et al., 2017).

Regulation of monoterpene biosynthesis, especially the pathways that contributed to the production

of camphor, are not yet fully understood in lavenders (Muñoz-Bertomeu et al., 2008). Thus, a genetic knowledge of camphor biosynthesis in lavenders is important for the biotechnological breeding of spike

lavender (Mendoza-Poudereux et al., 2017; Juan Segura et al., 2019). The biosynthetic route for camphor biosynthesis was previously defined in sage (*Salvia officinalis*) and results showed that camphor and borneol are biosynthetically related (Wise et al., 1998). GPP is converted by Bornyl diphosphate synthase (BPPS) to bornyl diphosphate (BPP) as a major prenyl diphosphate, and a few monoterpenes as minor compounds. BPP is then dephosphorylated by bornyl diphosphate diphosphatase, leading to borneol, and then oxidized to camphor by a borneol dehydrogenase (BDH) (Croteau et al., 1978; Mendoza-Poudereux et al., 2017; Sarker et al., 2012; Wise et al., 1998; Woronuk et al., 2011). In theory, the abundance of the intermediates and the end product depends on the activation or inactivation of the intermediating enzymes (Boeckelmann, 2008). Camphor accumulation depends on the expression of all the contributing genes and inactivation or absence of these enzymes might not only change the accumulation of GPP but also the intermediate and end products. Also, assuming the same route for camphor biosynthesis in lavenders, it seems likely that the high amount of camphor in spike lavender is due to the high activity of BPPS and BDH for the production of borneol followed by its conversion into camphor. In both *L. latifoila* and *L .x. intermedia*, camphor abundance is higher than borneol content (Boeckelmann, 2008; Lesage-Meessen et al., 2015; Lis-Balchin, 2002) assuming that although both BPPS and BDH are expressed in these plants, BDH has more activity than BPPS to convert borneol to camphor.

Recently, a *LiBPPS* cloned in our research group showed a high *in vitro* activity (unpublished) for the conversion of GPP to BPP. So, it was hypothesized that this gene would likely have a similar catalytic function *in planta.* To explore this hypothesis, we overexpressed *LiBPPS* gene using sense and antisense technology in spike lavender. We selected *L. latifolia* instead of *L. x intermedia* to transform with *LiBPPS* due to the following reasons: first, *L. latifolia* contained a higher abundance of camphor than *L. x intermedia* (Lesage-Meessen et al., 2015; Upson et al., 2004), thus by implementing antisense technology, we aimed to reduce camphor abundance in spike lavender for the first time. Furthermore, a reliable *Agrobacterium*-mediated transformation and regeneration of transgenic spike lavender were available.

In BPPS-transgenic plants, the BPPS cDNA was constitutively expressed under the control of the CaMV35s as a strong promoter. Thus, we expected an increase in the production of BPP and the consequent products such as borneol and camphor in BPPS-sense plants, and decrease in the biosynthesis of BPP and borneol and camphor in the BPPS-antisense plants. To confirm this, EO screening was performed in this study.

4.1 Cloning LiBPPS gene in sense and antisense orientation in *L. latifolia* plants

4.1.1 *L. latifolia* is sensitive to hygromycin

Transformation of spike lavenders was confirmed using callus induction and shoot regeneration under antibiotic supplemented culture medium, histochemical assays for β-glucuronidase activity in GUS plants and molecular analysis for the presence of the transgenes (*LiBPPS* and *HygR*) in BPPS-sense and BPPS-antisense plants.

In the present research, a protocol for the genetic transformation of *L. latilolia* using hygromycin as a selectable marker was developed. A reliable plant genetic transformation highly depends on implementing an appropriate antibiotic to select the transformants (Angenon et al., 1994). The sensitivity of *L. latifolia* explants to kanamycin was established prior to transformation experiments to determine the effective concentration for selecting transformed cells. In our work, Kanamycin did not suppress the growth of untransformed plants which was not in accordance with the kanamycin concentration (30 mg L^{-1}) reported in the previous works (Mendoza-Poudereux et al., 2014; Muñoz-Bertomeu et al., 2006; Nebauer et al., 2000). Various factors such as genotype, explants type, the developmental stage, and the tissue culture conditions may contribute to the sensitivity of plant cells to the selection agent (Angenon et al., 1994). We, therefore, studied the sensitivity of spike lavender to the selection agent under the actual conditions of the transformation and regeneration process. Results showed that kanamycin did not have an influence on callus induction, shoot and root induction in both WT and the putatively transformed

explants in the selection media. Thus, kanamycin was not a suitable selective agent for transforming spike lavender.

To test the sensitivity of spike lavender to hygromycin, we found that 7.5 mg L^{-1} hygromycin annulled root induction in the unrooted WT shoots and shoot regeneration in WT leaves. Thus, hygromycin at 7.5 mg L^{-1} was considered for the selection of transformed *L. latifolia* in tissue culture because concentration higher than 7.5 mg L^{-1} reduced shoot development. This result was not in line with 50 mg L^{-1} of hygromycin was used for the selection of transformed *L. latifolia* plants reported in the previous study (Mishiba et al., 2000).

We used a combination of both cefotaxime and TIMENTIN to suppress the growth of the remaining *Agrobacterium* in the elimination media. It was reported that 120 mg L^{-1} of cefotaxime killed the remaining bacteria without any influence on the frequency of bud and shoot regeneration, but concentrations higher than 160 mg L^{-1} slightly reduced shoot development (Nebauer et al., 2000). In our work, cefotaxime at a concentration of 120 mg L^{-1}combined with TIMENTIN at a concentration of 60 mg L^{-1} eliminated *Agrobacterium tumefaciens* in the elimination media.

Also, for the first time, we produced *L. latifoila* transgenic plants for the *BPPS-sense* and *BPPS-antisense* gene with the selectable marker *HygR* gene using *Agrobacterium*-mediated transformation. Since the purpose of regenerating GUS plants was validating the *in vitro Agrobacterium* transformation system, rooted plants expressing GUS were not transferred into the soil for further analysis. Production of transgenic plants through *Agrobacterium*-mediated transformation lasted for 150-160 days.

4.1.2 Hygromycin-resistant explants were regenerated under selection condition

The method used for regeneration of *L. latifolia* was based on protocol reported by previous studies (M C Calvo et al., 1988; Nebauer et al., 2000). We obtained a success rate of callus induction, shoot regeneration and elongation of the putative transgenic plants which was in line with the previous study (Nebauer et al., 2000). Rooting induction in transformed *L. latifolia* was rather difficult to be

obtained when we followed previous protocols, but was successfully obtained when using half-strength MS with 2.9 µM IAA reported by a previous study in our research group (Lauren A E Erland & Mahmoud, 2014). Although this mentioned concentration of IAA did not give the largest number of roots it did give better results than the previous treatments. We reported the success rate of organogenesis in transformed spike lavender measured based on the number of explants initially treated with *Agrobacterium* and the number of survived plants in the greenhouse. In this experiment, out of the initial treated 150 explants for each transformation experiment, 65 calli from transformed leaves with BPPS-senes gene and 80 calli from transformed leaves with BPPS-antisense gene emerged in selection media. Of these calli, 32 BPPS-senes and 59 BPPS-antisense plants were regenerated, of which 31.25% of BPPS-senes and 67% of BPPS-antisense plants were established in the greenhouse. This finding is comparable with the previous study (Nebauer et al., 2000). Although all BPPS-antisense plants were phenotypically indistinguishable from WT, 50% of the BPPS-sense plants showed severe growth retardation and did not survive in the soil in the greenhouse. This might be associated with the transgene (BPPS-sense) introduced into the plant genome which influenced structural and regulatory genes controlling the plant anatomical or physiological characteristics. Also, the 35S promoter used for the expression of BPPS in transgenic plants can induce massive expression in not only glandular trichome but also in various tissue (Endo et al., 2018). Thus, growth retardation in the BPPS-sense plants might be due to the expression of BPPS in other tissue such as root, leading to growth inhibition.

4.2 Expression analysis of putative transgenic plants

4. 2.1 Reporter gene (β-glucuronidase, GUS) activity

To test the *Agrobacterium*-mediated transformation system, the *gusA* gene under the control of CaMV 35S promoter was transformed into the spike lavender genome. Histochemical assay for β-glucuronidase activity showed the presence of blue precipitate in callus and regenerated shoots of transformed plants. Thus, a system for the genetic transformation of *L. latifolia* was developed using

hygromycin selection of *Agrobacterium*-inoculated leaf explants. In higher plants, the cauliflower mosaic virus 35S promoter is one of the most common promoters in the heterologous expression of plant genes (Endo et al., 2018).

4.2.2 Expression of selectable marker (HygR) gene in the transgenic plants

Because foreign DNA is integrated randomly into the plant genome (Beltrán et al., 2009), its molecular characterization becomes a primary tool for the presence of transgenes (*HygR and BPPS*). By approaching *Agrobacterium* transformation technology, although only a small proportion of target plant cells may receive the DNA, this method can introduce a large fragment of the foreign gene but with a small copy number into the host plant genome (Birch, 1997; Gelvin, 2003; Hwang et al., 2017; S. Li et al., 2017). Multiple integrations may lead to the inactivation of the transgene in the plant genome through the defence mechanism of the host plant ending in gene silencing. Thus developments of efficient tools with simple integrations and high levels of expression are preferred for plant transformation (Beltrán et al., 2009).

The *HygR* was amplified in 9 survived BPPS-sense plants, while this gene was amplified in 31 out of 40 putative BPPS-antisense plants. This indicated that the hygromycin resistance gene was present within the genome of transgenic plants and also showed the high transformation efficiency of the *HygR* gene in the transgenic plants. Although some putative transformants were able to grow in the presence of hygromycin, PCR amplification of the *HygR* gene was unsuccessful in these plants. This may due to the expression of this gene in these plants below a level which was detectable.

4.2.3 Expression of *BPPS* in the transgenic plants

As *BPPS* is an endogenous gene in spike lavender, quantitative PCR amplification from cDNA instead of genomic DNA from young leaves, was vital to evaluate the expression of BPPS in the transgenic plants. To examine the expression pattern of the transgene, both rt-PCR and qPCR were employed in our work.

Evident differences were observed at the level of BPPS expression according to amplification intensities by rt-PCR. Results revealed lower expression of BPPS in 11 out of 13 BPPS-antisense plants meaning that the gene was down-regulated confirming that antisense RNA technology can be used as a potential tool for reducing the expression of targeted genes in the transgenic plants. Although no studies have been reported regarding using antisense RNA in reducing the activity of a target gene in lavenders, some studies showed the application of this technique in other plants. For example, it was shown that the expression of an antisense chalchone synthase (*chsA*) cDNA driven by either the CaMV 35s promoter or the chsA promoter can result in a severe reduction of flower pigmentation in petunia(van der Krol et al., 1988). Previous studies also demonstrated the utility of antisense RNA to reduce nopaline synthase activity (Rothstein et al., 1987; Sandler et al., 1988), GUS activity (Cannon et al., 1990), small subunits of ribulose bisphosphate carboxylase (rbe) activity in tobacco (Rodermel et al., 1988). Also, knocking down the expression of limonene synthase by RNAi method in transgenic spearmint was reported (C. Li et al., 2020). Another study showed down-regulation of menthofuran synthase (*mfs*) by the antisense approach in transgenic peppermint (Mahmoud & Croteau, 2003).

We also used qPCR to determine the mRNA levels of the BPPS gene regulated by the promoter CaMV 35S. The differences were observed at the levels of transcription according to amplification intensities by rt-PCR agreed with the qPCR data for all the tested transgenic plants. 8 out of 10 tested BPPS-antisense plants showed a less transcriptional expression level of BPPS compared with BPPS-sense plants and WT. Surprisingly, 2 of the BPPS-antisense plants (BPPS-AS12 and BPPS-AS22), showed near-average BPPS expression compared with WT. Some authors have already reported no significant modification of monoterpene profile among the transgenic plants. This observation in our transgenic plants might be due to the silencing of the *LiBPPS* gene in the plant genome. The transgene-silencing effect can be interpreted in two general categories: positional effect in which the transgene may insert into a non-expressed portion in unfavourable chromosomal location (heterochromatin) or the repeat-sequence regions of telomers result in silencing of the transgene (M A Matzke & Matzke, 1998; Ye & Signer,

1996). The second class of transgene inactivation occurs when multiple copies of the transgene are present in a genome (Kumar & Fladung, 2001; M A Matzke & Matzke, 1998). This indicates that the host genome has some control over the expression of the transgene in defense against foreign DNA through DNA methylation and histone deacetylation at the transcriptional level (Marjori A Matzke et al., 2002) or promoter methylation in the posttranscriptional level. Another assumption is that the *BPPS* transgene might not be expressed due to the integration of truncated copies of the gene into the plant genome. The actual mechanism of gene silencing in our plants cannot not be fully elucidated from our data.

BPPS amplification by rt-PCR and qPCR showed that all BPPS-sense plants had a higher level of BPPS transcript compared with WT. When we compared the highest transcript levels of BPPS lines with the lowest transcript level of BPPS-antisense plants, we calculated differences of up to 10.6 times for BPPS. In BPPS-transgenic plants, the BPPS cDNA was constitutively expressed under the control of the CaMV35s as a strong promoter. Thus, we assumed that as a plant develops, the promoter remains active, resulted in increasing the level of BPPS transcript. Some studies showed a correlation between overexpression of transgene with a level of transcript in the transgenic plants however this depends on the plant tissue type, developmental stage, tissue position on the plant and transgenic lines (Diemer et al., 2001; Bernd Markus Lange et al., 2011; Mahmoud et al., 2004; Mendoza-Poudereux et al., 2014; Muñoz-Bertomeu et al., 2006, 2008). For example, it was shown that the expression of *LIS* transgene in spike lavender was dependent on the developmental stage of plant leaf as young leaves showed the highest expression (Mendoza-Poudereux et al., 2014). Also, in an another effort, up-regulation of DXR, an enzyme of the MEP pathway, some lines of transgenic spike lavender plants led to an increase in mRNA level (Muñoz-Bertomeu et al., 2006). In a separate study, a higher LS expression in some of the transgenic spike lavender lines was reported whereas the rest of the lines showed a moderate expression of the transgene (Muñoz-Bertomeu et al., 2008).

4.3 Essential oil screening in WT and transgenic plants

4.3.1 Oil yield and monoterpene composition in WT plants

To select the appropriate tissue for screening oil yield and monoterpene composition in transgenic plants, pre-screening of EO in different developmental stages (young, medium, and old) of WT spike lavender leaves were performed. Results showed that the oil content (per weight) decreased with the age of leaves from 36.3 % in young leaves to 11.2 % in old leaves. Also, we observed a higher abundance of borneol, camphor, 1,8 cineole and limonene in WT spike lavender leaf. This might be due to an increase in a higher density of essential oil glands in young leaves. The pattern of decreasing EO as the leaf matures was in line with the higher oil composition in young leaves of WT and mutant *L. x intermedia* (Lauren Alexandra Elizabeth Erland, 2015). Also, monitoring monoterpene abundance in both transgenic and WT spike lavender showed that the content of some monoterpenes such as limonene, pinene, myrcene, 1,8 cineole, camphor and borneol was decreased with leaf development (Muñoz-Bertomeu et al., 2007). Other studies in *Lamiaceae* showed a higher EO profile in young leaves. For example, in *Salvia officinalis*, camphor, and borneol levels were higher in expanding leaves compared to mature leaves (Croteau et al., 1981). Other studies in *Menta piperita* showed that monoterpene profiles like menthone and menthol increase with leaf age advancement (Maffei & Codignola, 1990; Turner et al., 2000).

4.3.2 Oil yield and monoterpene content in the BPPS-sense and BPPS-antisense plants

To confirm whether modification of *BPPS* expression influences the abundance of camphor, borneol and other monoterpenes, we monitored the EO of transgenic plants. EO screening of transgenic plants over a period of 9 months was accomplished by sonication of young leaves followed by gas chromatography separation of components and quantification by the internal standard. GC-MS analysis Since, lavender EO monoterpenes are produced mainly in the glandular trichomes (Guo et al., 2020), it was reasoned that alterations in mTPS gene expression should be observable at the level of essential oil

accumulation (Mahmoud & Croteau, 2001). The goal of our study was to investigate the influence of the sense and antisense version of *LiBPPS* on borneol and camphor abundance.

Since borneol diphosphate (BPP) produced by BPPS is not a volatile monoterpene due to its high molecular weight (314.21 g/mol) and having biphosphate polarity group, detection of this monoterpene was not feasible by GC-MS (Despinasse et al., 2017; Wise et al., 1998). Thus, we screened the abundance of borneol and camphor (which are derived from BPP), in addition to some other monoterpenes in our transgenic plants.

The analytical screen of BPPS-antisense plants showed that EO yield in most antisense plants was similar to that in WT except for BPPS-As33, which produced less EO. This may due to the positional effects of the transgene. It is possible that the transgene disrupted other genes that control plant growth vigour and essential oil production in BPPS-As33. Data obtained in this study cannot confirm this postulate, which can be further investigated through genome sequencing or other methods. In terms of monoterpene composition, 9 of the BPPS-antisense plants showed a reduction of ~ 80% in borneol and of ~55% in camphor abundance relative to WT. The results indicated that in all cases in which the expression of *BPPS* was transcriptionally downregulated in BPPS-antisense plants, a consistent correlation was observed between the reduction of borneol and camphor and downregulation of *BPPS*. This implies that camphor biosynthesis is preliminary controlled by the transcriptional regulation of *BPPS*. Our results parallel those previously reported in other plants. To date, numerous studies have shown that monoterpene production can be regulated through gene expression. For example, a positive correlation between menthofuran content and the level of the menthofuran synthase transcript in peppermint was reported, implying that menthofuran biosynthesis is controlled primarily by transcriptional regulation of menthofuran synthase (Mahmoud & Croteau, 2003). Also, a positive correlation between the level of camphor and the expression of its biosynthetic enzyme (BDH) in glandular trichomes of *L. x intermedia* was shown (Sarker et al., 2012). Further, it was reported that the changes in terpene synthase (TPS) expression led to changes in EO composition during lavender (*L.*

angustifolia and *L. x intermedia*) inflorescence developments (Guitton et al., 2010). Another study showed that the developmental expression of the limonene synthase (LS) transcript paralleled with limonene accumulation in spike lavender leaves (Muñoz-Bertomeu et al., 2008). Also, the abundance of linalool correlated with the transcription of the linalool synthase gene in *L. x intermedia* and *L. angustifolia* (Boeckelmann, 2008).

The decrease in borneol in conjunction with camphor reduction in our BPPS-antisense plants indicates that similar to sage (Sarker et al., 2012; Wise et al., 1998), camphor is produced from consecutive dephosphorylation of BPP and oxidation of borneol in spike lavender. Furthermore, the results may imply that downregulation of *BPPS* affected the downstream key genes such as BDH by a precursor availability so that they were not able to accelerate monoterpene production and accordingly led to a reduction of borneol and camphor in spike lavender. Although, the antisense approach for regulation of monoterpene synthase had not been reported in lavenders prior to this study, one study showed that down-regulation of menthofuran synthase gene (*mfs*) by antisense approach in peppermint led to a decrease in menthofuran by the precursor availability (Mahmoud & Croteau, 2001). Similarly, downregulation of *BPPS* gene in antisense plants resulted in the reduction of borneol (~40-80%) and camphor (32-54%) abundance. To date, only two studies were conducted for the identification and *in vitro* functional characterization of genes committed in camphor biosynthesis in lavender. However, the *in vivo* potential role of these genes was not confirmed in lavender plants yet. For example, *BDH* gene from glandular trichomes of *L. x Intermedia* cDNA library cloned in *E.coli* converted borneol into camphor (Sarker et al., 2012). Also, a BPPS has been identified from lavender plants (Despinasse et al., 2017) showing a potential role for *in vitro* converting of GPP to less BPP and other monoterpenes such as pinenes and camphene. It was assumed that this gene was not strongly expressed, or its mRNA was not very stable *in vitro* (Despinasse et al., 2017).

Screening of other monoterpenes in BPPS-antisense plants showed that 8 of the transgenic plants contained a significant increment of 1,8 cineole between 18.5% - 53% and of limonene ranged

from 22%-206% compared with WT. It is possible that the downregulation of *BPPS* in BPPS-antisense plants favored increasing the level of GPP as a precursor for other monoterpenes such as 1,8 cineole and limonene. It can be speculated that the accumulation of 1,8 cineole in BPPS-antisense plants is due to the accumulation of the GPP pool after the downregulation of BPPS in the transgenic plants. Accordingly, the GPP as the universal precursor of the monoterpenes will be recruited for the biosynthesis of other monoterpenes including 1,8 cineole, limonene, linalool, 3-carene or β-phelandrene (Zhao et al., 2020; Zhou et al., 2016). Our results were in accordance with previous studies showing that GPP as a precursor supply acts as a limiting factor in the biosynthesis of monoterpenes through the plastid MEP pathway. Such competition between enzymes sharing the same limiting substrate (GPP) has already been observed with some transgenic plants. For instance, competition for GPP between 4S-limonene synthase (4S-LS) and those enzymes catalyze the production of sabinene, 1,8-cineole and the ocimene led to altered level of compounds formed directly from GPP in pepermint and cornmint transgenic lines (Diemer et al., 2001).

BPPS-sense plants exhibited a range of phenotypes, and oil yield. Among 9 BPPS-sense plants, 4 showed growth retardation and did not survive in greenhouse. Considering a high level of BPPS expression in these transgenic lines (7-10 times compared with WT), it was assumed that strong expression of BPPS may lead to the conversion of GPP to levels of borneol and camphor that are lethal for transgenic plants. Also, it is possible that the presumably higher level of borneol and camphor in these transgenic lines, caused a dramatically reduced level of GPP for other monoterpenes or substrates such as terpenoid, growth regulators, chlorophylls or carotenoids that are important to plant growth and development (Aharoni et al., 2003; B Markus Lange & Ahkami, 2013). Among the 5 survived transgenic plants, BPPS-S2 and BPPS-S5 showed a significantly higher oil yield (8.1 and 10.2 % compared to WT plants respectively) which was consistent with their high level of BPPS transcript, while BPPS-S3 and BPPS-S4 showed a significant decrease in their oil yield with 9.3 and 7.5 % compared with WT, respectively. BPPS-S1 showed an oil of near-average yield compared with WT. Although BPPS-S1, BPPS-S3 and BPPS-S4, showed higher BPPS expression with 4-6 times transcript level compared with WT, EO yield

was drastically decreased in these transgenic plants. We did not anticipate this result, as overexpression of BPPS is not expected to lower oil yield. However, complications due to somoclonal variation and positional effects of the transgene could affect plant growth and development, and oil yield. In this context, similar results have been previously reported in other plants. For example, although the constitutive overexpression of the spearmint LS gene led to high transcript abundance and enzyme activity in glandular trichomes of transgenic peppermint plants, the oil yield and monoterpene composition were the same as in WT controls (Mahmoud et al., 2004). In another study, the elevated expression levels of spearmint limonene synthase gene (*4SLS*) led to up to 200% oil yield in some transgenic peppermint compared with WT while overexpression of the same transgene in cornmint resulted in similar or lower yield oil than that of WT (Diemer et al., 2001). The reduction in oil yield due to transgene expression could also be due to a decrease in glandular trichome (where oil is produced) numbers (B Markus Lange & Ahkami, 2013). In our case study, evaluating the density and size of glandular trichomes on the leaves of the highest, moderate and lowest yielding transgenic plants would help to investigate the mechanisms controlling EO yield in spike lavender.

Regarding the monoterpene profile of the BPPS-sense plants, we found that changes in the amount of borneol and camphor along with other monoterpenes such as 1,8 cineole and camphor were dependent on the transgenic lines and could not be clearly related to overexpression of *LiBPPS* gene on the variations of these compounds in the transgenic spike lavender lines. Although BPPS-S1 contained near-average EO compared with WT, it showed expected higher borneol and camphor with less 1,8 cineole and limonene compared to WT. BPPS-S1, BPPS-S2 and BPPS-S5 also showed an expected higher level of camphor and borneol with less quantity of limonene and 1,8 cineole compared with WT, suggesting the potential function of *LiBPPS* in regulating borneol and camphor production in spike lavender. It is possible that the introduced *LiBPPS* might improve the availability of BPPS for the steps leading to camphor biosynthesis in this transgenic line. This outcome is in accordance with the accumulation of borneol and camphor increased with the expression level of *LaBPPS* in *L. angustifolia* (Despinasse et al., 2017). The transgenic

lines BPPS-S3 and BPPS-S4 with lower EO yield showed a lower abundance of borneol and camphor compared with WT. Although these two lines contained higher 1,8 cineole than the other 3 transgenic plants, they accumulated lower quantities of 1,8 cineole compared with WT. Limonene, which is a minor monoterpene in EO of WT spike lavender leaves, showed a significant increase (~50 and ~ 88% respectively) in BPPS-S3 and BPPS-S4 compared to WT and other transgenic plants.

The monoterpene abundance results observed in some BPPS-sense lines were unexpected. This could be due to the introduction of a transgene that may not have 100 % homology with the endogenous gene (*LiBPPS*) lead to variation in the monoterpene profile in BPPS-sense plants. Such effects have previously been observed in other studies. For example, Some of the transgenic spike lavender lines overexpressing limonene synthase gene resulted in increased amounts of limonene without alteration the levels of other monoterpenes compared to control (Muñoz-Bertomeu et al., 2008). Also, it was found that overexpression of *Clarkia breweri* linalool synthase gene in spike lavender resulted in an increase in linalool content in leaves without modifying the total content of EO (Mendoza-Poudereux et al., 2014). It was reported that the constitutive expression of *4S-LS* transgene did not lead to altered oil composition (Krasnyanski et al., 1999). Moreover, it was shown that a decrease of total monoterpenes after the introduction of *4S-LS* transgene in two cornmint transgenic lines (Diemer et al., 2001). In a separate study, the attempt to overexpress the endogenous limonene-3-hydroxylase (*L3H*) gene in transgenic peppermint plants was unsuccessful, as none of the transgenics contained elevated levels of L3H activity or increased oil yield. However, cosuppression of *L3H* in peppermint promotes accumulation of limonene (up to 80% of the essential oil compared to about 2% of the oil in wild type plants), without influence on oil yield of the transgenic plants (Mahmoud et al., 2004).

Unexpected changes in monoterpene profile in our transgenic plants could be due to an influence on the availability of GPP as a precursor to other terpene synthases that produce multiple products in spike lavender. Numerous plant terpenoid synthases produce multiple products (Bohlmann et al., 1998). For example, identification, cloning and functional characterization of BPPS from floral-based

glandular trichomes of *L. angustifolia* (Despinasse et al., 2017) showed that LaBPPS produced BPP (30%) and some other terpenes such as pinenes and camphene. Also, the recombinant BPPS from *sage officinalis* produced mainly BPP (75%), but also limonene, camphene, pinene, 1,8 cineole, sabinene and myrcene in the lower level (Wise et al., 1998). The *in vitro* cloned and functionally characterized LiBPPS in our research group showed production of a high amount of borneol (61%) following alkaline phosphatase (AP) treatment and a lower amount of camphene, α-pinene, β-pinene, limonene and terpinolene. Thus, it is assumed that overexpression of LiBPPS in our transgenic spike lavenders altered GPP availability, and led to alteration in the abundance of monoterpenes other than borneol and camphor. Similar results were reported where the expression of spearmint LS in transgenic spike lavender led to the production of limonene as a main product, pinenes and myrcene in developing leaves of the transgenic plant (Muñoz-Bertomeu et al., 2008).

In summary, our results confirm that monoterpene metabolism can be modified through genetic manipulation of terpene synthase expression. In studies reported here, the introduction of sense and antisense versions of *LiBPPS* gene resulted in modifications of borneol and camphor in addition to other monoterpenes profiles in spike lavender. However, interpretation of some results is difficult, in particular since the regulatory mechanisms that control monoterpene production in plans are poorly understood

Chapter 5: Conclusion

Some of the most commercially important lavender species with high essential oil yield (e.g., *L. latifolia* and *L. x intermedia*) produce relatively high amounts of camphor and borneol, which render their EO undesired for use in perfumes and other cosmetics. Metabolic engineering can potentially be used to reduce camphor and borneol content in these plants. However, the relevant genes must be first cloned. One of the genes that can be used to alter borneol and camphor production is *BPPS*.

Recently, a floral-based *BPPS* was identified from *L. x intermedia* in Mahmoud's lab and the identity of this gene was confirmed through *in vitro* functional characterization of the BPPS. Results showed a high *in vitro* catalytic function of LiBPPS in the conversion of GPP to BPP (unpublished) but the function of this gene has not been detected *in planta*. The main goal of this thesis was to examine the *in planta* role of *LiBPPS* gene in camphor biosynthesis through altering its expression in transformed *L. latifolia* plants. Specific objectives were to overexpress *LiBPPS* in sense and antisense in stably transformed *L. latifolia* plants.

The objectives of our study were successfully met through cloning a full-length cDNA-encoding *LiBPPS* in sense and antisense version of BPPS driven by the CaMV 35S promoter separately into the pCambia1390 vector, transforming *Agrobacterium tumefacience* with pCambia1390:: *LiBPPS-antisense* (antisense plasmid) and with pCambia1390:: *LiBPPS-sense* (sense plasmid). The constructs were used to stably transform spike lavender using an *Agrobacterium*-mediated transformation protocol. Finally, we evaluated the transcriptional expression of BPPS *in planta* via molecular analysis, and screened the essential oil of BPPS-sense and BPPS-antisense plants by GC-MS technique.

For plant transformation, we used hygromycin as a selectable marker, and obtained a reasonable success rate of callus induction, shoot regeneration and elongation of the putative transgenic plants which was in line with the previous work (Nebauer et al., 2000). However, we found that regenerated putative BPPS-antisense plants had a higher survival rate than the putative BPPS-sense plants in the greenhouse,

most likely since ectopic expression of BPPS in sense affects plant growth via an unknown mechanism. Results of the molecular analysis by rt-PCR showed that 100% of survived BPPS-sense plants and 77.5% of survived BPPS-antisense plants showed expression of *HygR* gene. Results of normalized BPPS expression via rt-PCR and transcription level of BPPS via q-PCR showed a higher and lower transcriptional expression level of BPPS for all BPPS-sense plants and some of the BPPS-antisense plants, respectively, compared with that of WT. However, some of the BPPS-antisense plants showed a similar BPPS expression compared with WT. These results indicate that that sense and antisense technology can be used as a potential tool for altering the expression of targeted genes in transgenic plants. Our results support the hypothesis that the overexpression of a homologous sense version of *LiBPPS* will lead to increased expression of the gene in some transformed *L. latifolia* plants while the overexpression of a homologous antisense version of *LiBPPS* results in downregulation of *BPPS* in *L. latifolia.* However, some BPPS-antisense plants did not show the expected higher BPPS expression, likely due to positional effects.

EO yield was not substantially altered in most BPPS-antisense and BPPS-sense plants. However, as expected, most of the BPPS-antisense plants showed a reduction of borneol and camphor relative to WT. These results may imply that camphor biosynthesis is preliminary controlled by the transcriptional regulation of *BPPS,* and also that LiBPPS has a function in converting GPP to BPP in spike lavender. Our results parallel those previously reported in other plants (Guitton et al., 2010; Mahmoud & Croteau, 2003; Sarker et al., 2012). From these results, we also found that the decrease in borneol in conjunction with camphor reduction in our BPPS-antisense plants indicates that camphor is produced from consecutive dephosphorylation of BPP and oxidation of borneol in spike lavender. We also observed an expected higher level of camphor and borneol in sense plants compared with WT, confirming the potential function of *LiBPPS* in regulating borneol and camphor production in spike lavender. However, some of the BPPS-sense plants exhibited a decreased level of borneol and camphor despite showing higher BPPS expression. Although we did not anticipate this result, similar results have been previously reported in

other plants (Diemer et al., 2001; Mahmoud et al., 2004). Our results confirm the hypothesis that the overexpression of *LiBPPS* gene leads to increased production of BPP, borneol, and camphor, while downregulation of the gene will result in reduced biosynthesis of these metabolites in spike lavender. However, interpretation of some of EO screening results in our transgenic plants was difficult as the observed monoterpene concentrations and EO yield did not follow expected patterns. Thus, complementary studies such as evaluating the density and size of glandular trichomes on the leaves of the highest, moderate and lowest yielding transgenic plants would help to investigate the mechanisms controlling EO yield in spike lavender. Further studies, for example using Southern Blotting to detect the copy number of the transgene, identification and functional characterization of transcription factors (TF) that interact with the *BPPS* expression gene in spike lavender, and RNA sequencing to identify numerous candidate genes in transgenic plants which show the unique expression patterns. Also, some BPPS-sense lines showed growth retardation and did not survive in the soil in the greenhouse. Thus, we were unable to screen the EO of these lines. It is possible that ectopic expression of BPPS in sense interfered with the normal growth and development of plants. One way to avoid such complications is to use glandular trichome-specific promoters to overexpress or downregulate the BPPS gene in spike lavender. Thus, the target monoterpenes can accumulate in glandular trichomes and no other plant tissues.

In conclusion, our results clearly demonstrate that *LiBPPS* encodes a functional BPPS gene, and that production of camphor and borneol can be controlled through manipulating the expression this gene in lavenders.

Bibliography

Adal, A. M., Sarker, L. S., Lemke, A. D., & Mahmoud, S. S. (2017). Isolation and functional characterization of a methyl jasmonate-responsive 3-carene synthase from Lavandula x intermedia. *Plant Molecular Biology*, *93*(6), 641–657. https://doi.org/10.1007/s11103-017-0588-6

Adal, A. M., Sarker, L. S., Malli, R. P. N., Liang, P., & Mahmoud, S. S. (2019). RNA-Seq in the discovery of a sparsely expressed scent-determining monoterpene synthase in lavender (Lavandula). *Planta*, *249*(1), 271–290. https://doi.org/10.1007/s00425-018-2935-5

Aharoni, A., Giri, A. P., Deuerlein, S., Griepink, F., de Kogel, W.-J., Verstappen, F. W. A., Verhoeven, H. A., Jongsma, M. A., Schwab, W., & Bouwmeester, H. J. (2003). Terpenoid Metabolism in Wild-Type and Transgenic Arabidopsis Plants. *The Plant Cell*, *15*(12), 2866 LP – 2884. https://doi.org/10.1105/tpc.016253

Ait-Ouazzou, A., Cherrat, L., Espina, L., Lorán, S., Rota, C., & Pagán, R. (2011). The antimicrobial activity of hydrophobic essential oil constituents acting alone or in combined processes of food preservation. *Innovative Food Science & Emerging Technologies*, *12*(3), 320–329. https://doi.org/https://doi.org/10.1016/j.ifset.2011.04.004

Altpeter, F., Baisakh, N., Beachy, R., Bock, R., Capell, T., Christou, P., Daniell, H., Datta, K., Datta, S., Dix, P. J., Fauquet, C., Huang, N., Kohli, A., Mooibroek, H., Nicholson, L., Nguyen, T. T., Nugent, G., Raemakers, K., Romano, A., … Visser, R. (2005). Particle bombardment and the genetic enhancement of crops: myths and realities. *Molecular Breeding*, *15*(3), 305–327. https://doi.org/10.1007/s11032-004-8001-y

Alves, A. C., Quecini, V. M., & Vieira, M. L. C. (1999). PLANT TRANSFORMATION: ADVANCES AND PERSPECTIVES . In *Scientia Agricola* (Vol. 56, pp. 1–8). scielo .

Amaral, L. F. B., Camilo, N. S., Pereda, M., Levy, C. E., Moriel, P., & Mazzola, P. G. (2011). Evaluation

of antimicrobial effectiveness of C-8 xylitol monoester as an alternative preservative for cosmetic products. *International Journal of Cosmetic Science*, *33*(5), 391–397.

Angenon, G., Dillen, W., & Van Montagu, M. (1994). Antibiotic resistance markers for plant transformation. In *Plant molecular biology manual* (pp. 125–137). Springer.

Aprotosoaie, A. C., Gille, E., Trifan, A., Luca, V. S., & Miron, A. (2017). Essential oils of Lavandula genus: a systematic review of their chemistry. *Phytochemistry Reviews*, *16*(4), 761–799. https://doi.org/10.1007/s11101-017-9517-1

Attia, S., Lognay, G., Heuskin, S., & Hance, T. (2016). Insecticidal activity of Lavandula angustifolia Mill against the pea aphid Acyrthosiphon pisum. *Journal of Entomology and Zoology Studies*, *4*(1), 118–122.

Baker, J., Brown, K., Rajendiran, E., Yip, A., DeCoffe, D., Dai, C., Molcan, E., Chittick, S. A., Ghosh, S., Mahmoud, S., & Gibson, D. L. (2012). Medicinal lavender modulates the enteric microbiota to protect against Citrobacter rodentium-induced colitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *303*(7), G825–G836. https://doi.org/10.1152/ajpgi.00327.2011

Beltrán, J., Jaimes, H., Echeverry, M., Ladino, Y., López, D., Duque, M. C., Chavarriaga, P., & Tohme, J. (2009). Quantitative analysis of transgenes in cassava plants using real-time PCR technology. *In Vitro Cellular & Developmental Biology - Plant*, *45*(1), 48–56. https://doi.org/10.1007/s11627-008-9159-5

Benabdelkader, T., Guitton, Y., Pasquier, B., Magnard, J. L., Jullien, F., Kameli, A., & Legendre, L. (2015). Functional characterization of terpene synthases and chemotypic variation in three lavender species of section Stoechas. *Physiologia Plantarum*, *153*(1), 43–57. https://doi.org/https://doi.org/10.1111/ppl.12241

Birch, R. G. (1997). PLANT TRANSFORMATION: Problems and Strategies for Practical Application. *Annual Review of Plant Physiology and Plant Molecular Biology*, *48*(1), 297–326. https://doi.org/10.1146/annurev.arplant.48.1.297

Bird, C. R., & Ray, J. A. (1991). Manipulation of Plant Gene Expression by Antisense RNA. *Biotechnology and Genetic Engineering Reviews*, *9*(1), 207–228. https://doi.org/10.1080/02648725.1991.10647881

Blažeković, B., Vladimir-Knežević, S., Brantner, A., & Štefan, M. B. (2010). Evaluation of Antioxidant Potential of Lavandula x intermedia Emeric ex Loisel. "Budrovka": A Comparative Study with L. angustifolia Mill. In *Molecules* (Vol. 15, Issue 9). https://doi.org/10.3390/molecules15095971

Boeckelmann, A. (2008). *Monoterpene production and regulation in lavenders (Lavandula angustifolia and Lavandula x intermedia)*. University of British Columbia.

Bohlmann, J., Meyer-Gauen, G., & Croteau, R. (1998). Plant terpenoid synthases: molecular biology and phylogenetic analysis. *Proceedings of the National Academy of Sciences*, *95*(8), 4126–4133.

Boyle, A. D. H. D. G., Olsen, V., Boyle, D. B., Berger, L., Obendorf, D., Dalton, A., Kriger, K., Hero, M., Hines, H., & Phillott, R. (2007). Diagnostic assays and sampling protocols for the detection of Batrachochytrium dendrobatidis. *Diseases of Aquatic Organisms*, *73*(3), 175–192.

Burbott, A. J., & Loomis, W. D. (1967). Effects of Light and Temperature on the Monoterpenes of Peppermint. *Plant Physiology*, *42*(1), 20 LP – 28. https://doi.org/10.1104/pp.42.1.20

Calvo, M C, Jordan, A., & Segura, J. (1988). Plant regeneration from isolated cells ofLavandula latifolia medicus. *In Vitro Cellular & Developmental Biology*, *24*(9), 943–946.

Calvo, M C, & Segura, J. (1988). In vitro morphogenesis from explants of Lavandula latifolia and Lavandula stoechas Seedlings. *Scientia Horticulturae*, *36*(1), 131–137.

https://doi.org/https://doi.org/10.1016/0304-4238(88)90016-7

Calvo, Maria Carmen, & Segura, J. (1989). In vitro propagation of lavender. *HortScience*, *24*(2), 375–376.

Cannon, M., Platz, J., O'Leary, M., Sookdeo, C., & Cannon, F. (1990). Organ-specific modulation of gene expression in transgenic plants using antisense RNA. *Plant Molecular Biology*, *15*(1), 39–47. https://doi.org/10.1007/BF00017722

Cavanagh, H. tempspacetempspaceM tempspacetempspaceA, & Wilkinson, J. tempspacetempspaceM. (2002). Biological activities of lavender essential oil. *Phytotherapy Research*, *16*(4), 301–308.

Chakrabarty, R., Viswakarma, N., Bhat, S. R., Kirti, P. B., Singh, B. D., & Chopra, V. L. (2002). *Agrobacterium*-mediated transformation of cauliflower: Optimization of protocol and development of Bt-transgenic cauliflower. *Journal of Biosciences*, *27*(5), 495–502. https://doi.org/10.1007/BF02705046

Chamorro, E. R. (2012). *Study of the Chemical Composition of Essential Oils by Gas Chromatography* (S. N. Zambón (ed.); p. Ch. 15). IntechOpen. https://doi.org/10.5772/33201

Chen, F., Tholl, D., Bohlmann, J., & Pichersky, E. (2011). The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. *The Plant Journal*, *66*(1), 212–229.

Chen, W., Vermaak, I., & Viljoen, A. (2013). Camphor—a fumigant during the black death and a coveted fragrant wood in ancient Egypt and Babylon—a review. *Molecules*, *18*(5), 5434–5454.

Chen, X., Chen, H., Yuan, J. S., Köllner, T. G., Chen, Y., Guo, Y., Zhuang, X., Chen, X., Zhang, Y., Fu, J., Nebenführ, A., Guo, Z., & Chen, F. (2018). The rice terpene synthase gene OsTPS19 functions as an (S)-limonene synthase in planta, and its overexpression leads to enhanced resistance to the blast

fungus Magnaporthe oryzae. *Plant Biotechnology Journal*, *16*(10), 1778–1787. https://doi.org/https://doi.org/10.1111/pbi.12914

Chou, T. W., Biswas, S., & Lu, S. (2004). Gene delivery using physical methods: an overview. *Methods in Molecular Biology (Clifton, N.J.)*, *245*, 147–166. https://doi.org/10.1385/1-59259-649-5:147

Christianson, D. W. (2006). Structural biology and chemistry of the terpenoid cyclases. *Chemical Reviews*, *106*(8), 3412–3442.

Clark, R. J., & Menary, R. C. (1980). Environmental Effects on Peppermint (<I>Mentha piperita</I> L.). II. Effects of Temperature on Photosynthesis, Photorespiration and Dark Respiration in Peppermint With Reference to Oil Composition. *Functional Plant Biology*, *7*(6), 693–697. https://doi.org/10.1071/PP9800693

Crossway, A., Oakes, J. V, Irvine, J. M., Ward, B., Knauf, V. C., & Shewmaker, C. K. (1986). Integration of foreign DNA following microinjection of tobacco mesophyll protoplasts. *Molecular and General Genetics MGG*, *202*(2), 179–185. https://doi.org/10.1007/BF00331634

Croteau, R., Felton, M., Karp, F., & Kjonaas, R. (1981). Relationship of Camphor Biosynthesis to Leaf Development in Sage (Salvia officinalis). *Plant Physiology*, *67*(4), 820 LP – 824. https://doi.org/10.1104/pp.67.4.820

Croteau, R., Kutchan, T. M., & Lewis, N. G. (2000). Natural products (secondary metabolites). *Biochemistry and Molecular Biology of Plants*, *24*, 1250–1319.

Croteau, R., Lee Hooper, C., & Felton, M. (1978). Biosynthesis of monoterpenes: Partial purification and characterization of a bicyclic monoterpenol dehydrogenase from sage (Salvia officinalis). *Archives of Biochemistry and Biophysics*, *188*(1), 182–193. https://doi.org/https://doi.org/10.1016/0003-9861(78)90371-5

de Ruijter, N. C. A., Verhees, J., van Leeuwen, W., & van der Krol, A. R. (2003). Evaluation and Comparison of the GUS, LUC and GFP Reporter System for Gene Expression Studies in Plants. *Plant Biology*, *5*(2), 103–115. https://doi.org/https://doi.org/10.1055/s-2003-40722

Debnath, M., Malik, C. P., & Bisen, P. S. (2006). Micropropagation: a tool for the production of high quality plant-based medicines. *Current Pharmaceutical Biotechnology*, *7*(1), 33–49. http://www.ncbi.nlm.nih.gov/pubmed/16472132

Degenhardt, J., Köllner, T. G., & Gershenzon, J. (2009). Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. *Phytochemistry*, *70*(15–16), 1621–1637.

Demissie, Z. A., Cella, M. A., Sarker, L. S., Thompson, T. J., Rheault, M. R., & Mahmoud, S. S. (2012). Cloning, functional characterization and genomic organization of 1,8-cineole synthases from Lavandula. *Plant Molecular Biology*, *79*(4–5), 393–411.

Demissie, Z. A., Erland, L. A. E., Rheault, M. R., & Mahmoud, S. S. (2013). The biosynthetic origin of irregular monoterpenes in Lavandula: isolation and biochemical characterization of a novel cis-prenyl diphosphate synthase gene, lavandulyl diphosphate synthase. *The Journal of Biological Chemistry*, *288*(9), 6333–6341. https://doi.org/10.1074/jbc.M112.431171

Desautels, A., Biswas, K., Lane, A., Boeckelmann, A., & Mahmoud, S. S. (2009). Suppression of Linalool Acetate Production in Lavandula x intermedia. *Natural Product Communications*, *4*(11), 1934578X0900401115. https://doi.org/10.1177/1934578X0900401115

Despinasse, Y., Fiorucci, S., Antonczak, S., Moja, S., Bony, A., Nicolè, F., Baudino, S., Magnard, J.-L., & Jullien, F. (2017). Bornyl-diphosphate synthase from Lavandula angustifolia: A major monoterpene synthase involved in essential oil quality. *Phytochemistry*, *137*, 24–33. https://doi.org/https://doi.org/10.1016/j.phytochem.2017.01.015

Diemer, F., Caissard, J.-C., Moja, S., Chalchat, J.-C., & Jullien, F. (2001). Altered monoterpene

composition in transgenic mint following the introduction of 4S-limonene synthase. *Plant Physiology and Biochemistry*, *39*(7), 603–614. https://doi.org/https://doi.org/10.1016/S0981-9428(01)01273-6

Djenane, D., Aïder, M., Yangüela, J., Idir, L., Gómez, D., & Roncalés, P. (2012). Antioxidant and antibacterial effects of Lavandula and Mentha essential oils in minced beef inoculated with E. coli O157: H7 and S. aureus during storage at abuse refrigeration temperature. *Meat Science*, *92*(4), 667–674.

Dronne, S., Colson, M., Moja, S., & Faure, O. (1998). Plant regeneration and transient GUS expression in a range of lavandin (Lavandula × intermedia Emeric ex Loiseleur) cultivars. *Plant Cell, Tissue and Organ Culture*, *55*(3), 193–198. https://doi.org/10.1023/A:1006272520475

Dudareva, N, Cseke, L., Blanc, V. M., & Pichersky, E. (1996). Evolution of floral scent in Clarkia: novel patterns of S-linalool synthase gene expression in the C. breweri flower. *The Plant Cell*, *8*(7), 1137 LP – 1148. https://doi.org/10.1105/tpc.8.7.1137

Dudareva, Natalia, Martin, D., Kish, C. M., Kolosova, N., Gorenstein, N., Fäldt, J., Miller, B., & Bohlmann, J. (2003). (E)-β-Ocimene and Myrcene Synthase Genes of Floral Scent Biosynthesis in Snapdragon: Function and Expression of Three Terpene Synthase Genes of a New Terpene Synthase Subfamily. *The Plant Cell*, *15*(5), 1227 LP – 1241. https://doi.org/10.1105/tpc.011015

Dušková, E., Dušek, K., Indrák, P., & Smékalová, K. (2016). Postharvest changes in essential oil content and quality of lavender flowers. *Industrial Crops and Products*, *79*, 225–231. https://doi.org/https://doi.org/10.1016/j.indcrop.2015.11.007

Endo, S., Iwamoto, K., & Fukuda, H. (2018). Overexpression and cosuppression of xylem-related genes in an early xylem differentiation stage-specific manner by the AtTED4 promoter. *Plant*

Biotechnology Journal, *16*(2), 451–458. https://doi.org/https://doi.org/10.1111/pbi.12784

Erland, Lauren A E, & Mahmoud, S. S. (2014). An efficient method for regeneration of lavandin (Lavandula x intermedia cv. 'Grosso'). *In Vitro Cellular & Developmental Biology - Plant*, *50*(5), 646–654. https://doi.org/10.1007/s11627-014-9614-4

Erland, Lauren Alexandra Elizabeth. (2015). *Enhancement of specialized metabolism, regeneration efficiency and biological activity in lavandin (Lavandula x intermedia cv 'Grosso')*. https://open.library.ubc.ca/collections/24/items/1.0074431

Falk, L., Biswas, K., Boeckelmann, A., Lane, A., & Mahmoud, S. S. (2009). An Effcient Method for the Micropropagation of Lavenders: Regeneration of a Unique Mutant. *Journal of Essential Oil Research*, *21*(3), 225–228. https://doi.org/10.1080/10412905.2009.9700154

Fransz, P. F., & Schel, J. H. N. (1994). *Ultrastructural Studies on Callus Development and Somatic Embryogenesis in Zea mays L BT - Maize* (Y. P. S. Bajaj (ed.); pp. 50–65). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-57968-4_4

Froger, A., & Hall, J. E. (2007). Transformation of plasmid DNA into E. coli using the heat shock method. *Journal of Visualized Experiments : JoVE*, *6*, 253. https://doi.org/10.3791/253

Gelvin, S. B. (2003). Agrobacterium-Mediated Plant Transformation: the Biology behind the "Gene-Jockeying" Tool. *Microbiology and Molecular Biology Reviews*, *67*(1), 16 LP – 37. https://doi.org/10.1128/MMBR.67.1.16-37.2003

George, E. F., Hall, M. A., & Klerk, G.-J. De. (2008a). *Micropropagation: Uses and Methods BT - Plant Propagation by Tissue Culture: Volume 1. The Background* (E. F. George, M. A. Hall, & G.-J. De Klerk (eds.); pp. 29–64). Springer Netherlands. https://doi.org/10.1007/978-1-4020-5005-3_2

George, E. F., Hall, M. A., & Klerk, G.-J. De. (2008b). *The Components of Plant Tissue Culture Media*

II: Organic Additions, Osmotic and pH Effects, and Support Systems BT - Plant Propagation by Tissue Culture: Volume 1. The Background (E. F. George, M. A. Hall, & G.-J. De Klerk (eds.); pp. 115–173). Springer Netherlands. https://doi.org/10.1007/978-1-4020-5005-3_4

Germinara, G. S., Di Stefano, M. G., De Acutis, L., Pati, S., Delfine, S., De Cristofaro, A., & Rotundo, G. (2017). Bioactivities of Lavandula angustifolia essential oil against the stored grain pest Sitophilus granarius. *Bulletin of Insectology*, *70*(1), 129–138.

Gershenzon, J., McConkey, M. E., & Croteau, R. B. (2000). Regulation of monoterpene accumulation in leaves of peppermint. *Plant Physiology*, *122*(1), 205–214.

Glas, J. J., Schimmel, B. C. J., Alba, J. M., Escobar-Bravo, R., Schuurink, R. C., & Kant, M. R. (2012). Plant glandular trichomes as targets for breeding or engineering of resistance to herbivores. *International Journal of Molecular Sciences*, *13*(12), 17077–17103. https://doi.org/10.3390/ijms131217077

Gonçalves, S., & Romano, A. (2013). In vitro culture of lavenders (Lavandula spp.) and the production of secondary metabolites. *Biotechnology Advances*, *31*(2), 166–174. https://doi.org/https://doi.org/10.1016/j.biotechadv.2012.09.006

Green, P. J., Pines, O., & Inouye, M. (1986). THE ROLE OF ANTISENSE RNA IN GENE REGULATION. *Annual Review of Biochemistry*, *55*(1), 569–597. https://doi.org/10.1146/annurev.bi.55.070186.003033

Guitton, Y., Nicolè, F., Moja, S., Valot, N., Legrand, S., Jullien, F., & Legendre, L. (2010). Differential accumulation of volatile terpene and terpene synthase mRNAs during lavender (Lavandula angustifolia and L. x intermedia) inflorescence development. *Physiologia Plantarum*, *138*(2), 150–163. https://doi.org/https://doi.org/10.1111/j.1399-3054.2009.01315.x

Guo, D., Kang, K., Wang, P., Li, M., & Huang, X. (2020). Transcriptome profiling of spike provides

expression features of genes related to terpene biosynthesis in lavender. *Scientific Reports*, *10*(1), 6933. https://doi.org/10.1038/s41598-020-63950-4

Gutensohn, M., Nagegowda, D. A., & Dudareva, N. (2013). *Involvement of Compartmentalization in Monoterpene and Sesquiterpene Biosynthesis in Plants BT - Isoprenoid Synthesis in Plants and Microorganisms: New Concepts and Experimental Approaches* (T. J. Bach & M. Rohmer (eds.); pp. 155–169). Springer New York. https://doi.org/10.1007/978-1-4614-4063-5_11

Hampel, D., Mosandl, A., & Wüst, M. (2005). Biosynthesis of mono-and sesquiterpenes in carrot roots and leaves (Daucus carota L.): Metabolic cross talk of cytosolic mevalonate and plastidial methylerythritol phosphate pathways. *Phytochemistry*, *66*(3), 305–311.

Hassiotis, C. N., Ntana, F., Lazari, D. M., Poulios, S., & Vlachonasios, K. E. (2014). Environmental and developmental factors affect essential oil production and quality of Lavandula angustifolia during flowering period. *Industrial Crops and Products*, *62*, 359–366. https://doi.org/https://doi.org/10.1016/j.indcrop.2014.08.048

Hritcu, L., Cioanca, O., & Hancianu, M. (2012). Effects of lavender oil inhalation on improving scopolamine-induced spatial memory impairment in laboratory rats. *Phytomedicine*, *19*(6), 529–534. https://doi.org/https://doi.org/10.1016/j.phymed.2012.02.002

Hu, C.-Y., & Zanettini, M. H. B. (1995). *Embryo Culture and Embryo Rescue for Wide Cross Hybrids BT - Plant Cell, Tissue and Organ Culture: Fundamental Methods* (O. L. Gamborg & G. C. Phillips (eds.); pp. 129–141). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-79048-5_11

Hwang, H.-H., Yu, M., & Lai, E.-M. (2017). Agrobacterium-mediated plant transformation: biology and applications. *The Arabidopsis Book*, *15*, e0186–e0186. https://doi.org/10.1199/tab.0186

Jameson, P. E., & Song, J. (2016). Cytokinin: a key driver of seed yield. *Journal of Experimental Botany*, *67*(3), 593–606. https://doi.org/10.1093/jxb/erv461

Jones, M. P. A., Yi, Z., Murch, S. J., & Saxena, P. K. (2007). Thidiazuron-induced regeneration of Echinacea purpurea L.: micropropagation in solid and liquid culture systems. *Plant Cell Reports*, *26*(1), 13–19. https://doi.org/10.1007/s00299-006-0209-3

Jullien, F., Moja, S., Bony, A., Legrand, S., Petit, C., Benabdelkader, T., Poirot, K., Fiorucci, S., Guitton, Y., Nicolè, F., Baudino, S., & Magnard, J.-L. (2014). Isolation and functional characterization of a τ-cadinol synthase, a new sesquiterpene synthase from Lavandula angustifolia. *Plant Molecular Biology*, *84*(1), 227–241. https://doi.org/10.1007/s11103-013-0131-3

Jyothishwaran, G., Kotresha, D., Selvaraj, T., Srideshikan, S. M., Rajvanshi, P. K., & Jayabaskaran, C. (2007). A modified freeze–thaw method for efficient transformation of Agrobacterium tumefaciens. *Current Science*, *93*(6), 770–772. http://www.jstor.org/stable/24099118

Kara, N., & Baydar, H. (2012). Effects of Different Explant Sources on Micropropagation in Lavender (Lavandula sp.). *Journal of Essential Oil Bearing Plants*, *15*(2), 250–255. https://doi.org/10.1080/0972060X.2012.10644043

Koulivand, P. H., Khaleghi Ghadiri, M., & Gorji, A. (2013). Lavender and the nervous system. *Evidence-Based Complementary and Alternative Medicine : ECAM*, *2013*, 681304. https://doi.org/10.1155/2013/681304

Krasnyanski, S., May, R. A., Loskutov, A., Ball, T. M., & Sink, K. C. (1999). Transformation of the limonene synthase gene into peppermint (Mentha piperita L.) and preliminary studies on the essential oil profiles of single transgenic plants. *Theoretical and Applied Genetics*, *99*(3), 676–682. https://doi.org/10.1007/s001220051284

Kumar, S., & Fladung, M. (2001). Gene stability in transgenic aspen (Populus). II. Molecular characterization of variable expression of transgene in wild and hybrid aspen. *Planta*, *213*(5), 731–740. https://doi.org/10.1007/s004250100535

Landmann, C., Fink, B., Festner, M., Dregus, M., Engel, K.-H., & Schwab, W. (2007). Cloning and functional characterization of three terpene synthases from lavender (Lavandula angustifolia). *Archives of Biochemistry and Biophysics*, *465*(2), 417–429. https://doi.org/https://doi.org/10.1016/j.abb.2007.06.011

Lane, A., Boecklemann, A., Woronuk, G. N., Sarker, L., & Mahmoud, S. S. (2010). A genomics resource for investigating regulation of essential oil production in Lavandula angustifolia. *Planta*, *231*(4), 835–845.

Lane, W. A., & Mahmoud, S. S. (2008). Composition of Essential Oil from Lavandula Angustifolia and L. Intermedia Varieties Grown in British Columbia, Canada. *Natural Product Communications*, *3*(8), 1934578X0800300822. https://doi.org/10.1177/1934578X0800300822

Lange, B Markus, & Ahkami, A. (2013). Metabolic engineering of plant monoterpenes, sesquiterpenes and diterpenes—current status and future opportunities. *Plant Biotechnology Journal*, *11*(2), 169–196.

Lange, Bernd Markus, Mahmoud, S. S., Wildung, M. R., Turner, G. W., Davis, E. M., Lange, I., Baker, R. C., Boydston, R. A., & Croteau, R. B. (2011). Improving peppermint essential oil yield and composition by metabolic engineering. *Proceedings of the National Academy of Sciences*, *108*(41), 16944 LP – 16949. https://doi.org/10.1073/pnas.1111558108

Laule, O., Fürholz, A., Chang, H.-S., Zhu, T., Wang, X., Heifetz, P. B., Gruissem, W., & Lange, M. (2003). Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences*, *100*(11), 6866–6871.

Lesage-Meessen, L., Bou, M., Sigoillot, J. C., Faulds, C. B., & Lomascolo, A. (2015). Essential oils and distilled straws of lavender and lavandin: a review of current use and potential application in white biotechnology. *Applied Microbiology and Biotechnology*, *99*(8), 3375–3385.

https://doi.org/10.1007/s00253-015-6511-7

Li, C., Sarangapani, S., Wang, Q., Nadimuthu, K., & Sarojam, R. (2020). Metabolic Engineering of the Native Monoterpene Pathway in Spearmint for Production of Heterologous Monoterpenes Reveals Complex Metabolism and Pathway Interactions. In *International Journal of Molecular Sciences* (Vol. 21, Issue 17). https://doi.org/10.3390/ijms21176164

Li, S., Cong, Y., Liu, Y., Wang, T., Shuai, Q., Chen, N., Gai, J., & Li, Y. (2017). Optimization of Agrobacterium-Mediated Transformation in Soybean . In *Frontiers in Plant Science* (Vol. 8, p. 246). https://www.frontiersin.org/article/10.3389/fpls.2017.00246

Lis-Balchin, M. (2002). Chemical composition of essential oils from different species, hybrids and cultivars of Lavandula. *Lavender: The Genus Lavandula. Taylor & Francis, London*, 251–262.

Liu, Y., Wang, H., YE, H., & LI, G. (2005). Advances in the plant isoprenoid biosynthesis pathway and its metabolic engineering. *Journal of Integrative Plant Biology*, *47*(7), 769–782.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Maffei, M., & Codignola, A. (1990). Photosynthesis, Photorespiration and Herbicide Effect on Terpene Production in Peppermint (Mentha piperita L.). *Journal of Essential Oil Research*, *2*(6), 275–286. https://doi.org/10.1080/10412905.1990.9697886

Mahmoud, S. S., & Croteau, R. B. (2001). Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. *Proceedings of the National Academy of Sciences*, *98*(15), 8915 LP – 8920. https://doi.org/10.1073/pnas.141237298

Mahmoud, S. S., & Croteau, R. B. (2002). Strategies for transgenic manipulation of monoterpene biosynthesis in plants. *Trends in Plant Science*, *7*(8), 366–373. https://doi.org/https://doi.org/10.1016/S1360-1385(02)02303-8

Mahmoud, S. S., & Croteau, R. B. (2003). Menthofuran regulates essential oil biosynthesis in peppermint by controlling a downstream monoterpene reductase. *Proceedings of the National Academy of Sciences*, *100*(24), 14481–14486.

Mahmoud, S. S., Williams, M., & Croteau, R. (2004). Cosuppression of limonene-3-hydroxylase in peppermint promotes accumulation of limonene in the essential oil. *Phytochemistry*, *65*(5), 547–554. https://doi.org/https://doi.org/10.1016/j.phytochem.2004.01.005

Markus Lange, B., & Turner, G. W. (2013). Terpenoid biosynthesis in trichomes—current status and future opportunities. *Plant Biotechnology Journal*, *11*(1), 2–22. https://doi.org/https://doi.org/10.1111/j.1467-7652.2012.00737.x

Matzke, M A, & Matzke, A. J. (1998). Epigenetic silencing of plant transgenes as a consequence of diverse cellular defence responses. *Cellular and Molecular Life Sciences : CMLS*, *54*(1), 94–103. https://doi.org/10.1007/s000180050128

Matzke, Marjori A, Aufsatz, W., Kanno, T., Mette, M. F., & Matzke, A. J. M. (2002). Homology-dependent gene silencing and host defense in plants. *Advances in Genetics*, *46*, 235–275. https://doi.org/10.1016/s0065-2660(02)46009-9

Mayrhofer, S., Teuber, M., Zimmer, I., Louis, S., Fischbach, R. J., & Schnitzler, J.-P. (2005). Diurnal and Seasonal Variation of Isoprene Biosynthesis-Related Genes in Grey Poplar Leaves. *Plant Physiology*, *139*(1), 474 LP – 484. https://doi.org/10.1104/pp.105.066373

McConkey, M. E., Gershenzon, J., & Croteau, R. B. (2000). Developmental Regulation of Monoterpene Biosynthesis in the Glandular Trichomes of Peppermint. *Plant Physiology*, *122*(1), 215 LP – 224.

https://doi.org/10.1104/pp.122.1.215

Mendoza-Poudereux, I., Kutzner, E., Huber, C., Segura, J., Arrillaga, I., & Eisenreich, W. (2017). Dynamics of monoterpene formation in spike lavender plants. *Metabolites*, *7*(4), 65.

Mendoza-Poudereux, I., Muñoz-Bertomeu, J., Navarro, A., Arrillaga, I., & Segura, J. (2014). Enhanced levels of S-linalool by metabolic engineering of the terpenoid pathway in spike lavender leaves. *Metabolic Engineering*, *23*, 136–144. https://doi.org/https://doi.org/10.1016/j.ymben.2014.03.003

Mishiba, K.-I., Ishikawa, K., Tsujii, O., & Mii, M. (2000). Efficient transformation of lavender (Lavandula latifolia Medicus) mediated by Agrobacterium. *The Journal of Horticultural Science and Biotechnology*, *75*(3), 287–292. https://doi.org/10.1080/14620316.2000.11511239

Moon, T., Cavanagh, H. M. A., & Wilkinson, J. M. (2007). Antifungal Activity of Australian Grown Lavandula spp. Essential Oils Against Aspergillus nidulans, Trichophyton mentagrophytes, Leptosphaeria maculans and Sclerotinia sclerotiorum. *Journal of Essential Oil Research*, *19*(2), 171–175. https://doi.org/10.1080/10412905.2007.9699253

Muñoz-Bertomeu, J., Arrillaga, I., Ros, R., & Segura, J. (2006). Up-regulation of 1-deoxy-D-xylulose-5-phosphate synthase enhances production of essential oils in transgenic spike lavender. *Plant Physiology*, *142*(3), 890–900.

Muñoz-Bertomeu, J., Arrillaga, I., & Segura, J. (2007). Essential oil variation within and among natural populations of Lavandula latifolia and its relation to their ecological areas. *Biochemical Systematics and Ecology*, *35*(8), 479–488. https://doi.org/https://doi.org/10.1016/j.bse.2007.03.006

Muñoz-Bertomeu, J., Ros, R., Arrillaga, I., & Segura, J. (2008). Expression of spearmint limonene synthase in transgenic spike lavender results in an altered monoterpene composition in developing leaves. *Metabolic Engineering*, *10*(3), 166–177. https://doi.org/https://doi.org/10.1016/j.ymben.2008.04.002

Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum*, *15*(3), 473–497. https://doi.org/https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nabors, M. W., Daniels, A., Nadolny, L., & Brown, C. (1975). Sodium chloride tolerant lines of tobacco cells. *Plant Science Letters*, *4*(3), 155–159. https://doi.org/https://doi.org/10.1016/0304-4211(75)90089-9

Nebauer, S. G., Arrillaga, I., del Castillo-Agudo, L., & Segura, J. (2000). Agrobacterium tumefaciens-mediated transformation of the aromatic shrub Lavandula latifolia. *Molecular Breeding*, *6*(6), 539–552. https://doi.org/10.1023/A:1011332904024

Okamoto, Y., Yamaji, K., & Kobayashi, K. (2011). Allelopathic activity of camphor released from camphor tree (Cinnamomum camphora). *Allelopathy Journal*, *27*(1).

Oseni, O. M., Pande, V., & Nailwal, T. K. (2018). A Review on Plant Tissue Culture, A Technique for Propagation and Conservation of Endangered Plant Species. *International Journal of Current Microbiology and Applied Sciences*, *7*(07), 3778–3786. https://doi.org/10.20546/ijcmas.2018.707.438

Peana, A. T., D'Aquila, P. S., Panin, F., Serra, G., Pippia, P., & Moretti, M. D. L. (2002). Anti-inflammatory activity of linalool and linalyl acetate constituents of essential oils. *Phytomedicine*, *9*(8), 721–726.

Pierik, R. L. M. (1988). * IN VITRO CULTURE OF HIGHER PLANTS AS A TOOL IN THE PROPAGATION OF HORTICULTURAL CROPS. *Acta Horticulturae*, *226*, 25–40. https://doi.org/10.17660/ActaHortic.1988.226.1

Polichuk, D. R., Zhang, Y., Reed, D. W., Schmidt, J. F., & Covello, P. S. (2010). A glandular trichome-specific monoterpene alcohol dehydrogenase from Artemisia annua. *Phytochemistry*, *71*(11), 1264–

1269. https://doi.org/https://doi.org/10.1016/j.phytochem.2010.04.026

Quazi, M. H. (1980). In vitro multiplication of Lavandula spp. *Annals of Botany*, *45*(3), 361–362.

Reed, S. M. (1996). *DNA amplification in doubled haploids of Nicotiana BT - In Vitro Haploid Production in Higher Plants: Volume 2 — Applications* (S. M. Jain, S. K. Sopory, & R. E. Veilleux (eds.); pp. 111–125). Springer Netherlands. https://doi.org/10.1007/978-94-017-0477-9_5

Rodermel, S. R., Abbott, M. S., & Bogorad, L. (1988). Nuclear-organelle interactions: Nuclear antisense gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. *Cell*, *55*(4), 673–681. https://doi.org/https://doi.org/10.1016/0092-8674(88)90226-7

Rothstein, S. J., DiMaio, J., Strand, M., & Rice, D. (1987). Stable and heritable inhibition of the expression of nopaline synthase in tobacco expressing antisense RNA. *Proceedings of the National Academy of Sciences*, *84*(23), 8439 LP – 8443. https://doi.org/10.1073/pnas.84.23.8439

Rozman, V., Kalinovic, I., & Korunic, Z. (2007). Toxicity of naturally occurring compounds of Lamiaceae and Lauraceae to three stored-product insects. *Journal of Stored Products Research*, *43*(4), 349–355. https://doi.org/https://doi.org/10.1016/j.jspr.2006.09.001

Salido, S., Altarejos, J., Nogueras, M., Sánchez, A., & Luque, P. (2004). Chemical Composition and Seasonal Variations of Spike Lavender Oil from Southern Spain. *Journal of Essential Oil Research*, *16*(3), 206–210. https://doi.org/10.1080/10412905.2004.9698698

Sánchez-Gras, M. C., & del Carmen Calvo, M. (1996). Micropropagation of Lavandula latifolia through nodal bud culture of mature plants. *Plant Cell, Tissue and Organ Culture*, *45*(3), 259–261. https://doi.org/10.1007/BF00043639

Sandler, S. J., Stayton, M., Townsend, J. A., Ralston, M. L., Bedbrook, J. R., & Dunsmuir, P. (1988). Inhibition of gene expression in transformed plants by antisense RNA. *Plant Molecular Biology*,

11(3), 301–310. https://doi.org/10.1007/BF00027387

Sangwan, N. S., Farooqi, A. H. A., Shabih, F., & Sangwan, R. S. (2001). Regulation of essential oil production in plants. *Plant Growth Regulation*, *34*(1), 3–21. https://doi.org/10.1023/A:1013386921596

Santos Tozin, L. R. dos, de Melo Silva, S. C., & Rodrigues, T. M. (2016). Non-glandular trichomes in Lamiaceae and Verbenaceae species: morphological and histochemical features indicate more than physical protection. *New Zealand Journal of Botany*, *54*(4), 446–457. https://doi.org/10.1080/0028825X.2016.1205107

Sarker, L. S., Demissie, Z. A., & Mahmoud, S. S. (2013). Cloning of a sesquiterpene synthase from Lavandula x intermedia glandular trichomes. *Planta*, *238*(5), 983–989. https://doi.org/10.1007/s00425-013-1937-6

Sarker, L. S., Galata, M., Demissie, Z. A., & Mahmoud, S. S. (2012). Molecular cloning and functional characterization of borneol dehydrogenase from the glandular trichomes of Lavandula x intermedia. *Archives of Biochemistry and Biophysics*, *528*(2), 163–170. https://doi.org/https://doi.org/10.1016/j.abb.2012.09.013

Segura, J, & Calvo, M. C. (1991). *Lavandula spp. (Lavender): In Vitro Culture, Regeneration of Plants, and the Formation of Essential Oils and Pigments BT - Medicinal and Aromatic Plants III* (Y. P. S. Bajaj (ed.); pp. 283–310). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-84071-5_18

Segura, Juan, Muñoz-Bertomeu, J., Mendoza-Poudereux, I., & Arrillaga, I. (2019). *Biotechnological Approaches to Increase Essential Oil Yield and Quality in Aromatic Plants: The Lavandula latifolia (Spike Lavender) Example. Past and Recommendations for the Future BT - Essential Oil Research: Trends in Biosynthesis, Analytics, Industri* (S. Malik (ed.); pp. 301–325). Springer International

Publishing. https://doi.org/10.1007/978-3-030-16546-8_11

Sharafati Chaleshtori, R., Rokni, N., Rafieian-Kopaei, M., Deris, F., & Salehi, E. (2015). Antioxidant and antibacterial activity of basil (Ocimum basilicum L.) essential oil in beef burger. *Journal of Agricultural Science and Technology*, *17*(4), 817–826.

Sharif, I., Farooq, J., Chohan, S. M., Saleem, S., Kainth, R. A., Mahmood, A., & Sarwar, G. (2019). Strategies to enhance cottonseed oil contents and reshape fatty acid profile employing different breeding and genetic engineering approaches. *Journal of Integrative Agriculture*, *18*(10), 2205–2218. https://doi.org/https://doi.org/10.1016/S2095-3119(18)62139-2

Sharifi-Rad, J., Sureda, A., Tenore, G. C., Daglia, M., Sharifi-Rad, M., Valussi, M., Tundis, R., Sharifi-Rad, M., Loizzo, M. R., Ademiluyi, A. O., Sharifi-Rad, R., Ayatollahi, S. A., & Iriti, M. (2017). Biological Activities of Essential Oils: From Plant Chemoecology to Traditional Healing Systems. *Molecules (Basel, Switzerland)*, *22*(1), 70. https://doi.org/10.3390/molecules22010070

Sharkey, T. D., & Yeh, S. (2001). ISOPRENE EMISSION FROM PLANTS. *Annual Review of Plant Physiology and Plant Molecular Biology*, *52*(1), 407–436. https://doi.org/10.1146/annurev.arplant.52.1.407

Sheng, J., & Citovsky, V. (1996). Agrobacterium-plant cell DNA transport: have virulence proteins, will travel. *The Plant Cell*, *8*(10), 1699–1710. https://doi.org/10.1105/tpc.8.10.1699

Singsaas, E. L., Lerdau, M., Winter, K., & Sharkey, T. D. (1997). Isoprene Increases Thermotolerance of Isoprene-Emitting Species. *Plant Physiology*, *115*(4), 1413 LP – 1420. https://doi.org/10.1104/pp.115.4.1413

Skoog, F., & Armstrong, D. J. (1970). Cytokinins. *Annual Review of Plant Physiology*, *21*(1), 359–384. https://doi.org/10.1146/annurev.pp.21.060170.002043

Skoog, F., & Miller, C. (1957). Chemical regulation of growth and organ formation in plant tissues cultured. *Vitro Symp Soc Exp Biol*.

Slavova, Y., Zayova, E., & Krastev, S. (2004). Vegetative Propagation of Lavender (Lavandula vera V.) by Using Green Cuttings. *Bulgarian Journal of Agricultural Science*, *10*(4), 518–520.

Stirk, W. A., Bálint, P., Tarkowská, D., Novák, O., Maróti, G., Ljung, K., Turečková, V., Strnad, M., Ördög, V., & van Staden, J. (2014). Effect of light on growth and endogenous hormones in Chlorella minutissima (Trebouxiophyceae). *Plant Physiology and Biochemistry*, *79*, 66–76. https://doi.org/https://doi.org/10.1016/j.plaphy.2014.03.005

Tholl, D. (2006). Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Current Opinion in Plant Biology*, *9*(3), 297–304. https://doi.org/https://doi.org/10.1016/j.pbi.2006.03.014

Tholl, D., Chen, F., Petri, J., Gershenzon, J., & Pichersky, E. (2005). Two sesquiterpene synthases are responsible for the complex mixture of sesquiterpenes emitted from Arabidopsis flowers. *The Plant Journal*, *42*(5), 757–771. https://doi.org/https://doi.org/10.1111/j.1365-313X.2005.02417.x

Thorpe, T. A. (2007). History of plant tissue culture. *Molecular Biotechnology*, *37*(2), 169–180. https://doi.org/10.1007/s12033-007-0031-3

Tiwari, M., Sharma, D., & Trivedi, P. K. (2014). Artificial microRNA mediated gene silencing in plants: progress and perspectives. *Plant Molecular Biology*, *86*(1), 1–18. https://doi.org/10.1007/s11103-014-0224-7

Trigiano, R. N., & Gray, D. J. (2016). *Plant tissue culture, development, and biotechnology*. CRC Press.

Turner, G. W., & Croteau, R. (2004). Organization of Monoterpene Biosynthesis in Mentha. Immunocytochemical Localizations of Geranyl Diphosphate Synthase, Limonene-6-Hydroxylase,

Isopiperitenol Dehydrogenase, and Pulegone Reductase. *Plant Physiology*, *136*(4), 4215 LP – 4227. https://doi.org/10.1104/pp.104.050229

Turner, G. W., Gershenzon, J., & Croteau, R. B. (2000). Distribution of Peltate Glandular Trichomes on Developing Leaves of Peppermint. *Plant Physiology*, *124*(2), 655 LP – 664. https://doi.org/10.1104/pp.124.2.655

Upson, T., Andrews, S., & Royal Botanic Gardens, K. (2004). *The Genus Lavandula*. Richmond, Surrey, U.K. : Royal Botanic Gardens, Kew.

Vakili, A., Sharifat, S., Akhavan, M. M., & Bandegi, A. R. (2014). Effect of lavender oil (Lavandula angustifolia) on cerebral edema and its possible mechanisms in an experimental model of stroke. *Brain Research*, *1548*, 56–62. https://doi.org/https://doi.org/10.1016/j.brainres.2013.12.019

van der Krol, A. R., Mol, J. N. M., & Stuitje, A. R. (1988). Antisense genes in plants: an overview. *Gene*, *72*(1), 45–50. https://doi.org/https://doi.org/10.1016/0378-1119(88)90126-6

Verpoorte, R., van der Heijden, R., Hoge, J. H. C., & ten Hoopen, H. J. G. (1994). Plant cell biotechnology for the production of secondary metabolites. *Pure and Applied Chemistry*, *66*(10–11), 2307–2310. https://doi.org/doi:10.1351/pac199466102307

Vranová, E., Coman, D., & Gruissem, W. (2012). Structure and dynamics of the isoprenoid pathway network. *Molecular Plant*, *5*(2), 318–333.

Wang, D., Yuan, X., Liu, T., Liu, L., Hu, Y., Wang, Z., & Zheng, Q. (2012). Neuroprotective activity of lavender oil on transient focal cerebral ischemia in mice. *Molecules (Basel, Switzerland)*, *17*(8), 9803–9817. https://doi.org/10.3390/molecules17089803

Wang, G. (2014). Recent progress in secondary metabolism of plant glandular trichomes. *Plant Biotechnology*, *31*(5), 353–361.

Wang, P.-J., & Charles, A. (1991). *Micropropagation Through Meristem Culture BT - High-Tech and Micropropagation I* (Y. P. S. Bajaj (ed.); pp. 32–52). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-76415-8_3

Wang, Q., Reddy, V. A., Panicker, D., Mao, H.-Z., Kumar, N., Rajan, C., Venkatesh, P. N., Chua, N.-H., & Sarojam, R. (2016). Metabolic engineering of terpene biosynthesis in plants using a trichome-specific transcription factor MsYABBY5 from spearmint (Mentha spicata). *Plant Biotechnology Journal*, *14*(7), 1619–1632. https://doi.org/https://doi.org/10.1111/pbi.12525

Wells, R., Truong, F., Adal, A. M., Sarker, L. S., & Mahmoud, S. S. (2018). Lavandula Essential Oils: A Current Review of Applications in Medicinal, Food, and Cosmetic Industries of Lavender. *Natural Product Communications*, *13*(10), 1934578X1801301038. https://doi.org/10.1177/1934578X1801301038

Williams, D. C., McGarvey, D. J., Katahira, E. J., & Croteau, R. (1998). Truncation of limonene synthase preprotein provides a fully active 'pseudomature'form of this monoterpene cyclase and reveals the function of the amino-terminal arginine pair. *Biochemistry*, *37*(35), 12213–12220.

Wise, M. L., Savage, T. J., Katahira, E., & Croteau, R. (1998). Monoterpene synthases from common sage (Salvia officinalis): cDNA isolation, characterization, and functional expression of (+)-sabinene synthase, 1, 8-cineole synthase, and (+)-bornyl diphosphate synthase. *Journal of Biological Chemistry*, *273*(24), 14891–14899.

Woronuk, G., Demissie, Z., Rheault, M., & Mahmoud, S. (2011). Biosynthesis and therapeutic properties of lavandula essential oil constituents. *Planta Medica*, *77*(1), 7–15. https://doi.org/10.1055/s-0030-1250136

Xu, J., Zhang, J., & Zhang, W. (2018). Antisense RNA: the new favorite in genetic research. *Journal of Zhejiang University-SCIENCE B*, *19*(10), 739–749. https://doi.org/10.1631/jzus.B1700594

Ye, F., & Signer, E. R. (1996). RIGS (repeat-induced gene silencing) in Arabidopsis is transcriptional and alters chromatin configuration. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(20), 10881–10886. https://doi.org/10.1073/pnas.93.20.10881

Zhang, L., Xiao, W.-H., Wang, Y., Yao, M.-D., Jiang, G.-Z., Zeng, B.-X., Zhang, R.-S., & Yuan, Y.-J. (2017). Chassis and key enzymes engineering for monoterpenes production. *Biotechnology Advances*, *35*(8), 1022–1031.

Zhanji, L., Xue, S., Pingli, X., Guangcun, L., & Xiuli, W. (2001). Advances in genetic transformation of graminaceous crops by particle bombardment. In *Yournal of Shenyang Agricultural University* (Vol. 32, Issue 6, pp. 465–468). http://europepmc.org/abstract/CBA/354727

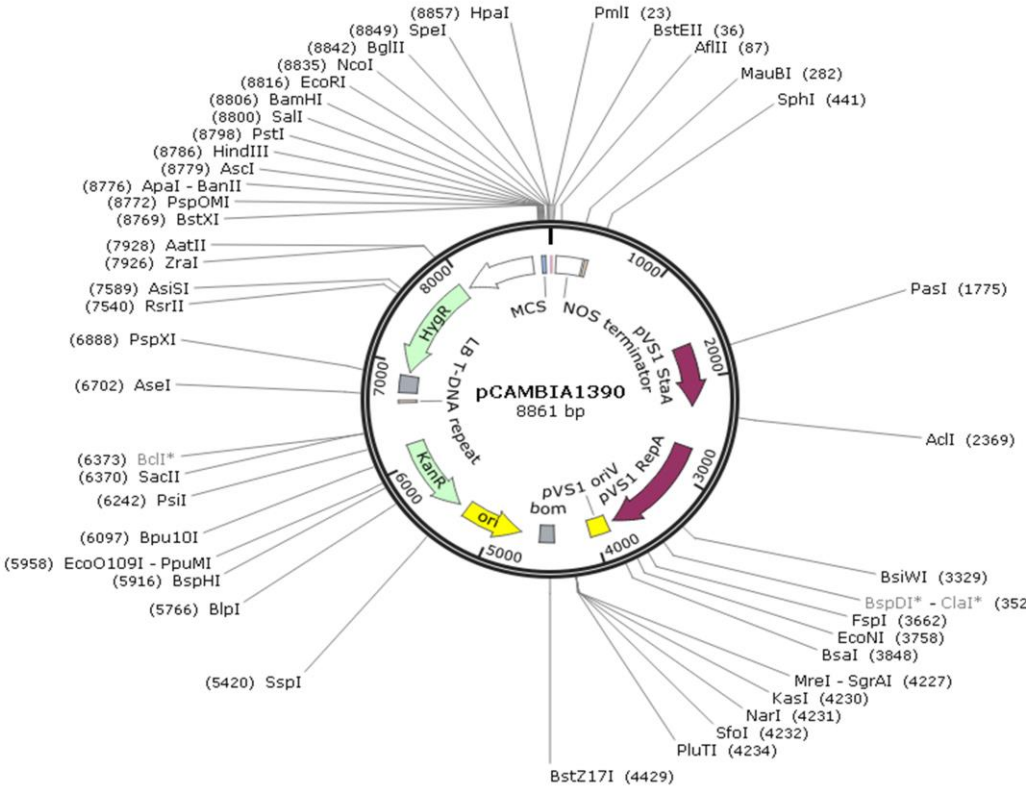
Zhao, Y., Chen, Y., Gao, M., Yin, H., Wu, L., & Wang, Y. (2020). Overexpression of geranyl diphosphate synthase small subunit 1 (LcGPPS.SSU1) enhances the monoterpene content and biomass. *Industrial Crops and Products*, *143*, 111926. https://doi.org/https://doi.org/10.1016/j.indcrop.2019.111926

Zhou, W., Huang, F., Li, S., Wang, Y., Zhou, C., Shi, M., Wang, J., Chen, Y., Wang, Y., Wang, H., & Kai, G. (2016). Molecular cloning and characterization of two 1-deoxy-d-xylulose-5-phosphate synthase genes involved in tanshinone biosynthesis in Salvia miltiorrhiza. *Molecular Breeding*, *36*(9), 124. https://doi.org/10.1007/s11032-016-0550-3

Zupan, J. R., & Zambryski, P. (1995). Transfer of T-DNA from Agrobacterium to the plant cell. *Plant Physiology*, *107*(4), 1041–1047. https://doi.org/10.1104/pp.107.4.1041

Appendices

Appendix A: Map of Pcambia 1390 plant expression vector (NovoPro)



Appendix B: Bacteria strains used in this thesis

Organism	Strains	Antibiotic resistant	Purpose
E. coli	*DH5α*	Kanamycin	Cloning
A. tumefaciens	GV3101	Rifampicin	Plant transformation

Appendix C: PCR programs used in this study

Table C. 1. The PCR program for amplification of *BPPS* in sense plasmid and antisense plasmid and for amplification of *35s:gus A* genes in GUS plasmid

Stage	Temperature	Time	Cycle
Stage 1			
Pre-denaturation	95 °C	5 min	9
Denaturation	95 °C	30 sec	
Annealing	65 °C	30 sec	
Extension	72 °C	2 min	
Stage 2			
Denaturation	95 °C	30 sec	31
Annealing	56 °C	30 sec	
Extension	72 °C	2 min	
Stage 3			
Final extension	72 °C	10 min	1

Table C. 2. The PCR program for validation of hygromycin resistance gene (*HygR*) in transformed plants

Stage	**Temperature**	**Time**	**Cycle**
Stage 1			
Pre-denaturation	95 °C	5 min	8
Denaturation	95 °C	30 sec	
Annealing	59 °C	30 sec	
Extension	72 °C	1.45 sec	
Stage 2			
Denaturation	95 °C	30 sec	32
Annealing	51°C	30 sec	
Extension	72 °C	1.45 sec	
Stage 3			
Final extension	72 °C	10 min	1
Hold	4 °C	∞	

Table C. 3. The PCR program for validation of actin in transformed plants

Stage	**Temperature**	**Time**	**2. Cycle**
Stage 1			
Pre-denaturation	95 °C	5 min	30
Denaturation	95 °C	30 sec	
Annealing	56	30 sec	
Extension	72 °C	45 sec	
Stage 2			
Final extension	72 °C	7 min	1
Hold	4 °C	∞	

Table C. 4. The PCR program for validation of the *BPPS* gene in transformed plants

3. Stage	Temperature	Time	Cycle
Stage 1			
Pre-denaturation	95 °C	5 min	8
Denaturation	95 °C	30 sec	
Annealing	60 °C	30 sec	
Extension	72 °C	2 min	
Stage 2			
Denaturation	95 °C	30 sec	22
Annealing	53 °C	30 sec	
Extension	72 °C	2 min	
Stage 3			
Final extension	72 °C	10 min	1
Hold	4 °C	∞	

Appendix D: Media compositions used for *L. latifolia* transformation and regeneration

For *L. latifolia* transformation

a) Suspension medium (BM medium) plus acetosyringtone:

- 4.43 g L^{-1} Murashige and Skoog salts with vitamins (PhytoTechnology laboratories)
- 3.0 % (w/v) sucrose (Fisher scientific)
- pH 5.5

After autoclaving:

- 100 μM Acetosyringone (PhytoTechnology laboratories)

b) Co-cultivation medium (BRM without antibiotics):

- 4.43 g L^{-1} Murashige and Skoog salts with vitamins
- 0.4 % Gellan gum (PhytoTechnology laboratories, USA)
- 3.0 % (w/v) sucrose
- 0.6 μM indole acetic acid (IAA) (Sigma Aldrich)
- 8.8 μM 6-benzylaminopurine (BA) (Sigma Aldrich)
- pH 5.5

For *L. latifolia* organogenesis:

a) Elimination medium:

- 4.43 g L^{-1} Murashige and Skoog salts with vitamins

- 0.4 % Gellan gum

- 3.0 % (w/v) sucrose

- 0.6 μM indole acetic acid (IAA)

- 8.8 μM 6-benzylaminopurine (BA)

- pH 5.7

After autoclaving:

- 60 mg L^{-1} TIMENTIN (ticarcillin and clavulanate) (Gold Biotechnology, USA),

- 120 mg L^{-1} cefotaxime (Gold Biotechnology, USA)

b) Selection medium:

- 4.43 g L^{-1} Murashige and Skoog salts with vitamins

- 0.4 % Gellan gum

- 3.0 % (w/v) sucrose

- 0.6 μM indole acetic acid (IAA)

- 8.8 μM 6-benzylaminopurine (BA)

- pH 5.7

After autoclaving:

- 60 mg L^{-1} TIMENTIN

- 120 mg L^{-1} cefotaxime

- 7.5 mg L^{-1} hygromycin B (AG.Scientific)

SEM (Shoot Elongation Medium):

- 4.43 g L^{-1} Murashige and Skoog salts with vitamins

- 0.4 % Gellan gum

- 3.0 % (w/v) sucrose

-0.06 μM IAA

-8.9 μM BA

-pH to 5.7

After autoclaving:

- 60 mg L^{-1} TIMENTIN

- 120 mg L^{-1} cefotaxime

- 7.5 mg L^{-1} hygromycin

c) Rooting medium:

- 2.21g L^{-1} Murashige and Skoog salts with vitamins,

- 0.8 % Gellan gum

- 3.0 % (w/v) sucrose

- 2.9 μM IAA

-pH to 5.7

After autoclaving:

- 60 mg L^{-1} TIMENTIN

- 120 mg L^{-1} cefotaxime

- 7.5 mg L^{-1} hygromycin

Note: all antibiotics used in regeneration and rooting media were added after autoclaving

Appendix E: Supplementary table for EO screening of transgenic plants

Oil yield (mg oil/g leaf) and relative percent composition of major oil constituents in young leaves of wild-type (WT), BPPS-sense (BPPS-S) and BPPS-antisense (BPPS-As) plants. Values are compared to WT using the student's t-test. Data represented as mean ± standard error for three biological replicates and three technical repetitions, * P< 0.05.

Line	Camphor	SD	Borneol	SD	1,8 Cineole	SD	Limonene	SD	Oil Yield	SD
WT	23.5	1.45	4.18	0.23	23.74	0.76	1.22	0.19	37.3	2.81
BPPS-As4	13.4*	0.52	0.87*	0.12	35.31*	1.46	2.41*	0.24	36.8	2.78
BPPS-As9	14.6*	1.42	1.85*	0.09	35.86*	2.34	1.96*	0.14	37.91	1.21
BPPS-As11	12.8*	0.39	1.32*	0.05	34.89*	1.9	2.3*	0.05	36.5	2.1
BPPS-As12	22.22	1.33	3.98	0.20	23.91	0.36	1.13	0.1	38.0	2.55
BPPS-As14	11.6*	1.24	1.87*	0.1	35.18*	1.72	3.74*	0.18	37.8	1.5
BPPS-As18	13.5*	1.95	2.31*	0.17	32.46*	2.64	2.39*	0.13	36.5	0.58
BPPS-As19	12.9*	2.17	1.89*	0.2	36.33*	0.97	2.42*	0.25	38.7	2.69
BPPS-As20	16.06*	3.13	2.14*	0.17	28.15*	2.66	1.53*	0.09	35.8	1.33
BPPS-As22	22.61	2.22	4.03	0.06	23.51	2.96	1.31	0.2	37.22	1.90
BPPS-As30	10.8*	2.9	1.39*	0.16	33.6*	3.39	2.04*	0.19	36.1	2.61
BPPS-As33	15.9*	1.23	2.63*	0.34	25.24	2.71	1.49*	0.15	32.21**	2.86
BPPS-S1	30.11*	2.24	6.88*	0.67	19.2*	1.74	0.56*	0.14	39.89	3.58
BPPS-S2	32.81*	1.24	7.38*	0.41	17.19*	1.9	1.01*	0.09	40.33*	3.58
BPPS-S3	18*	3.43	1.87*	0.27	20.15*	2.06	1.83*	0.3	33.81*	1.33
BPPS-S4	20.8*	2.39	2.32*	0.62	19.89*	3.8	2.3*	0.05	34.5*	2.1
BPPS-S5	36.38*	3.39	9.24*	1.01	16.79*	2.11	1.01*	0.08	41.12*	2.34