Evaluation of Rosemary (*Salvia rosmarinus* Spenn.) taxon for Commercial Horticultural Production

The assessment of beneficial traits, varietal selection, and characterization of volatile organic compounds to improve aroma and taste.

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Declaration of Authorship

I Emily Leggatt hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Signed: _____

Date: 13th October 2022

Abstract

Rosemary (*Salvia rosmarinus* Spenn.) is one of the biggest selling herbs in the UK with five million pots, bunches and packs being sold each year. This culinary herb produces a range of volatile compounds that give its characteristic aroma and taste. Rosemary cultivars produce different blends of essential and volatile oils, which give each variety a different scent. The availability of different cultivars also make rosemary a popular home gardening herb. This research assessed the morphological, genetic, and physiological traits and identified beneficial traits to inform horticultural production and selection of rosemary varieties with better aroma and taste for the consumer in both the domestic garden market and the commercial herb retail market.

Analysis of morphological traits showed that the taxa rosemary has high morphological diversity, which allows for a selection of plants with desirable visual and commercial qualities. Growth habit traits differed, with some cultivars upright and tall, others semi-upright, low growing, with multiple branching. The second trait with high diversity is leaf size. Rosemary is characterised by thin needle-like leaves, the leaves of different cultivars varied in shape from linear to ovate. Leaf size, colour and density on a single branch was also variable. This variability proved beneficial for the selection of visual quality traits in rosemary for the consumer.

Gas Chromatography Mass Spectrometry (GCMS) was undertaken for the volatile profiling of cultivars and to assess the impact of various environmental conditions on rosemary cultivars. The variation in varietal aroma profiles was assessed to select rosemary varieties with enhanced volatile production and improved aroma and taste. Results showed that rosemary cultivars have distinctive aroma profiles. Based on the chemical composition of oil, rosemary can be classed in four groups: group 1. 1,8 cineole, eucalyptol, group 2. Camphor, group 3. Myrcene and group 4. Verbenone types. The results show that the commercially available Perigord variety is a 1,8 cineole type.

Environmental conditions were investigated to enhance volatile production. Light quality is known to improve to improve quality traits such as aroma and taste in rosemary and supplementary lighting is often used I glasshouse horticulture to maximise plant growth and plant quality. However, less is known about how light spectrums such as far-red and UV-C affect the molecular pathways involved in aroma production in rosemary. The aim of this work was to investigate the effects of far-red and UV-C lighting on the expression of genes in the aromatic portion of the terpenoid synthesis pathways. Far red and UV-C supplementary light treatments showed significant changes in the aroma profile of rosemary, including changes in quantity of volatile emissions and differences in the overall chemical composition of the aroma profile. Supplementary far-red light treatment caused increases in phenolic content and gene expression of terpene synthases, boosting volatile organic compound production. This treatment also altered the morphology of prostate plants to a more desirable upright visual retail appearance for supermarkets through the action of shade avoidance on gravitropic responses.

RNA sequencing was used to produce an annotated Rosemary transcriptome and investigate differences in terpene synthase expression between cultivars. A selection of terpene synthase genes were assessed as quality markers for improved volatile production and aroma. Supplementary far-red lighting changed levels of

gene expression in terpene synthases as well as altering gene expression along the non-mevalonate (MEP) and mevalonate (MVA) pathways. Quality markers were designed based on seven terpene synthases found in the RNA sequencing. Quantitative PCR was used for further assessment of gene expression in UV-C light and with the addition of soil microbes, arbuscular mycorrhizae fungi (AMF) treatment. This revealed gene expression patterns of terpene synthases were different between cultivars. The use of AMF increased gene expression of some terpene synthases. The addition of AMF to the rhizosphere was found to increase the rate of root striking, growth rate of cuttings, and enhance the aroma of rosemary through increased phenolic content and upregulation of sesquiterpene synthase.

Rosemary leaves contain antioxidants, such as carnosic acid, which are known to be beneficial for human health. Antioxidant content of 19 rosemary cultivars was measured to identify cultivars with high quantities of antioxidants. . This study showed that antioxidant and phenolic assays can be used as a preliminary assessment of specialised metabolism and can inform the selection of cultivars with high production of secondary metabolites. The preliminary screening in this study allowed for more cultivars to be evaluated for antioxidant content, and correlate high performing cultivars to higher production of specialised metabolites which may indicate an improvement to aromatic volatile production. Selected cultivars were further analysed with headspace-GCMS to quantify aromatic volatile production in each of the cultivars and under different environmental conditions.

The selection of rosemary cultivars was based on morphological characteristics important to the horticultural industry, a high production of antioxidants and other specialised metabolites, and the expression of terpene synthases responsible for the synthesis of aromatic volatiles The combined approach of screening cultivars for desirable traits and evaluating their performance under changing environmental conditions lead to a complete analysis that determined which traits should be targeted to enhance horticulturally grown rosemary Overall, the use of this multi-disciplinary approach provided a wealth of information to improve the horticultural production of rosemary for culinary use.

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Table of Contents

Table of Figures

Chapter 1

Chapter 3

Chapter 4

Chapter 1: Literature review

Introduction

Salvia rosmarinus Spenn. (Fridolin Carl Leopold Spenner 1798-1841, abbreviated to Spenn.), rosemary, is a woody evergreen perennial shrub belonging to the plant family Lamiaceae (Zhao *et al.*, 2021). Rosemary is one of the biggest selling culinary herbs in the UK with five million pots, bunches and packs being sold each year. Rosemary is a popular herb due to its characteristic aroma and is commonly used in home cooking. Trials of rosemary at RHS Wisley (2016 to 2021) collected a selection of 90 named cultivars in cultivation. These rosemary cultivars produce different blends of essential and volatile oils, which give each variety of differing scents (Huang, Y. *et al.*, 2023). The leaf extract of rosemary is also used in pharmaceuticals and as a food preservative due to its antioxidant and antimicrobial compounds, such as phenolics, flavonoids and rosmarinic acids (Veenstra and Johnson, 2021). The diversity of cultivars also makes rosemary a popular herb for home gardening. Rosemary has both upright and prostrate habits, with different leaf sizes and branching which gives cultivars their shape. This diversity of morphological shapes allows for selection of desirable traits which will be discussed in this first chapter. The first chapter of this review will also summarise the biochemical diversity among rosemary cultivars, provide detail on the two biochemical pathways that lead to the production of aromatic volatiles, and will evaluate potential candidate genes that would promote aroma production in plants.

Rosemary - Desirable traits

Salvia rosmarinus Spenn., rosemary has many desirable traits for humans and is used for medicinal, antimicrobial, antioxidative purposes due to the composition of its leaf extracts and essential oil (Christopoulou *et al.*, 2021). Rosemary is produced by commercial horticulture for culinary purposes due to its aroma and taste and is grown in pots or packaged stems that are produced for supermarkets. The agricultural and horticultural industry have focused on traits contributing to the ease of propagation and production on a large scale. In addition to this there are three traits which should be observed to enhance horticulturally produced rosemary, these are: Volatile production, Antioxidant capacity and Plant Morphology. These three traits will be discussed below.

1.1 Volatile Production

1.1.1 Volatile Biosynthesis takes place in the Leaf Glandular Trichomes

Volatile compounds, particularly monoterpenes and sesquiterpenes, are synthesised and stored in secretory tissues. The *Lamiaceae* plant family have specialised glandular trichomes for the storage and secretion of volatile organic compounds (VOCs). Volatile Organic Compounds are organic chemicals that have a high vapor pressure and low water solubility at room temperature, they easily evaporate into the air and dissolve in water. The VOCs are released when herbivore feeding or movements on the plant's surface ruptures the cuticle of the glandular trichome (Maffei, 2010). Glandular trichomes found on the leaf surface of Rosemary plants have been found to be the primary site of secondary metabolite biosynthesis, storage and secretion (Boix *et al.*, 2011). The leaf abaxial epidermis is covered in glandular trichomes, where they occur in great numbers in epidermal grooves. The density of glandular trichomes is important for high yields of plant leaf extracts, however the size of the glands is another factor that influences biosynthesis and storage of VOC's. Glandular trichomes are classified into two types: capitate Figure 1.1 (**c**) and peltate Figure 1.1. (**p**) glandular trichomes being the main source of volatile production and storage. The capitate trichomes consist of a basal cell, a short unicellular stalk and a one or two celled secretory head. The peltate trichomes consist of one basal epidermal cell, a wide unicellular stalk cell and a multicellular secretory head. The quantity of head cells in peltate trichomes may contribute to an increased production of volatiles. Secretion of VOCs from peltate glandular trichomes occurs when volatiles and essential oils accumulate in the subcuticular space above the cell walls of the secretory cells, the build-up of pressure causes rupture of the cuticle, which then releases the volatiles and finally new cuticle is generated (Sharma *et al.*, 2003). Research by Boix *et al.* (2011) to investigate the production of VOCs in rosemary demonstrated the importance of peltate glands in aromatic volatile synthesis. They performed histochemical tests and phytochemical analysis to locate the secondary metabolites in glandular trichomes. Their results indicated that glandular trichomes of rosemary leaves are involved in producing and secreting VOCs, with terpenes and phenolic compounds only being produced in peltate glandular trichomes. As terpenes are the main constituent of volatile oil, it was concluded that peltate trichomes are the main site of volatile biosynthesis and secretion. This conclusion is agreed with Marin *et al*, 2006 who observed that secreted material from peltate trichomes consisted of a mixture containing terpenes, phenolic compounds and other classes of VOCs. They observed that peltate were more abundant than capitate trichomes on the leaf surface. This indicates that the abundance and size of peltate glandular trichomes could be used as a classification factor for rosemary varieties based on their production of VOCs.

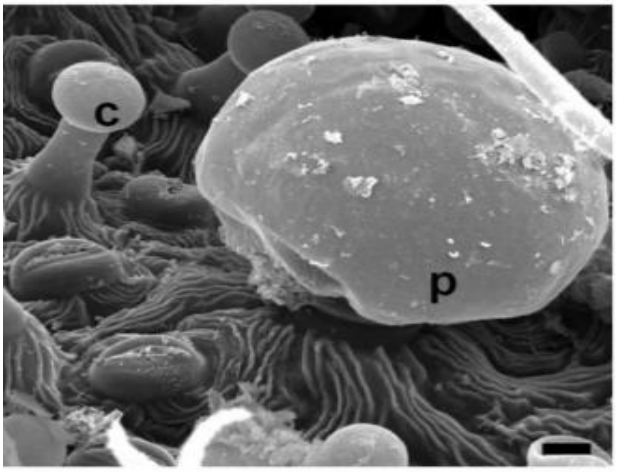


Figure 1. 1 Electron scanning of leaf surface of *Salvia rosmarinus* Spenn.

Leaf surface of *Salvia rosmarinus* Spenn., imaged by scanning electron microscopy, showing detail of capitate (**c**) and peltate (**p**) glandular trichomes. Scale bar = 10µm. Image from: [Boix et al., 2011]

1.1.2 Plant Volatiles: Chemical Composition, Biosynthesis and Function

Plants produce thousands of structurally diverse volatile signalling compounds to attract pollinating insects and seed-dispersing animals, to protect against plant pathogens and to mediate interactions with other plants (Dong *et al.*, 2016). Plant volatiles are produced in the leaf, flower and roots of the plant. Chemically, VOCs (Volatile Organic Compounds) contain different types of terpenoids, phenylpropanoid aromatic compounds, as well as certain alkenes, alcohols, esters, aldehydes and ketones (Maffei, 2010). Different blends of VOCs are emitted by plants, such as in response to herbivore attack. An herbivore-induced VOC blend may contain more than 200 compounds to perform indirect or direct defence mechanisms against the herbivore attack. Indirect defences involve attracting natural enemies of the insect herbivore to predate the insects. In direct defence, VOCs serve as insect repellents and decrease oviposition rates (Dong *et al.*, 2016). VOC's have many functions in plants, and there are many types of chemicals that class as volatiles. The following section focuses in on terpenoids, which are a major group of volatile organic compounds in plants. This section explains the biosynthesis and classification of terpenoids.

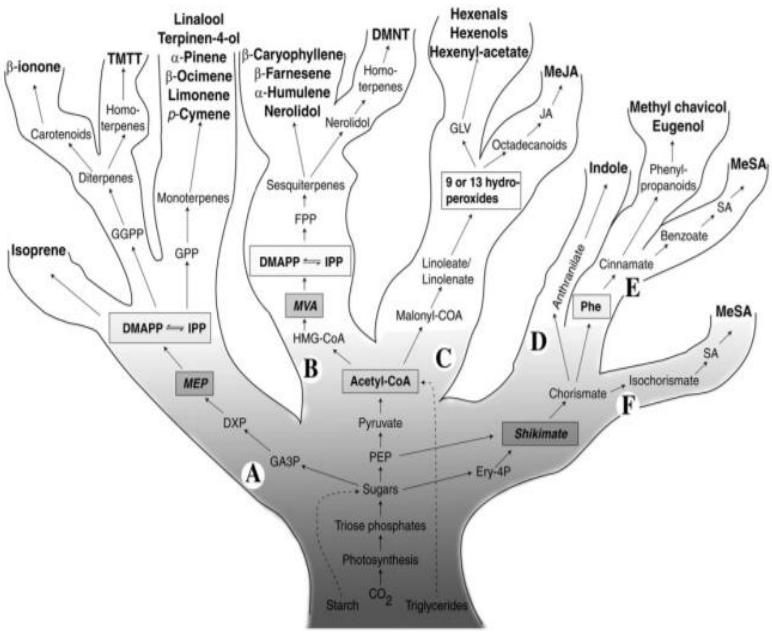


Figure 1. 2 Map of biosynthetic pathways for voaltile production in higher plants.

Volatilome tree depicting plant biosynthetic pathways for volatile secondary metabolite production. Terpenes and phenylpropanoids are the main constituents of rosemary volatile oil, pathways A, B and E pathways focussed on in this review. Pathway A involves the MEP pathway for the synthesis of monoterpenes from a common precursor, DMAPP. The addition of IPP units to DMAPP forms FPP, the precursor of sesquiterpenes through the MVA pathway (B). Pathway E involves the synthesis of phenolic aromatic compounds. Figure from: [Maffei, 2010]

Figure 1.2 illustrates the main volatile organic compounds found in plants. The main three types of terpenes found in rosemary volatile oil include: monoterpenes - highly volatile, found in leaves (e.g. α-pinene), sesquiterpenes - less volatile, found in flower aromas (e.g. β-caryophyllene) and terpenoids -volatiles found in the leaves (e.g. camphor). Monoterpenes are the main constituents of rosemary volatile oils, synthesised by a large family of terpene synthase enzymes. These are synthesised via two main pathways: the plastidial MEP pathway (methylerythritol 4-phosphate pathway) and the cytosolic MVA pathway (mevalonate pathway). The MVA pathway obtains its precursor molecule, acetyl-CoA from the Krebs cycle whereas the MEP pathway utilizes glyceraldehyde phosphate from the Calvin cycle as its precursor molecule (Webb *et al.*, 2015). All terpenoids are derived from the precursors DMAPP and IPP during the first catalytic steps of both pathways (Webb *et al.*, 2015; Tetali, 2019). IPP and DMAPP are further condensed by prenyltransferases, resulting in intermediates of different chain lengths and cyclization (Yazaki *et al.*, 2009). Figure 1.3 highlights key steps in the biosynthetic pathways of monoterpenes by the MEP pathway. The MVA pathway synthesises the precursors for triterpenes and sesquiterpenes.

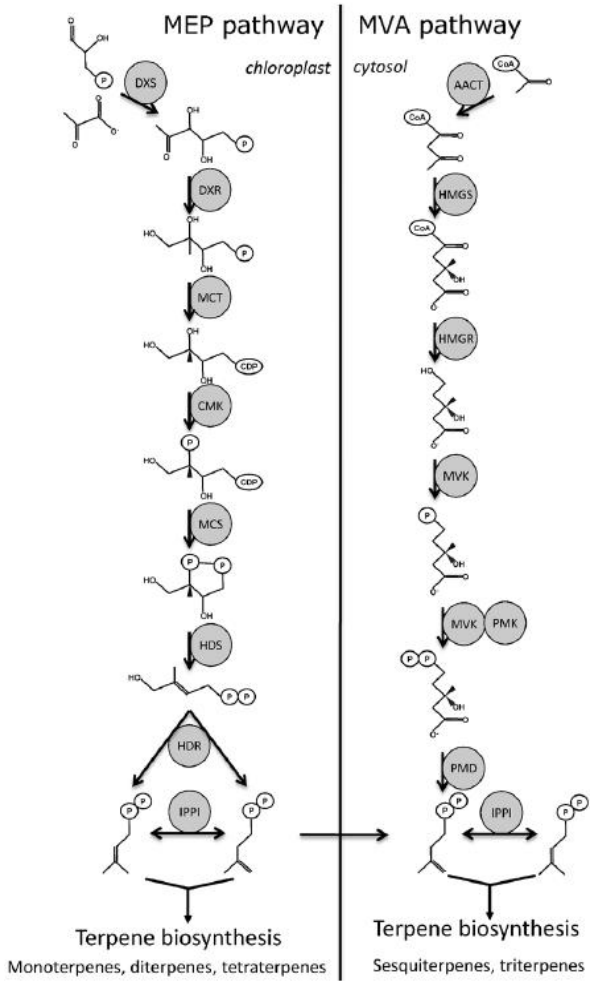


Figure 1. 3 The two terpenoid biosynthetic pathways in higher plants.

Enzymatic steps in the isoprenoid pathways; MEP and MVA isoprenoid pathways for the biosynthesis of terpenes. Each enzyme in the pathway is shown. MEP pathway takes place in the cell's chloroplast and the MVA pathway in the cytosol. MEP pathway leads mainly to biosynthesis of mono-, di- and tetra-terpenes. The MVA pathway synthesises sesquiterpenes and triterpenes. [Webb *et al.,* 2015].

Rosemary produces various types of isoprenoids, however, the biosynthesis of these isoprenoids in rosemary is not yet fully understood. The exact enzymes, intermediates, and regulation mechanisms of the IPP pathway in rosemary are still unclear, and more research is needed to elucidate them. All terpenes are synthesized from a common precursor, isopentyl pyrophosphate (IPP) also known as isoprenoids. IPP can be reversibly transformed to its isomer dimethylallyl pyrophosphate (DMAPP). IPP and DMAPP are further condensed by prenyltransferases, resulting in intermediates of different chain lengths and cyclization (Yazaki *et al.*, 2009). Rosemary uses both the MVA and the MEP pathways to produce IPP and DMAPP. These intermediates are then used by terpene synthases to produce aromatic volatiles. Prenylation of IPP or DMAPP leads to the precursor geranyl pyrophosphate (GPP) of mono-di- and tetraterpenes in the MEP pathway, depending on the activity of prenyl phosphate synthases. Prenyl phosphate forms the backbone of all terpenes, the three substrates used by terpene biosynthesis are GPP that is used for monoterpene synthesis, and geranylgeranyl pyrophosphate (GGPP) which is use for di and tetraterpene biosynthesis (Webb *et al.*, 2015). A list of genes important to substrate synthesis in the MEP pathway is presented on table 1.1. GPP is then the substrate of

terpene synthases which produce the terpenes that for part of the volatile fraction of essential oils. The second pathway of interest is the mevalonate (MVA) pathway for the biosynthesis of sesquiterpenes as seen on Figure 1.3. Farnesyl pyrophosphate (FPP) is the substrate for sesquiterpenes, and triterpenes products synthesised by terpene synthases in the MVA pathway, enzymes important for substrate synthesis for the MVA pathway are presented on table 1.2.

The diversity of terpenoids produced is attributed to the enzymatic activity of these pathways but also to the large family of multi-substrate terpene synthases (Pazouki and Niinemets, 2016). The main volatile constituents of rosemary that contribute to aroma are α-pinene, linalool, cymene, and eucalyptol (Ozcan and Chalchat, 2008) among others and are all synthesised by terpene synthases. Altering the expression of terpene synthases *in planta* would likely alter the aroma profile. Terpene synthases use Prenyl phosphate as substrate for the terpene production. The products of terpene synthases can undergo several rounds of modification (Blerot *et al.*, 2018), this combined with the multi-substrate functionality of terpene synthases and the genetic diversity withing the terpene synthase family contributes to the terpene diversity observed in plants.

Table 1.1 MEP pathway genes which produce the main substrates or are involved in key catalytic steps towards GPP production for the isoprenoid and terpenoid pathways.

Gene name	***Arabidopsis* ortholog of the common ancestral gene**	**Description**
Geranylgeranyl reductase	GGR	Geranyl geranyl-diphosphate synthase small subunit. With the large subunit GGPS1 catalyses the production of geranyl-diphosphate (GPP).
Geranylgeranyl pyrophosphate synthase 1	GGPS1	Geranyl diphosphate synthase catalyses the trans-addition of IPP onto DMAPP to form geranyl diphosphate (GPP).
Geranyl reductase	CHLP	Catalyses the reduction of GPP to phytyl diphosphate, providing phytol for tocopherol and chlorophyll synthesis.
Geranyl transferase	RGTB1	Catalyses the transfer of geranylgeranyl diphosphate to both cysteines of RAB proteins.
4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	GCPE	Important for isoprenoid biosynthesis and is involved in step five of the sub

		pathway, synthesises isopentenyl diphosphate (IPP).
1-deoxy-D-xylulose-5-phosphate reductoisomerase	DXR	Enzyme of the MEP pathway that catalyses the reduction of DXP to MEP, MEP is used and modified sequentially through the MEP pathway towards terpene synthesis.
1-deoxy-D-xylulose-5-phosphate synthase	DXPS1	First step enzyme that synthesises DXPS from pyruvate and glyceraldehyde pyruvate.
4-hydroxy-3-methylbut-2-en-1-yl diphosphate reductase	CLB6	Essential in the conversion of hydroxy methyl butenyl diphosphate to IPP and DMAPP, leading to isoprenoid biosynthesis.

Table 1.2 MVA pathway genes involved in the catalysis of substrates for isoprenoid production, mainly towards FPP production for sesquiterpene and triterpene synthesis.

Gene name	***Arabidopsis* ortholog of the common ancestral gene**	**Description**
acetyl-CoA C-acetyltransferase	MVA1 (also known as HMGS)	Involved is step 2 of the MVA pathway. This enzyme condenses acetyl-CoA to form HMG-CoA, substrate for HMG-CoA reductase.
mevalonate diphosphate decarboxylase	MVD	Performs the first committed step in the biosynthesis of isoprene compounds, such as terpenoids. Synthesises isopentenyl diphosphate from mevalonate.
isopentenyl-diphosphate delta-isomerase	IPP1 / IPP2	Coverts IPP to DMAPP. DMAPP is then used for sesquiterpene and triterpene synthesis.
hydroxymethylglutaryl-CoA reductase	HMGR1 / HMGR 2	Catalyses the synthesis of mevalonate, the precursor of isoprenoid compounds.

Farnesyl pyrophosphate synthase	FPS1 / FPS2	Catalyses the sequential condensation of IPP with dimethylallyl pyrophosphate to result in FPP.

1.1.3 Terpene synthases are a diverse gene family responsible for terpenoid production.

Plants synthesise, including those in the Lamiaceae family, a diverse array of terpenoid metabolites, these are synthesised by enzymes in the terpenoid pathways called terpene synthases. Terpene synthases are responsible for the synthesis of many different terpenes, at the final steps of the MVA and MEP pathways. Terpene synthases are specialized enzymes responsible for catalysing the synthesis of monoterpenes, sesquiterpenes and diterpenes from the substrates GPP and FPP from the terpenoid pathways (Filipe *et al.*, 2017). They are a large, diverse group of enzymes and each plant taxon has a unique pattern of expression accounting for their distinctive aromas. The diversification of rosemary has led to an expansion of the terpene synthase gene family. The result of the large terpene synthase gene family is the array of terpene products synthesised, which leads to a diverse and unique metabolite blends even within a plant species (Karunanithi and Zerbe, 2019). Terpene synthases are common to all plants, but many are found in one taxon presumed to have evolved in response to specific ecological pressures, such as herbivory, defence against microbial pathogens and mitigation of drought stress (Zhou and Pichersky, 2020; Vaughan *et al.*, 2015). As well as having a large gene family, terpene synthases themselves are multi-substrate; terpene synthases have the capacity to bind and use multiple different substrates. The diversity of products catalysed depends on the terpene synthase, some have high specificity for catalysing a limited number of products while others have low specificity and catalyse a large number of volatile products (Pazouki and Niinemets, 2016). Terpene synthases are capable of this large diversity by catalysing complex carbon cyclization, rearrangement, and elimination reactions to enable the conversion of diphosphate substrates into a vast array of terpene scaffolds (Karunanithi and Zerbe, 2019).

Recent research tends to focus on specific terpene synthases responsible for catalysing terpene products that are useful to the agricultural, pharmaceutical, and medical industries. The genetic engineering of bacteria or plants to produce large quantities of terpenoid products has been the focus of research, underpinning this is the functional characterization of specific terpene synthases (Li *et al.*, 2017; Blerot *et al.*, 2018; Filipe *et al.*, 2017). It is this functional and genetic characterization research which allows for precise gene identification in other plants, such as rosemary, to elucidate the terpene gene family. Filipe et al. (2017) characterized 1,8 cineole synthase expression in the aromatic shrub *Thymus albicans* Hoffmann's & Link. Genetic analysis and recombinant expression of Cineole synthase in bacterial cultures, increased the production of the monoterpene Cineole as well as other minor products and because of the multiple synthesised products it was concluded to be a multi-substrate terpene synthase (Filipe *et al.*, 2017). Overexpression of terpene synthases has also been investigated in plants, such as tomato to enhance the flavour of the fruits (Davidovich-Rikanati *et al.*, 2008). In their research Davidovich-Rikanati*, et al.* (2008) overexpressed the sesquiterpene synthase α-zingiberene synthase directed towards the tomato fruit to enhance terpene production. They found that the phenotype of the fruit was unchanged and there was an increased accumulation of mono- and sesquiterpenes

in the tomato fruit, provided there is sufficient terpenoid precursors available in the cytosol. Current research states that there is a rate limitation along the terpenoid pathway either through lack of GPP/FPP precursors (Estévez *et al.*, 2001) or through bottlenecks in the enzymatic steps (Mani *et al.*, 2021). Further research has been conducted into the rate limitation steps along the pathway to increase substrate availability for terpene synthases and increase accumulation of desirable volatile products. (Gutensohn *et al.*, 2021; Webb *et al.*, 2013).

Gutensohn et al (2021) investigated the availability of GPP and FPP precursors for sesquiterpene production in tomatoes by genetically engineering plants expressing two terpene synthases (nerolidol/linalool synthases -1 and -2) which would specifically use GPP and FPP as precursors in the cytosol and chloroplast, respectively. In this way they were able to indirectly quantify the pool of GPP and FPP available in both cytosol and plastid cell compartments. The terpene synthase nerolidol/linalool synthase -2 which used GPP as a precursor was a monoterpene synthase and synthesised large quantities of linalool by a 60-fold increase compared to the other nerolidol/linalool synthase -1. The sesquiterpene synthase (nerolidol/linalool synthase -1) only catalysed from the precursor FPP which indicated that there was a small pool of available FPP for sesquiterpene formation in the cytosol. The subsequent step in their research was to co-express enzymes along the terpenoid pathway leading to more FPP precursors. Overexpression of HMGR and IPK lead to an increased availability of substrate for nerolidol/linalool synthase -1 and increased sesquiterpene production of nerolidol by 2.9-fold (Gutensohn *et al.*, 2021). They concluded the MVA pathway had lower metabolic activity than the MEP pathway, supported by the increased nerolidol accumulation once co-overexpression of pathway genes was introduced. Both pathways have crosstalk and transportation between pathways is not yet fully understood. Since the MEP pathway has a higher metabolic rate, the genetic engineering of monoterpene synthases has been more successful than of sesquiterpene synthases. The MEP pathway metabolic activity was earlier investigated by Webb et al (2013) in tea tree oil (*Melaleuca alternifolia* ((Maiden & Betch) Cheel). Bottlenecks in the MEP pathway have been correlated with the genes DXR and DXS, as overexpression of these genes leads to larger terpene production and subsequently essential oil yield. Webb *et al.,* (2013) also suggested that after analysis of the whole MEP pathway DXS and DXR could be acting as bottlenecks, or their overexpression could initiate a cascade effect that results in the upregulation of other genes within the pathway (Webb *et al.*, 2013). Since Rosemary is also a woody plant, it may be of interest to investigate expression along the MEP and MVA pathways as this would form the base of the research for enhancing volatile production.

1.1.4 Aroma profiles of several terpene synthases identified in rosemary cultivars have different aroma profiles as a result of the different aromatic compounds synthesised by the MEP and MVA pathways. The last steps of the MVA and MEP pathways involves terpenoid synthases. These gene produce a variety of terpenoid compounds, including monoterpenes, diterpenes and sesquiterpenes which contribute to the aromatic volatile profiles. The terpene synthases presented in table 1.3 are involved in the synthesis of aromatic volatiles present in rosemary volatilome.

Table 1.3 Terpene synthase (TPS) genes, synthesised products and aromatic descriptions of volatile products associated with each TPS. Descriptors of aromas from the Good Scents Database (The Good Scents Company - Flavor, Fragrance, Food and Cosmetics Ingredients information)

Gene Name	*Arabidopsis* ortholog	Products	Description
Ocimene synthase	TPS03	β-ocimene, terpinolene, β-myrcene, β-pinene	Monoterpene synthase, the products are all monoterpenes. β-pinene is a pinene isomer and has a pine aroma. β-ocimene and terpinolene have a citrus, woody aroma. β-myrcene has a peppery and woody aroma.
β-caryophyllene synthase	TPS12	α-terpinene, gamma bisabolene, caryophyllene, (E)-nerolidol and α-bisabolol	Sesquiterpene synthase. The VOC's have distinct aromas: gamma bisabolene sweet-spicy-balsamic odour. Caryophyllene has aroma of spice and pepper. Nerodol has a woody, bark aroma. α-bisabolol has a weak sweet floral aroma
Cineole synthase	TPS27	1,8 cineole (eucalyptol)	Also known as eucalyptol for its eucalyptus aroma. This compound is a monoterpenoid.
Linalool synthase	TPS14	linalool	Linalool has a fresh, sweet, pine and floral aromas. Linalool is a monoterpenoid.
Myrcene synthase	TPS24	β-ocimene, α-farnesene	Monoterpene synthase. β-ocimene has a citrus and woody aroma. α-farnesene has a sweet green-floral woody aroma.
Lupeol synthase	LUP2	β-amyrin, lupeol	β-amyrin is a triterpene with little to no scent. Lupeol is a pentacyclic triterpene with no aroma but has anti-inflammatory and is used in pharmaceuticals.
TPS07 synthase	TPS07	NA	General putative terpene synthase, currently not associated with any products

A few of the terpene synthases presented on table 1.3 are multiproduct; producing multiple VOC's simultaneously. Ocimene synthase is an example of multiproduct terpene synthases and has been found to be involved in plant defence against herbivory (Fäldt *et al.*, 2003). Ocimene synthase was found to be upregulated in response to mechanical wounding or treatment with jasmonic acid in *Arabidopsis thaliana* (L.) Heynh. As a result of increased gene expression, there was increased accumulation of β-ocimene. Most research reports a direct relationship between upregulation of terpene synthase gene and the volatile product, and this is usually proven through over-expression experiments. In addition, research focuses on one specific terpene synthase and if upregulated, it would be beneficial to the pharmaceutical industry. For example, the overexpression of α-zingiberene from basil (*Ocimum basilicum* L.) caused an increase in the accumulation of α-zingiberene and other sesquiterpene products when the gene was introduced into tomato fruits (Davidovich-Rikanati *et al.*, 2008).

There has been less focus on the expression of the terpene gene family in plants to evaluate the overall blend of aromatics produced. In basil the correlation between several aromatic terpenes and terpene synthases in the peltate glands was investigated by Iijima et al. (2004). They investigated three basil chemotypes and found nine terpene synthases expressed differently across the chemotypes and reported that the level of expression of each correlated with the total amount of terpene present in the essential oil (Iijima *et al.*, 2004). But for other plants the correlation was weaker which may indicate more complexity to terpene synthase and their regulation in plants. In tea tree (*Mealeuca alternifolia* ((Maiden & Betche) Cheel). Terpene synthases contributed to the patterns in aroma profiles, however they concluded that terpene synthase gene expression was not enough on its own to explain all the variation in the chemotypes suggesting that other regulatory factors were influencing expression (Padovan *et al.*, 2017). The link between terpene synthase gene expression and the aroma profile, the regulation of gene expression and the impact of environmental factors has yet to be explored in rosemary.

1.2 Antioxidant Production by Plants

Plants biosynthesise compounds with antioxidative properties, and are mainly phenolic compounds, carotenoids and vitamins. These compounds are produced by plants as defence against free radicals produced during photosynthesis and environmental stressors (Xu *et al.*, 2017). Antioxidants act to scavenge these radicals before they damage DNA or oxidise lipids and proteins in cells. Antioxidants act in similar ways in the human body, including fruit, vegetables, and herbs rich in antioxidants is important in our own physical and neurological health (Sohag *et al.*, 2022).

1.2.1 Rosemary antioxidant content, antioxidative capacity and use in industry.

Rosemary essential oil has been used by the food industry as a preservative due to its high antioxidant content mainly attributed to the essential oil compounds carnosic acid, carnosol and rosmarinic acid (Thorsen and Hildebrandt, 2003). Terpenes also play important roles in protecting the plant from abiotic stress through their antioxidant activity. Carnosic acid is a phenolic diterpene belonging to the isoprenoids plant secondary metabolites and has antimicrobial and antioxidant properties. Carnosic acid is produced through the isoprenoid pathway, using the diterpene precursor GGPP, it then has multiple enzymatic steps in the pathway and the final step is catalysed by diterpene synthase. The exact diterpene pathway has yet to be proposed, but it is thought that precursors for carnosic acid are from the MEP pathway catalysed by DXP or from crosstalk between the MEP and MVA pathway leading to the diterpene biosynthetic pathway (Brückner *et al.*, 2014).

Carnosic acid is abundant in rosemary essential oil and the oil is used as a food preservative. Its antioxidative capacity was not well known in planta and was investigated by Loussouarn et al (2017). The antioxidant system consists of carnosic acid and its oxidised form, carnosol. *Arabidopsis* plants subjected to stress conditions of high light and high temperature showed a decrease in carnosic acid and an increase in carnosol. It was found that reactive oxygen species (ROS) causes oxidative degradation of carnosic acid and carnosol protects the chloroplast from oxidative damage. Therefore, carnosic acid acts as a ROS scavenger that can be oxidised and converted into carnosol and other metabolites. Supplementing chloroplast membranes with carnosic acid

during high light stress reduced oxidative damage. This was confirmed by the consumption of the exogenously supplied carnosic acid during stress. *Arabidopsis* thylakoid membranes were subjected to high light stress and the oxidative damage caused a loss of chlorophyll by 30%. However, when thylakoid membranes were treated with carnosic acid chlorophyll loss was only 5%. Carnosic acid can protect chloroplast membranes against high light-induced oxidation (Loussouarn *et al.*, 2017). The concentration of carnosic acid varies between varieties of Rosemary plants, indicating that some varieties may be more tolerant to oxidative stress than other varieties. Finding varieties with a higher carnosic acid content may be of interest as the varieties can be used for the production of essential oils high in antioxidants. The antioxidant content and other secondary metabolite production will be used as a measure to select varieties for horticultural production of commercial rosemary. As previously mentioned, the MEP and MVA pathway activity produces the GGPP precursors for antioxidants such as carnosic acid, therefore antioxidant content in the leaves may be able to indicate a higher activity of the MEP / MVA pathways and the subsequent synthesis of aromatics through the monoterpene branch of the pathway.

1.3 Morphology

Rosemary shows high morphological variation across its natural range. From upright forms to low growing prostrate plants, highly compact shrubs to sparse stems, over branching or with strong apical dominance leading to few branches. There is variation in leaf and flower colour. Leaves are long and thin, rigid, and rolled over at the margins with a thick cuticle. These leaf adaptations are characteristic for warm Mediterranean climates, allowing rosemary to survive prolonged summer droughts and herbivory (Mateu-Andrés *et al.*, 2013). The observed morphology by De Mastro et al 2004, highlights the traits contributing the most to differences among rosemary taxa. Taller cultivars achieve height through two traits, a higher number of nodes and a long internode length (De Mastro *et al.*, 2004). De Mastro et al. 2004 classified rosemary cultivars by observing two leaf traits, leaf size and number of leaves per branch, with small leaf cultivars having many leaves and cultivars with large leaves with a lower number of leaves. Observation of morphological traits in rosemary allows for a greater understanding of available traits in rosemary for cultivar selection and breeding. The Sandy Mush herb nursery described the horticulturally cultivated rosemary in terms of plant habit, flowering, quality of the leaf, aroma and taste (The Sandy Mush Herb Nursery, 2004). The Sandy Mush herb nursery hold a collection of herbs including rosemary and have been collecting and selling herbs for 45 years in the UK. They have recorded the morphological characteristics of rosemary cultivars and provides a detailed description of the horticultural traits. These traits should be considered when selecting rosemary cultivars for horticultural production and can inform the variation within the taxa for breeding purposes.

A more detailed study of rosemary morphology was performed by Carrubba *et al*., 2020 studying the morphological trial observations of rosemary cultivars in Sicily to characterise cultivars. The morphological assessment included observing growth habit, flower colour, number and size of leaves, length and number of internodes. The characterisation, which was paired with GCMS of the volatile organic compounds and the genome size (via flow cytometry), allowed them to distinguish between wild and cultivated rosemary. The differences seen between wild and cultivated species may be because of cultivation practices that influence

the morphological characteristics, such as fertilizer use to maximise yield. Carrubba *et al*., 2020 also argued that the differences seen could also be due to trait breeding, as seen in traits such as leaf size which is important to growers as larger leaves was thought to correlate with higher yields of rosemary oil, and so most cultivars in Sicily have a larger leaf than the wild relatives. The characterisation of morphological traits in rosemary allows for better understanding of the traits available in cultivated and wild populations and will be useful for future breeding programs to create desirable cultivars (Carrubba *et al.*, 2020). In this study a similar assessment of rosemary cultivars was performed, and this forms the basis of selecting cultivars with the desired morphology for commercial horticultural production in supermarkets and for the domestic gardener.

1.4 Approaches to Plant Quality improvement

1.4.1 Light Quality Changes to Plant Morphology and Specialised metabolism

Light treatments are being used to manipulate plant growth of commercially grown herbs. Plants sense and respond to light throughout development and can quickly adapt to changes in light conditions. Plants are able to sense and respond to a broad range of the light spectrum, from UV-C to the far-red regions. The combination of wavelengths in the light source effects plant growth, development, morphology and metabolism (Carvalho *et al.*, 2016a). Red and Far-red light has been shown to improve essential oil production in rosemary (Mulas *et al.*, 2006). The addition of end of day light treatment with red and far-red light had a significant effect on the constituents of rosemary essential oil, compared with control plants not exposed to light treatments. Far-red light promoted the synthesis of the terpenes a-pinene, camphene and p-cymene. Red light was found to have the opposite effect of inhibiting the biosynthesis of these oil constituents but promoted the synthesis of other compounds such as limonene and bornyl-acetate which could also change the aroma. Far-red light was also found to increase oil production and this treatment also induced elongation of plat stems, resulting in taller plants (Mulas *et al.*, 2006).

UV-B light conditions have been shown to change the morphology of rosemary plants and improve the visual quality. UV-B radiation improved the growth through increasing the overall height and shoot length as well as increasing the number of branches and leaves. This improved the overall aesthetic value, by promoting elongation of stems and new leaf growth. However, high levels of UV-B radiation were found to decrease chlorophyll content decreasing photosynthetic efficiency which would lead to slower growth rates as a long-term effect (Hamidi Moghaddam *et al.*, 2019). UV-B radiation could be used as a supplementary treatment but, before this is possible, more information on how this lighting affects volatile synthesis needs to be investigated in rosemary. In basil seedlings, the volatile profiles grown under green, yellow or far-red light showed different volatile constituents. The addition of green and yellow lighting induced the accumulation of monoterpenes and phenylpropanoids. Far-red light was shown to increase the biosynthesis of sesquiterpenes in basil seedlings. There have been few reports on how light quality effects the aroma and taste of plant products. Understanding changes to the volatile and essential oil constituents is a first step to optimizing rosemary aroma and taste.

UV-C is widely known and used in industry as a treatment for fungal infections of crop plants. Rosemary grown in glasshouse conditions have been known to show powdery mildew infection. UV-C is commonly used as supplementary lighting in low, brief doses in order to kill fungal infections of powdery mildew in the leaves. Powdery mildew is caused by the fungus *Golovinomyces biocellatus* ex*. Salvia rosmarinus* which infects the leaves, stems, flowers and fruits of plants. The ultraviolet light penetrates the top epidermis of the leaf surface and can kill fungal cells. Subsequently UV-C light can be used to control and prevent powdery mildew infection as it is able to kill fungal cells and conidia germination (Pathak *et al.*, 2020).

UV-C light has also shown to have beneficial effects on the specialised metabolism in post-harvest fruits. Shen et al (2012) found that post-harvest UV-C light treatments increased flavonoid and phenolics quantities in mandarin fruit (Shen *et al.*, 2013). At 1.5kJ/m2 of irradiation the phenolic content of the treated fruit increased total phenolic content by 31.3% after two minutes of exposure. There is currently little research into the effect of UV-C light on specialised metabolism in herbs, but there is potential for this treatment to be used to enhance aroma and taste of rosemary. UVC lighting has been used in agricultural settings to boost phenolic content and it was found to have this effect for strawberries (F. Nigro *et al.*, 2000). They were treated with *vapour* lamps emitting UVC at a wavelength of 253.7nm, dosage ranged from 0.25 up to 4 kJ m-2. UVC has also been found to increase flavanol content in Lettuce, they used a pulse light system at lower intensity for a smaller surface area and would not be applicable to rosemary. (Fgaier *et al.*, 2019) found a great increase in flavanols in the leaves of lettuce grown under UVC. Tomato was found to have a significant increase in phenolics, mainly chlorogenic acid, when treated with UVC light at 3.7kJ/m2 of radiation for 16 minutes post-harvest (Urban *et al.*, 2016) and they determined total phenolic content (TPC) by the Folin assay and for individual phenolics using HPLC system against standards to ID. They also used qPCR on three specific phenolic compound synthases for flavanols. There is currently little evidence of UVC light effect on the volatile fraction of essential oils.

1.4.2 Isopropenoid Pathway is Regulated by Internal and External Signals

Plants emit volatiles “on demand” in response to external factors such as herbivore attack or oxidative stress. In response to herbivory, the leaves of *Melaleuca alternifolia* (Maiden & Betche) Cheel., plants released more volatile emissions compared to plants with no herbivore stress. Mechanical stress was also found to increase volatile emissions (Bustos-Segura and Foley, 2018). Mujiono et al (2021) found that certain volatiles are were strongly upregulated in rice in response to herbivore stress. Linalool synthase transcripts increased in the period of 1-3 hours post herbivore exposure (Mujiono *et al.*, 2021).Understanding the control mechanisms of terpene synthases is important for future improvement of volatile production and the overall aroma of the plant. Causing plant stress to improve aroma in plants is not ideal for the horticultural industry. Instead, the focus will be to alter the environment in a way that is non detrimental to the growth of the plant. Photoreceptors such as the phytochromes can also mediate an effect of light on isoprenoid pathway activities. In the MVA pathway, HMGR (3-hydroxy-3-methylglutaryl-coenzyme A reductase) mRNA levels and enzyme activity are induced in a *phyB* mutant while accumulation of products from the MEP pathway are repressed. Light intensity is an important factor for the production of antioxidant and phenolic compounds in the leaves. When aromatic plants are shaded, and have a low light intensity, the plants accumulated phenolic and

flavonoid compounds under light intensity of 630 µmol m^{-2} s^{-1} (Karimi *et al.*, 2013). Part of the shading response in plants is the perception of red: far-red light ratio, altering this ratio with supplementary lighting can lead to changes in phenolic compounds as found by Tegelberg *et al.,* (2004). They found that a higher ration of far-red: red light increased the concentration of chlorogenic acids and cinnamic acid compared to other light ratios (Tegelberg *et al.*, 2004).

Plants also emit volatiles on a rhythmic basis, controlled by the endogenous circadian clock timed by external light cues (Zeng *et al.*, 2017). Emission of volatile compounds are controlled rhythmically over a daily light/dark cycle and there is a diurnal emission pattern for rosemary volatiles from leaves peaking around midday (Nagegowda *et al.*, 2010). Knowing when rosemary plants would be at their most fragrant point throughout the day is important for harvesting and cooking. The regulation of volatile biosynthesis and emission is controlled by the endogenous circadian clock (Alexandra Pokhilko *et al.*, 2015). The circadian clock is a biological oscillator that maintains a 24 hour rhythm under normal environmental conditions and allows organisms to time their physiological processes in response to predictable day/night cycles (C. Robertson McClung, 2006). The MEP pathway and part of the MVA pathway is under control of the circadian clock to regulate the biosynthesis of terpenes. The circadian clock regulates certain enzymes in the volatile pathways. The circadian clock genes have different degrees of connectivity with the MVA and MEP pathway genes. Mostly all genes in the MEP pathway, with the exception of HDS, are significantly correlated with circadian clock genes from the morning loop. This indicates that expression of genes from the MEP pathway are switched on in the morning by circadian clock genes (Nagegowda *et al.*, 2010). There are fewer genes in the MVA pathway under the control of the circadian clock, with a few genes being controlled by clock genes in the evening loop (Vranová *et al.*, 2012). The circadian clock not only regulates the biosynthesis of terpenes in the isoprenoid pathways, but also regulates the emission of volatile terpenes to follow a diurnal pattern. The first report of monoterpene biosynthesis being transcriptionally regulated by the circadian clock was published by Lu *et al*, 2002. The expression of DXR and β-pinene synthases was investigated in *Artemisa annua* L., during a daily light/dark cycle. The levels of mRNA transcripts for both genes peaked around afternoon, showing a diurnal rhythm of expression. These levels of expression were retained during constant light or constant dark conditions, confirming the involvement of the circadian clock in the expression of these isoprenoid pathway genes (Lu *et al.*, 2002).

1.4.3 The role of Gravitropism in Determining Plant Form

Many rosemary varieties display a prostrate habit, because rosemary originates from arid Mediterranean regions and prostrate growth habits are an adaptation to high light intensities and drought. Such a habit is also commonly associated with an absence of gravitropic responses. Phototropism and gravitropism play important roles in providing plant organs with the ability to seek out and grow towards sunlight to promote photosynthetic efficiency. In complete darkness gravitropism is the dominating response guiding growth orientation, until the plant encounters a light source. Then photo-responses interact with gravi-responses to direct plant growth and the orientation of lateral organs. Gravity provides an almost constant stimulus that is the source of crucial spatial information about the surroundings and provides important cues for orientating plant growth during seedling emergence. Gravity also influences plant form during later stages of development

through its effect on lateral organs and supporting structures. A glance at mature plants is enough to show that the majority of plant organs grow at angles that are not parallel to the gravity vector. Thus, gravitropism plays an important role in determining whole-plant form in addition to the vertical positioning of the main axis of the stem root (Hangarter, 1997).

The effect of light on the orientation of lateral organs has been well investigated in *Arabidopsis* among other higher plants such as tomato (Sessa *et al.*, 2018). Shoot reorientation in response to light is a response present in most higher plants including Rosemary and the genetic pathway, controlled by phytochromes, is preserved in higher plants. When *Arabidopsis* plants are placed in darkness the petioles of rosette leaves bend up after several hours, the leaves become more vertically orientated as opposed to the more horizontal orientation they typically display in light (Hangarter, 1997). This indicates a light induced change in leaf angle is at least partly dependent on gravity and the perception of R:FR ratio through the action of phytochromes. Phytochromes photosensory systems act to modulate gravitropism responses, with light being the stimulus. The action of phytochromes in controlling gravitropism has mainly been studied in hypocotyls and in the *lazy-2* mutant of Tomato(Gaiser and Lomax, 1993). Phytochromes are red light photoreceptors that regulate negative gravitropism in hypocotyls, the activation of which leads to the loss of hypocotyl gravitropism and results in random growth direction (Kim *et al.*, 2011). Plant gravitropic responses can be divided into four steps consisting of gravity sensing, signal generation, signal transmission to the responding tissues and asymmetric elongation. Activation of signalling through phytochromes leads to loss of gravity sensing in responding tissues and non-directional growth of the hypocotyl occurs (Kim *et al.*, 2011). This study investigates if treating semi-upright forms of rosemary with far-red light would alter the orientation of shoots as gravi-responses are active when far-red light is sensed by the plant and phytochromes are inactive.

Lazy genes are thought to be involved in mediating gravitropic responses in plants and give genetic traits such as prostrate shoot growth. The function of *AtLazy1* was investigated in *Arabidopsis* to elucidate the role of Lazy genes in shoot gravitropism (Yoshihara and Spalding, 2020) They investigated the role of AtLazy1 in rescuing knockout mutants exhibiting increased branching angle and a less upright phenotype to the floral shoot. To investigate the protein function of *AtLazy1* Yoshihara and Spalding altered five regions of the AtLazy1 protein, resulting in dysfunctional proteins and were able to evaluate the phenotypical effects and sub-cellular localisation of the protein. They found altering region II with two conservative amino acid substitutions resulted in a switch of shoot gravity responses from negative to positive shoot gravitropism resulting in prostrate phenotype to the inflorescence. Further to this, they found that this alteration of region II reversed the auxin flow gradient normally established across the stems of the plant by the gravity-sensing mechanism. This indicates Lazy genes play a role in establishing and altering the auxin gradient, a new discovery that indicates mutation along the Lazy region II can lead to altered phenotypes in shoot growth. The disruption of auxin signalling in the shoot may influence the terpenoid pathways, as auxin gradient is necessary to induce flowering.

1.4.4 Edaphic factors for the Improvement of Volatile Production

Environmental factors have been known to influence the quality and quantity of essential production. Most studies concern the improvement of essential oil production and not VOCs. Yet the research presented here could be applied to volatile production and may show key improvements that can be made to the environment for the enhancement of rosemary volatile production. The use of organic fertilizers based on seaweed extract has been found to improve rosemary growth and essential oil yield (Tawfeeq *et al.*, 2016). Tawfeeq *et al*, 2016 found that seaweed fertilizers caused a significant increase in oil yield and caused an expansion in leaf area, when compared with inorganic fertilizers. Organic fertilizer showed a reduction in leaf biomass with an increase in leaf expansion. The decrease in leaf biomass was explained by a reduction in non-photosynthetic tissue in the leaf. The lack of sclerophyll (thick, non-photosynthetic tissue) allowed for an increased rate of photosynthesis as the photosynthetic tissue is not diluted by layers of non-photosynthetic tissue. The spray organic fertilizer aided fast absorption of nutrients into the leaf and allowed for quick cell division, explaining the increase in leaf area. As the leaves were capable of more photosynthetic activity, the plant was able to produce more essential oils. The observed increase in rosemary plant growth can be attributed to the contents of organic seaweed fertilizer. Organic seaweed fertilizers contain growth hormones, minerals, proteins and different polysaccharides that are not found in inorganic fertilizers. The organic fertilizers can positively affect cellular metabolism to give more vegetative growth and increase the number of secretory glandular trichomes. As this study focused on essential oil production it may be interesting to investigate the effects of organic fertilizers on VOC production in rosemary, particularly spray fertilizers which would be absorbed and used by the leaf to produce VOCs.

Salinity stress can also affect the composition of rosemary essential oils. Salinity reduces rosemary plant growth and effects essential oil components, such as a decrease in α-pinene and cineole and an increase in production of camphor and linalool (El-Esawi *et al.*, 2017). El-Esawi *et al*, 2017 found that salicylic acid significantly alleviated the morphological stress effects of salinity and increased the number of branches, plant height and fresh and dry weights of rosemary plants. Salicylic acid plays a crucial role in salinity and drought stress tolerance as well as increasing the production of secondary metabolites. The application of salicylic acid altered the composition of essential oils of saline stressed plants, compared with the control of SA-untreated plants. Under salinity stress, essential oil yield decreases but this effect was alleviated by the application of salicylic acid. Salicylic acid was found to increase the antioxidant capacity of rosemary essential oil, this is thought to be a result of stimulation of the antioxidant mechanism pathway by activating antioxidant enzymes (SOD, APX and CAD). Plant responses to abiotic stress, such as salinity, effect the yield and composition of essential oils through alteration of the specialised metabolism. Salinity stress has a negative impact on rosemary plants, as it reduces their essential oil yield and alters their secondary metabolism, which affects the composition and diversity of their VOCs. Therefore, the production of VOC's should also be affected by salinity stress and more stress tolerant rosemary varieties could have a higher VOC production and a different volatile oil composition. Rosemary varieties that are more resistant to salinity stress may have higher VOC production and a different volatile oil composition than those that are more sensitive to salinity stress. Mitigating salt

stress in rosemary plants is important to prevent changes in the quantity but also the quality and constituents of the volatile profile.

The presence of secondary metabolites and their antimicrobial activities makes colonization of rosemary leaves by phyllosphere bacteria difficult. Epiphytic bacteria have been reported on the leaves in extremely low numbers. The distribution of bacterial colonies on the leaf surface is heterogenous, due to the availability of hospitable sites on the leaf surface. There is a negative correlation between epiphytic bacteria colonisation and the total phenolic content of the leaves and the thickness abaxial epidermis. Rosemary leaves have a rolled edge towards the abaxial epidermis creating a thickness at the edge of the leaf. Due to the low quantity of colonizers on the leaf surface, rosemary can be classified as an incidental host rather than as a systematic host. VOC's have been shown to play a role in plant defence against pathogens by preventing colonization of the bacteria on the leaf surface and the higher the amount of phenolics and essential oils, the greater the defence against plant pathogens (Yadav *et al.*, 2005). As the phyllosphere of rosemary plants is limited due to the antibacterial properties of VOCs, the rhizosphere became the focus of investigation for biotic factors that could enhance volatile production.

The increased uptake of nutrients allows for an increased production of secondary metabolites, such as essential oils and VOCs. Yet adding more inorganic fertilizers to the soil do not always have the desired effects on phenolic and terpenoid compounds in Rosemary. Bustamante *et al* (2020) evaluated the effects of different fertilizer treatments on greenhouse grown Rosemary and had conflicting results. They reported that production of phenolic compounds was lower in fertilized rosemary plants, they concluded the fertilisation effects are a direct nitrogen trade-off between growth and the terpenoid pathways by which volatiles are synthesised (Bustamante *et al.*, 2020). Furthermore, for horticultural growers the additional use of inorganic fertilizers is not a viable solution because of rising costs and environmental challenges. The use of AMF as a plant stimulant as well as providing increased nutrient uptake for the soil would benefit commercial horticulture growing plants for enhanced aroma. Arbuscular mycorrhizal fungi (AMF) form an extensive hyphal network around the roots of the plant and extends the surface area for nutrient uptake from the soil. AMF also work by exchanging nutrients with the plants, such as nitrogen and phosphorus, in exchange for sugars and other carbohydrates (Parihar *et al.*, 2019). Rosemary forms associations with soil borne fungi from the phylum Glomeromycota, these fungi modify the plant metabolome and effect the antioxidant properties of the essential oil (Seró *et al.*, 2019). Inoculation of rosemary roots with AMF can increase shoot and root growth as well as increase essential oil yield (Camprubi *et al.*, 2015). The fungi-plant association enhances the acquisition of water and nutrients by the roots, through increasing root hydraulic conductivity or by modification of the root architecture (Sánchez-Blanco *et al.*, 2004). A meta-analysis performed by Beltrame *et al* (2019) found that AMF increased phosphorous accumulation on the shoots of Lamiaceae plants. The study found that AMF increases plant height and biomass through accumulation of nutrients in the shoots aided by the AMF nutrient uptake from the soil (Beltrame *et al.*, 2019). AMF has been shown to alter the polyphenolic profile of Rosemary by increasing the production of four antioxidative compounds ferulic acid, asiatic acid, carnosol and vanillin (Seró *et al.*, 2019).

Table 1.4 Summary of mycorrhizal supplementation research on different plant species.

AMF species	Plant Species	Reference
*Rhizoglomus irregulare (*syn. *Glomus intraradices)*	*Salvia rosmarinus*	Seró *et al.*, 2019
Rhizophagus irregularis (syn, *Glomus intraradices), Glomus mosseae*	*Salvia rosmarinus*	Camprubi *et al.*, 2015
Glomus deserticola	*Salvia rosmarinus*	Sánchez-Blanco *et al.*, 2004
Rhizoglomus irregulare, Funneliformis mosseae	*Ocimum basilicum* L.	Saia *et al.*, 2021
Glomus fasciculatum	*Lactuca sativa L.*	Baslam *et al.*, 2013
Funneliformis mosseae, Septoglomus viscosum	*Saliva rosmarinus, Salvia oficionalis* L., Sage, *Thymus vulgaris* L., Thyme and *Origanum vulgare* L., Oregano	Tarraf *et al.*, 2015

Arbuscular mycorrhizae are also being used in research to help mitigate the effects of abiotic stressors for field grown Rosemary. Previous studies have investigated the effect of AMF bio-stimulants on Rosemary growth and aroma while under environmental stressors, such as high salinity, drought, or nutrient deficiencies as these are the challenges that face field grown rosemary crops (Shenata *et al.*, 2019; Begum *et al.*, 2021). In glasshouse herbs, such as basil (*Ocimum basilicum*), the addition of fungal bio-stimulants to the substrate mitigated the effects of salinity stress and improved the production of aromatic products in the plant (Saia *et al.*, 2021). This study investigated the effect of AMF bio-stimulants in plants that were not saline stressed, they found the antioxidant content, through increases in rosmarinic acid production, in basil increased with AMF addition (Saia *et al.*, 2021). As the rosemary will be grown in a glasshouse under optimal conditions, they are not subject to abiotic stressors such as salinity or drought. Therefore, mycorrhizal associations can be used to improve volatile synthesis rather than mitigate the effects of environmental stressors. Using arbuscular mycorrhiza in a glasshouse with conditions for optimal growth has been shown to improve the volatile synthesis and shoot biomass of three species in the Lamiaceae family (*Salvia oficionalis* L., Sage, *Thymus vulgaris* L., Thyme and *Origanum vulgare* L., Oregano). The addition of arbuscular mycorrhizae to the soil increased shoot biomass, density of glandular trichomes and essential oil content of all three plant species. The AMF species *Glomus viscosum* showed a significantly higher increase in essential oil content and shoot biomass compared to the control (Tarraf *et al.*, 2015). The chemical profile of the essential oil also changed with arbuscular mycorrhizae association, changing the percentage composition of essential oil constituents of these plants (Tarraf *et al.*, 2015). The effect AMF has on the specialised metabolism to stimulate production of antioxidants and essential oil constituents (phenolics, carotenoids and flavonoids) is well researched (Baslam *et al.*, 2013; Begum *et al.*, 2021; Shenata *et al.*, 2019; Welling *et al.*, 2016). Baslam et al (2013) found that carotenoids and phenolics accumulated in lettuce with AMF addition to the soil. What is less known is how AMF addition to the soil influences terpene gene expression in the terpenoid pathways, particularly if AMF can increase, directly or indirectly, the expression of terpene synthases for volatile production.

Another consideration is the method of AMF application to the growing media, with focus on optimizing the method for commercial growing practices. Since the introduction of AMF into commercial markets for promoting plant growth and plant health, there has been no research to evaluate different application methods. Horticultural growers that add beneficial fungi also require a straightforward application method which can be incorporated into their current pipeline of commercial plant production.

There has been very little research on the effect of AMF on the volatile production and gene expression of terpene synthases, up until now the focus has been stress mitigation, increase plant growth and to deter potential plant pathogens. Some research reports an increase in phenolic compounds and essential oils in rosemary inoculated with AMF (Tarraf *et al.*, 2015; Seró *et al.*, 2019). Yet the interaction between AMF and the gene expression along the volatile pathway is not well understood. Therefore, this research undertaken would further the understanding of the link between terpene gene expression and volatile production as well as investigate the effect of AMF on terpene gene expression.

Summary

The traits involved in characterizing and selecting for a desirable rosemary cultivar extend from morphological characteristics to physiological characteristics; among the important traits for growers being leaf size, upright growth habit, green colour, high essential oil yield, aroma, and taste. These factors are determined by the genetics of each cultivar, and research into the morphological traits and the specialised metabolism of rosemary has revealed areas of improvement for rosemary horticultural production, as reviewed in sections 1.1 and 1.2. This research focused on the improvement of aroma production in rosemary, this is a quality improvement most desired by consumers and horticultural growers are looking for aromatic cultivars that will be desirable to the consumer. This research was undertaken because aroma is an important quality trait for rosemary, as it effects its market value. However, the aroma production in rosemary is not well understood, and there is a lack of information on how different environmental factors affect the biosynthesis and emission of aromatic compounds. The targeted areas for improvement include the production of aromatic compounds by rosemary, which is synthesised by terpene synthases in the MEP and MVA pathways under genetic control of environmental sensing genes and the circadian rhythm of gene expression (see sections 1.1.1 and 1.3.2). Since the aromatic production and the specialised metabolism is influenced by environmental factors (reviewed above in section 1.3) the growing conditions such as lighting, nutrient content of the substrate, AMF-root interactions, and temperature have an influence on the production of aromatic terpenoid compounds. The underlying mechanism of how aromatic production changes in response to different environmental conditions is not well understood. In this research the aim was to investigate the effect of environmental changes on the production of aromatic compounds in rosemary cultivars. The different lighting conditions, Far-Red and UV-C, were used to further investigate the molecular nature of the changes seen at the level of the aromatic volatile profile and the expression of genes involved in the production of aroma in the MEP and MVA pathways. The association between changing environmental conditions, and genetic expression in the MEP and MVA pathways, and its subsequent impact on aroma profile of several rosemary cultivars will be investigated in this research.

In this research AMF was chosen because they are natural root symbionts that can enhance the plant growth and health by providing nutrients and increasing the tolerance to stress. The use of AMF could therefore improve the quality and quantity of the aromatic compounds in the plant tissues. AMF can also affect the specialized metabolism of the plant, such as the biosynthesis and emission of terpenoids. AMF can also induce the production of antioxidants and other secondary metabolites, such as phenolics, carotenoids, and flavonoids, which can protect the plant from oxidative stress and enhance the aroma quality (See section 1.3.4). AMF can be applied to the growing media in different ways, in this research soil application was chosen because rosemary has been shown to make root associations with AMF and it the method of addition to the growing media can be optimized for commercial growing practices and can be compatible with the current pipeline of plant production.

By studying the effect of different environmental conditions on the aroma production in rosemary, this research aims to identify the key genes and pathways involved in the regulation of aroma synthesis, and to provide insights into the genetic and environmental factors that modulate the aroma profile in rosemary. This research can help to improve the breeding and cultivation of rosemary for enhanced aroma quality, and to increase the understanding of the specialized metabolism of terpenoids in plants.

2. Chapter 2: Materials and Methods

2.1 Total Phenolic Content and Antioxidant Content Assays

Total phenolic content was determined using the Folin-Ciocalteu assay, modified by (Sánchez-Rangel *et al.*, 2013). Plant extracts were prepared by grinding 0.2 g of rosemary leaf tissue in 2 ml of Acetate buffer using a pestle and mortar. The extract was transferred to a 1.5 ml Eppendorf tube and centrifuged at 10000 rpm for five minutes. The supernatant was then used either directly in the assay or diluted with acetate buffer. The dilution factor was accounted for during data analysis. Preparation of buffer; F-C reagent was diluted from the stock of 2 N to 0.25 N before use in the assay. Then the assay solution is prepared adding distilled water and sodium carbonate at 1 N. Then 300 µl of reagent was added to 15 µl of the extracted sample onto the plate and incubated for two hours. Gallic acid half serial dilution from 1000 µmol stock was used as a standard for comparison. The absorbance was read using a plate-reading spectrophotometer at 765 nm (Spectramax Plus 384 Microplate Reader, Molecular Devices LLC, UK). The Gallic Acid Equivalent (GAE) was calculated from the absorbance in mg of GAE/ g of fresh weight tissue.

The ferric reducing antioxidant power assay was used to determine the antioxidant capacity of rosemary extracts in ascorbic acid equivalents. The reagent was prepared fresh, consisting of 50 mmol/L acetate buffer at pH 3.6, 10 mmol/L TPTZ dissolved in 40 mmol/L HCl and 20 mmol/L Ferric Chloride Hexahydrate dissolved in 25 ml of water. Then 300 µl of this solution is then pipetted into each well containing 30 µl of the extracted sample. The absorbance was read immediately using a plate-reading spectrophotometer at 595 nm (Spectramax Plus 384 Microplate Reader, Molecular Devices LLC, UK). The Ascorbic Acid Equivalent (AAE) was then calculated in mg of AAE/ g of fresh weight leaf tissue.

2.2 Experimental Design for Supplementary Far-Red Light Treatment on Two Varieties of Rosemary

Two growth cabinets (Procema GmbH, Germany) labelled A and B were set to 20^{o}C, 12 hours light dark cycle set 7 am to 7 pm and plants were watered equally every two days when growing media was nearly dry. The supplementary Far- Red light treatment used LED strip lights and was set to the same conditions. Three plants of each variety were placed in the cabinets. Two plant varieties were used *Salvia rosmarinus* Spenn., 'Green Ginger' and 'Perigord'. Plants were pot grown in peat-based growing medium. Three replicate plants were kept in the cabinets for five days before taking leaf samples for analysis. Three samples of mature leaves were taken from the same place in each plant and were immediately frozen in liquid nitrogen. Samples used for determining antioxidant content and for RNA extraction were taken at 11 am to coincide with the circadian control of the MEP pathway. Light intensity levels in both cabinets were 50 µmol m-2 s-1. Upper cabinet had a red: far red ratio of 5.1, lower cabinet with red light treatment had a ratio of 0.2.

2.3 RNA extraction Protocol and Sequencing

RNA extraction was performed using the Qiagen RNeasy Plant Mini Kit and followed as instructed. Initial leaf tissue disruption (from leaves of three replicate plants) was by grinding leaf samples in liquid nitrogen using a pestle and mortar. An alternative lysis buffer was used to improve RNA yield, the lysis buffer was developed by (MacKenzie *et al.*, 1997) for plant tissues with high levels of phenolics and polysaccharides. The lysis buffer consisted of: 4M guanidine isothiocyanate, 0.2 M sodium acetate at pH 5.0, 25 mM PVP-40 (polyvinylpyrollidone) and 1% b-mercaptoethanol was added immediately before use. Then 20% sarkosyl was added to the sample lysate and incubated at 70 ^{o}C for 10 minutes. The extracted RNA was quantified using spectrophotometer NanoDrop (Thermo Scientific) and RNA integrity was checked by running on an RNA denaturing 1% agarose gel, with 37% formaldehyde, TAE was used as the buffer, and RNA was stained with ethidium bromide. Bands were visualised under UV light with the NuGenius (Syngene) imaging system. RNA sequencing was performed on illumina NextSeq500 Sequencer producing around 30 M reads per sample (3 samples per treatment, and 2 technical replicates per sample).

2.4 RNA Sequence Analysis

The illumina seq reads were processed using the online platform USEGALAXY eu, produced by Freiburg Galaxy Team (Online platform for UseGalaxy available at https://usegalaxy.org/). The following software was used in succession to determine differential expression of genes between two varieties of rosemary ('Perigord' and 'Green Ginger') and under white light vs supplementary far-red light conditions. The software used was as follows: FastQC by Babraham Bioinformatics (Labsquare Team *et al.*, 2017), Trimmomatic by USADELLAB, Trinity , DESEQ2 . The Trinity annotation was performed in a local server using the BLAST database (Camacho *et al.*, 2009) against the *Arabidopsis* Proteome obtained from UNIPROT accession code ID UP0000006548.

Function classification of gene expression was performed using MapMan software by Gabi and Julich, David Bioinformatics Resources (Huang *et al.*, 2009) and the iPath Interactive Pathway Explorer (Letunic *et al.*, 2008). Statistical analysis was performed in R-studio.

2.5 Design for Arbuscular Mycorrhizal Fungi (AMF) Addition to the growing media of Rosemary

A large-scale trial was conducted at Vitacress glasshouses under constant ambient conditions of 18 ^{o}C in the day and 19 ^{o}C during the night with a night/day schedule of 12 hours. Rosemary cuttings are pre-rooted, and the formed plugs are grown on in pots. The AMF mixture used was the RGPRO HORTI 2 (PlantWorks Ltd., Sittingbourne, UK) mixture composed of 5 AMF Glomus species: *Funneliformis mosseae*, *Funneliformis geosporus, Claroideoglomus clarodeum, Rhizophagus intraradices, Glomus micoraggregarum*. The soil capacity of each pot was 0.4L. Three conditions were used in this trial: a control condition of peat growing media. Method 1 involved a treatment of direct application of five grams of AMF directly to the planting hole, surrounding the root plug, and then planting the rosemary root plug on top of the AMF mixture. Method 2 involved a treatment of 25g of AMF mixed into the peat growing media, then then pot is filled with the media and the rosemary plugs planted into the pot. The current supermarket variety 'Perigord' was trialled with both AMF treatments, 12 pots were used as control of peat growing media, 12 pots were treated with 5g direct application, and 26 pots were treated with an AMF mixture. Plants were left to grow under controlled

conditions for 60 days. Random sampling was performed to take measurements of plant height, plant width, fresh and dry weight (g) of stem growth. Total phenolic and antioxidant content of the leaves was also taken, leaf samples were taken from the same area of each plant. Root samples were also taken from each condition to observe AMF colonization absence/presence to estimate percentage abundance. Roots from plants grown in the control peat growing media without AMF inoculations were also taken to verify absence of AMF.

2.6 Root Staining for AMF and Microscopy

The roots from the rosemary plants treated with the two AMF application methods were stained for fungal structures and observed under a microscope to estimate percentage colonisation. To prepare the roots for staining, rosemary plants were taken out of the pots, soil was removed, and the roots were cut from the stem. Then, the roots were washed with tap water to removing remaining soil. The following protocol was adapted to clear the roots of rosemary and stain AMF structures within the roots of the AMF treated plants. The roots of un-supplemented plants were also stained for AMF presence as a control. 5 g of root material was taken from the plant and washed in water to remove the substrate. Roots were placed in a 50 ml Falcon tube with 50 ml of 10 % w/v KOH and given a heat pre-treatment of 60 °C in a water bath for 1 hour. The roots were left to clear at room temperature for 24 hours. The cleared roots were rinsed and 5 % v/v HCl was added to the roots for 1 minute. The HCl solution was removed, and the stain trypan blue was added (0.01 % trypan blue, 2.5 % acetic acid, 50 % glycerol). Whole roots were measured then mounted on microscope slides and observed under a compound microscope. Colonization was quantified using the crosshair eyepiece method (McGonigle *et al.*, 1990). The roots were arranged on microscope slides and photographed using an eyepiece camera to validate quantification according to methods set out by Giovannetti and Mosse, 1980.

2.7 AMF trials on Different Cultivars of Rosemary

The trial was conducted at the Vitacress commercial glasshouse in Runcton, UK, under controlled ambient conditions in glasshouse, an average temperature of 20.2 °C (± 0.8 in the day) and average temperature of 18.3 °C (± 0.9) during the night over nine weeks. The experiment was carried out from February to April 2020 during springtime whereby rosemary as a perennial is growing well with the season, with natural light supplemented by SON-T high pressure sodium lamps (6000 lux) when the natural light was less than 8000 lux to maintain a light schedule of 12h light/dark cycle. Pots were filled with TPS peat substrate mix (Jiffy ProductsInternational, Moerdijk, The Netherlands). Pots were irrigated with potable water as required to keep the substrate moist. The AMF mixture used was the RGPRO HORTI 2 (PlantWorks Ltd, Sittingbourne, Kent UK) mixture. Two AMF application methods were used. The direct application method (method 1) involved 5 g of AMF mixture added directly to the planting hole of each pot at the repotting stage, then the rosemary root plug was planted directly into the hole containing AMF. The AMF mixture application method (method 2) involved 25 g of AMF mixture mixed into the peat-based compost, and dispersed evenly, per pot prior to planting the root plug. The control condition was peat substrate without the AMF mixture. In Trial 1, the cultivar Perigord was grown with both AMF application methods. Twelve pots were used as a control of just peat substrate, 12 pots were treated with the direct application (Method 1), and 26 pots were treated with an AMF mixture (Method 2). Plants were grown under controlled conditions for 64 days. In Trial 2, five rooted

plugs of each cultivar were potted in peat substrate containing the AMF mixture and were grown for 64 days in controlled conditions. At the end of the trials random sampling was performed to take measurements of plant height, plant width, fresh weight of the leaves and stems, dry weight of leaves and stems, total phenolic and total antioxidant content of the leaves. Root samples from five plants were taken from each substrate condition to assess AMF colonization.

2.8 RT- qPCR analysis of Terpene Synthase genes

Reverse transcription of extracted RNA was performed with the QuantiTect Reverse Transcription Kit (Qiagen, Maryland). gDNA was removed as per instructions, then 50 µL per sample was reverse transcribed at a final concentration of 1 µg of RNA. The cDNA synthesis was performed in a thermocycler with the following temperatures:42 ^{o}C for 60 minutes, followed by 70 ^{o}C for 10 minutes and a final hold of 4 ^{o}C. qPCR was performed on a Rotorgene 6000 (Qiagen, Maryland) using the primer sequences in table 2.1 and using GAPDH as the housekeeping gene. Three biological replicates of fully expanded leaves take from three individual rosemary plants (genetically the same through cultivated propagation) and two technical replicates of each sample were used. 0.5 µg of cDNA per sample was pipetted along with 1X SYBR Green master mix (QuantiTect SYBR Green RT-PCR kit from Qiagen, Maryland) with each primer set at a final concentration of 200 nM. A QIAgility robot (Qiagen, Maryland) automated pipette was used for pipetting accuracy. The following temperature programme was used for qPCR: an initial denaturing of 94 ^{o}C for 2 min, followed by a cycle of 94 ^{o}C for 15 secs, 58 ^{o}C for 45 sec and 72 ^{o}C sec for 30 cycles. Cycle threshold values were used to calculate relative expression using the ΔΔCt method (Rao *et al.*, 2013).

Table 2.1 qPCR primers for seven terpene synthases (Ocimene synthase, β-caryophyllene synthase, Cineole synthase, Linalool synthase, Myrcene synthase, Lupeol synthase, Terpene Synthase 07) and the Housekeeping gene GAPDH. Table shows sequences for the Forward and Reverse primers.

Gene	*Arabidopsis* ortholog	Forward Primer	Reverse Primer
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	AAGCATCGGAGACCAAGCTC	CGCGAGAACTGTAACCCCAT
Ocimene Synthase	TPS03	GGTACCACACGGGGCATAAA	CAAGATCATCTGCAAGCCGC
β-caryophyllene synthase	TPS12	AGACTGGCCGTAGCAAACTC	CCGATTGTTCAGGCAACACG
Cineole synthase	TPS27	CAGGCATCCTTGCCACATGA	GCCAAACGTTGAGAAAGCCC
Linalool synthase	TPS14	GCCAAATTCAGAGAGGCCCTT	TTGTCCGAGAAGGAAGCACG
Myrcene synthase	TPS24	TGACGCGAACCCTATTCTGG	CAAACCCCAACTTTTCCGGC
Lupeol synthase	LUP2	CTGGCTCTTCCCTTCCGTTT	TAAAACGACGTCGGTGAGGG
Terpene Synthase 07	TPS07	CGATGTTCGTGTTCTTGCCC	CCTTCAAATCTCCTCCCCCG

2.9 Experimental Design to assess current practices in rosemary cultivation.

UV-C light and cold storage were investigated for their effects on specialised metabolism. Three plant replicates from each condition (Control, treated with UV-C lighting, or with Cold Storage) were randomly selected from uniform plants (clonally propagated rosemary of same cutting age) three conditions; condition one was supplementary UV-C lighting, condition two was cold temperature storage in ambient lighting and condition three a control condition of the Vitacress glasshouse (18 ^{o}C in the day and 19 ^{o}C during the night with a night/day schedule of 12 hours, grown in peat soil, controlled irrigation, and biological pest management). The low dosage UV-C light treatment was additional to the control factors and was applied at 3am at night, with 6mJ/m^2 applied to the plants. A separate round of sampling was performed with high dosage UV-C light used. The High dosage UV-C light was also applied at 3am at night, with 11mJ/m^2. Plants kept in cold storage for multiple weeks, in this condition the plants are kept in storage glasshouses with natural light supplemented with glasshouse lighting on a 12 hour night/day cycle, and low temperature of 5-10^{o}C. Measurements and samples of the plants were taken in situ to monitor the effect of each condition. Leaf samples were taken and stored in liquid nitrogen for RNA extraction, antioxidant, and phenolic content.

Table 2.2 Summary of environmental conditions used to assess current practices in rosemary cultivation

Treatment	Controlled parameters	Condition of variable being tested
Control	18 ^{o}C in the day and 19 ^{o}C during the night with a night/day schedule of 12 hours Natural daylight supplemented by SON-T high pressure sodium lamps, output 140 mmol m^{-2}s^{-1} photosynthetically active radiation, PAR. Grown in peat substrate, TPS peat substrate mix (Jiffy Products International, Moerdijk, The Netherlands) controlled irrigation, and biological pest management	
Low Dosage UV-C Light	Same as above	Additional to control factors was application of UV-C light with 6mJ/m^2 applied over the top of the plants at 3 am.
High Dosage UV-C light	Same as above	Additional to control factors was application of UV-C light with 11mJ/m^2 applied over the top of the plants at 3 am.
Cold Storage	Same as above	Storage temperature of 5-10^{o}C

2.10 Headspace capture of VOC's for GCMS analysis

Volatile headspace profiling was performed by placing a potted plant in a polyethylene terephthalate bag and sealed at the top. Three replicates were taken from three plants of each cultivar grown in controlled

glasshouse conditions, and two replicates of the ambient air were taken. Air samples were collected and volatiles in the headspace were collected into thermal desorption tubes using Easy-VOC Grab Sampler hand operated pump by Markes Ltd (England). After collection desorption tubes were tightly sealed and stored at -20 ^{o}C to await processing. Volatile headspace profiling was also performed for the environmental treatments. Three plant replicates were collected in each condition (1. Supplementary far-red light, 2. Supplementary UV-C light, 3. Cold storage and 4. Ambient conditions in white light) along with a control consisting of air from the room in which samples were taken. Air samples were analysed by high spec gas chromatography mass spectrometry (Waters MALDI-TOF micro mx, MA USA) to determine volatile content by Dr Carsten Muller, Cardiff University School of Biological Sciences. The GC-MS VOC analysis was carried out as described by (Aros *et al.*, 2020). Samples underwent processing with the AMDIS 32 data analysis package and output was compared to a pre-existing library of volatiles (analysis carried out by Carsten T. Müller, School of Biosciences at Cardiff University, Cardiff, Wales, UK).

Chapter 3

3. Assessment of Rosemary Cultivars for Horticultural Production

3.1 Introduction

Rosemary as a taxon demonstrates a large range of morphological characteristics. The bio-morphological phenotypes of seven individuals from wild rosemary species have previously been characterized by De Mastro *et al*., (2004) and evaluated their usefulness for the horticultural industry. Although their work is not fully representative of the rosemary taxon, it does provide some insight into important traits presented in wild species of the Mediterranean region. They observed some correlation between traits, such as how plants with smaller leaf types have a larger abundance of leaves. Based on the leaf size the plants were grouped into three different types; type 1 is many and small leaves, type 2 is an intermediate with medium sized leaves, and type 3 is a large leaf size and low number of leaves. The defining of groups based on morphological characteristics is useful for rosemary selection for horticultural production. In this results chapter below there was an evaluation of the shape of rosemary plants and density groupings were designed to groups morphological types, similarly to the work of (De Mastro *et al.*, 2004). The density groupings were designated to include more features than just leaf size and leaf count. The density groups also include traits such as internode spacing, branching and the overall visual density of the plant.

Morphological characteristics have an influence over the selection process of rosemary varieties. The ideal rosemary varieties for horticultural production would be upright, taller than they are wide, be green in colour, have large leaves that are not too densely spaced and most importantly have a strong aroma of rosemary (Simon Budge Vitacress Herbs and Alistair Griffiths RHS Garden Wisley, personal communication, 2018). The following measurements were taken from all the living rosemary varieties present at RHS Garden Wisley and, as a comparison, measurements were taken from the current rosemary variety being grown at Vitacress. The measurements taken were height and width of the plant, mature leaf density, leaf width, leaf length, internode length, growth form (either prostrate, semi-prostrate or upright), colour comparison with the RHS Colour Chart and an overall plant density index was designed to group rosemary into one of three categories. Notes on aroma or distinguishing characteristics were also taken and the current list of varieties of interest can be viewed in appendix 1, table 1.

Since rosemary is valued for the properties of its essential oils, a key aspect of this evaluation is focused on the antioxidant and phenolic content of the leaf extracts. The antioxidant content is important to the food preservation industry and pharmaceutical industry and cultivars with high antioxidant content should be noted. The biologically active compounds in rosemary essential oil have multiple industry uses. As such, there are some economically important cultivars due to their chemical compositions (Sharifi-Rad *et al.*, 2020). In this chapter, the antioxidant content of selected rosemary varieties was assessed to investigate the differences in antioxidant capacity and secondary metabolite production. Assessing the antioxidant capacity of each variety will give an indication of secondary metabolite production and will also form the basis to evaluate the effect of

different environmental conditions on antioxidant production. Volatile organic chemicals are part of secondary metabolite production in Rosemary and contribute to the aroma and taste. Therefore, a variety with a higher secondary metabolite production may indicate higher production of aromatic compounds.

The aroma profile of rosemary has been investigated more in recent years as demand for aromatic cultivars by consumers increases. The aroma and taste have since become important traits to improvement of cultivars for supermarkets, and in other herbs such as basil the focus has been plant breeding to improve the aroma (Patel *et al.*, 2021). Patel *et al.,* (2021) linked the aromatics present in the volatilome of different basil cultivars to descriptions of aroma. The aromatic descriptors are important for industry as it gives an indication of the aromas of each basil cultivar and can be selected based on aromatic preferences, this work is also relevant to plant breeding and the creation of new cultivars with desirable aroma profiles. This breeding work for aromatics had been performed for basil (Ocimum basilicum L.) by da Costa *et al.,* (2014). They crossedthree types of aromatic basil with contrasting profiles and found a hybrid that exhibited a strong global aroma with a good sensorial profile (da Costa *et al.*, 2014). Since this aromatic breeding yielded results in basil, a similar approach can be applied to rosemary starting with aromatic volatile profiling. In this chapter the volatile profiles of several rosemary cultivars were analysed, furthermore the expression of terpene synthases responsible for volatile production of these aromatic compounds was evaluated in all the cultivars. To date, this side by side comparison of gene expression and biochemical profiling has not been seen before for multiple rosemary cultivars. There are possibilities in using terpene synthase gene expression as genetic markers of aroma production for rosemary, this could have the potential to be applied to marker-assisted breeding.

3.2 Methods

Morphological traits and grouping beneficial traits have an influence over the selection process of rosemary varieties. A conversation was held with Vitacress, Senior Agronomist Simon Budge and the RHS Director of Science and Collections, Alistair Griffiths and a review of the literature to ascertain the ideal rosemary varieties for commercial horticultural production and retail (Agriculture and Horticulture Development Board, 2018; Carrubba *et al.*, 2020; De Mastro *et al.*, 2004; Mateu-Andrés *et al.*, 2013) . The morphological assessment of rosemary was undertaken with a field trial of 87 representative cultivars located at RHS Garden Wisley, Royal Horticultural Society Wisley UK. Measurements were taken of plant height, plant width, leaf length, leaf width, internode length and the overall visual density of the plant was compared to the index below.

A Plant Density index was designed to group the rosemary into three categories based on the overall density of the shrub. This grouping is similar to the work of de Mastro *et al.,* (2004) who grouped rosemary into three groups based on leaf size, however the density groupings also include other traits that contribute to the grouping of varieties with similar visual characteristics. For prostrate varieties, the density groupings take into account the internode spacing and density of leaves, allowing these types to be grouped not solely on number of branches.

- Density 1: Sparse looking plants, with visible stems between leaves. Almost no side branching on the stem, indicating apical dominance.
- Density 2: the leaves are closely spaced but the stem is still visible and there is some side branching.
- Density 3: Very dense with compact foliage, the stem is barely visible and plenty of side branches to from a compact shrub.

The two supermarket cultivars of rosemary were also morphologically assessed and cultivars with similar visual aspects were selected. Measurements taken included height and width (cms) of the plant, mature leaf density (%), leaf width (cms), leaf length (cms), internode length (cms), growth form (either prostrate, semi-prostrate or upright) and colour comparison was obtained using the RHS Colour Chart. The method of measurements for potted rosemary are visualised in figure 3.1 .All 87 cultivars were included in the analysis of morphological traits and their similarities and differences were assessed by Principal component analysis (PCA) with six variables taken into account, performed on statistical software Past3, version 4.04 (Hammer *et al.,* 2013). PCA is a technique used to capture the maximum variance in the data, it standardizes each variable so that each variable contributes equally to the analysis and is not affected by the scale. The analysis of covariance included in PCA identifies the degree of correlation between each pair of variables. PCA was chosen over other methods to preserve the variance in the data set. PCA was chosen to analyse the morphological data because of the different scales involved in the capture of morphological traits, the PCA can standardize this and was used to approach the morphology of each cultivar in a holistic manner.

18 cultivars from the field trial were selected based on visual similarities. Through further physiological assessments (antioxidant assays and headspace GCMS, as described in sections 2.1 and 2.10) of the specialised metabolism, the cultivars with high levels of antioxidant activity and phenolic content were selected for volatile analysis using headspace capture GCMS. All plants were kept in in glasshouse at 22 ^{o}C in a 12-hour light/dark cycle for 2 weeks in peat-based soil before taking new samples taken from fully expanded leaves (from the third internode down from the apical bud of the stem) for antioxidant testing. Therefore, observed differences in antioxidant content are attributed to varietal difference in secondary metabolite biosynthesis. Antioxidant content was measured by FRAP assay (mg AAE/g FW leaf tissue) and the total phenolic content by Folin-Ciocalteu assay (mg GAE/g FW leaf tissue). Preliminary assessment of the secondary metabolites present was necessary due to financial restraints, best performing cultivars were selected for further analysis with GCMS. Gene expression of seven terpene synthase genes was evaluated by qPCR in the selected cultivars and expressed as relative gene expression using GAPDH as the housekeeping gene. Five cultivars were trialled for aroma testing at RHS Garden Wisley, Wisley UK. The aroma experiment was a pilot experiment set up for one week at RHS Garden Wisley, Wisley UK and invited the members of the public to vote for their preferred rosemary cultivar based on aroma alone. The cultivars were unlabelled, as to not hint at which aroma profile was sampled. Due to the covid-19 pandemic further aroma and taste trials could not be undertaken.

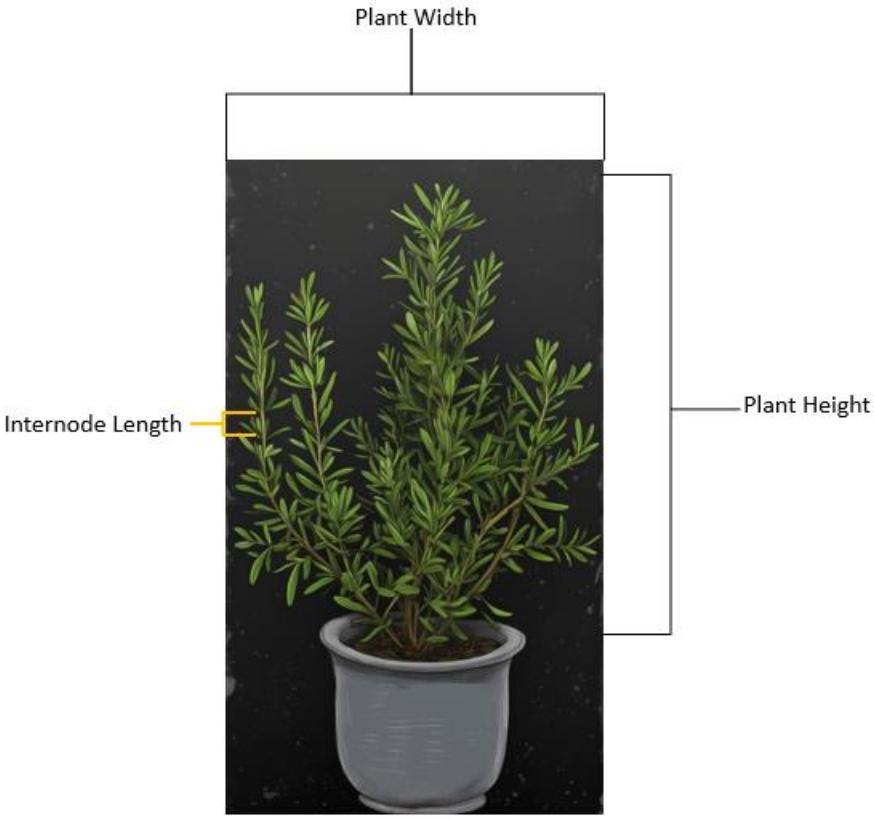


Figure 3. 1 Representation of the methods of morphological measurements taken for rosemary grown in a pot.

Plant height, plant width and internode length are visually represented. Plant width was taken in two dimensional planes, one of which is represented in the image. Plant height was taken from the top of the pot to the tip of the longest stem. Internode length was measured between two internodes, near the middle portion of the stem.

3.3 Results

3.3.1 Observation of Morphological characteristics

The Principal Components Analysis (PCA) (as defined in section 3.3.) of the measured characteristics for all 87 rosemary varieties shows some clustering of varieties based on the density index (figure 3.2). Clustered varieties have similar measurements. Two principal components were used to produce the scatter plot (figure 3.2) accounting for 60.34% of total variance observed in the data. The results of the principal component analysis show that component 1 (also referred to as eigenvector 1, contributes to 40.15 % of variance) correlates with leaf density. The results show as the mean internode length increases leaf density decreases, therefore, varieties with long internode lengths are less dense. Component 2 (contributing 20.19% of variance) correlates with leaf length, rosemary varieties 29 and 30 and Vitacress rosemary have long leaves. Leaf length varies from 6.3 mm to 30 mm. Leaf width varies between 0.5 mm and 4 mm, with the median being 2mm, and therefore has a weak correlation along component 2. Height/width measurements also has a weak correlation along component 1. Both height/width and mean leaf width measurements contribute less to clustering than the other three measurements. Clustering of varieties with similar characteristics can be observed, such as the clustering of varieties 10, 24 and 31 along component 1 all of which are prostrate or semi-prostrate form and of Density 3. Flattening along component 2 shows another clustering of Upright form density 2 for varieties 42, 45, 63, 68 and 80. Variety 72 is on the current variety of interest list (described below) and can be seen in proximity to current supermarket varieties, 'Abraxas' and 'Perigord' (represented by; A and P), on the scatter plot, indicating that variety 72 has similar characteristics to Perigord. This similarity between Perigord and variety 'Israel' was confirmed with a p value of $9x10^{-4}$ using the Pearson's correlation coefficient the results of which are presented in appendix 1, table 2. The principal components analysis can be used to find varieties with similar morphological characteristics to those of interest and, therefore, can be used later to find similar varieties that may have faster growth rates, have better cutting establishment or a more desirable aroma while retaining a similar appearance.

The following varieties were selected using the PCA. From the PCA, similar cultivars to the current supermarket cultivar were selected as they have a similar morphology desirable to the horticultural industry. These include varieties 'Israel' (72) and 'Primley Blue' (87), and 'Vatican blue' (83). Other cultivars retain desirable characteristics and were selected for their distinctive aromas, these include cultivars 'Logee Blue' (41), 'Stravordale' (83), 'Blue boy' (59), and 'Green Ginger' (38). A full list of all 18 chosen cultivars can be found in appendix 1.

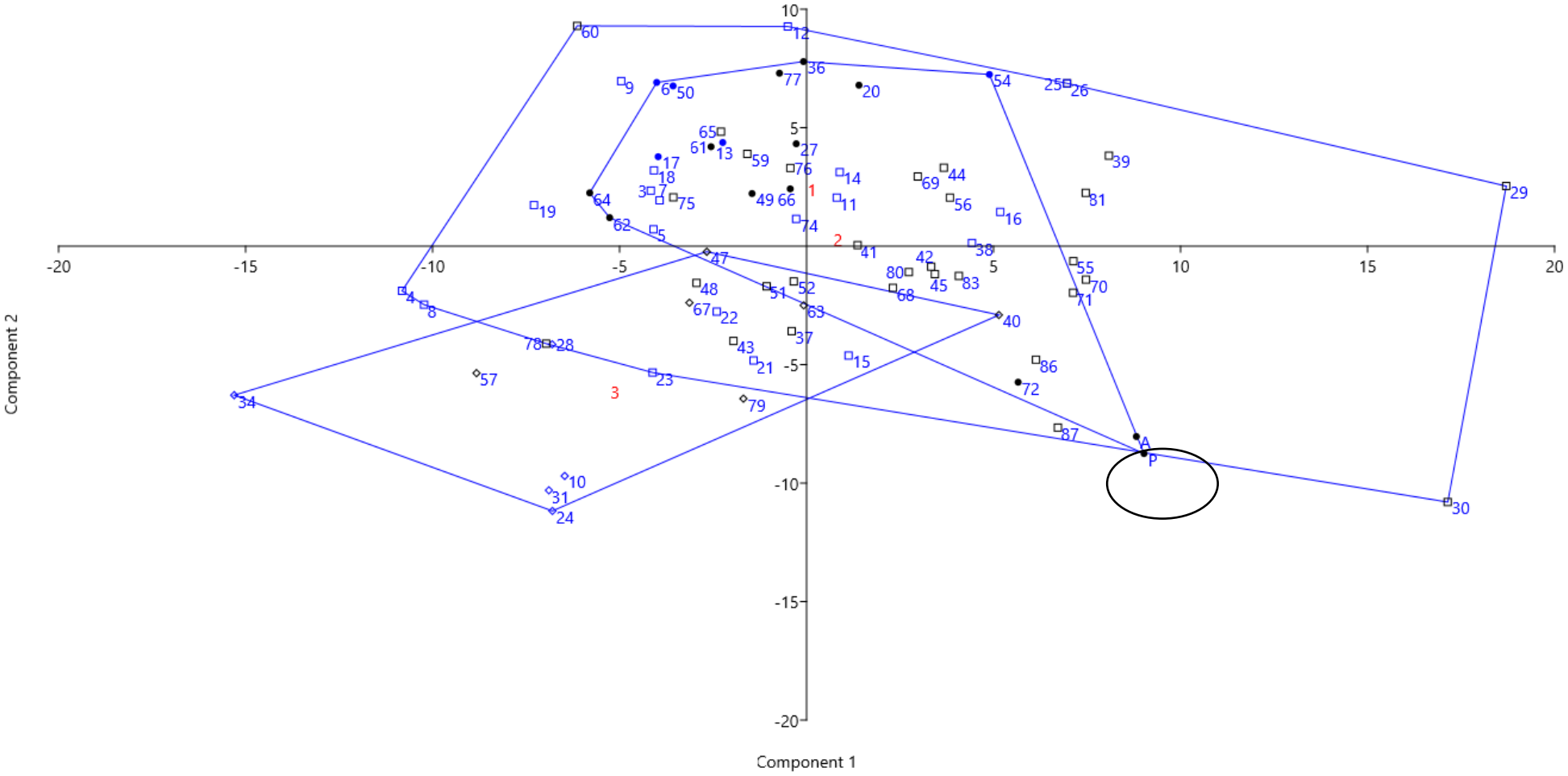


Figure 3. 2 PCA indicating similarities and differences of morphological characterstics in 87 rosemary cultivars.

Principal components plot of measured morphological characteristics for all 87 rosemary varieties and the current rosemary varieties being grown at Vitacress (represented by A, 'Abraxas' and P, 'Perigord'). Convex hulls represent the three density groupings, from highest density rated as 3 and lowest density rated a 1. Density 1 and 2 overlap and density 3 shows the most difference. Component 1 contributes 40.15% and component 2 20.91%.

3.3.2 Varietal differences in Antioxidant Capacity and Secondary Metabolites

The selected cultivars from the morphological selection are compared to the current supermarket variety called Perigord. The assays show varietal differences in antioxidant content. Rosemary cultivar Arp has double the antioxidant capacity of Perigord, while total phenolic content (TPC)varies among cultivars (figure 3.3). A high Antioxidant content tends to indicate the cultivar is a good producer of secondary metabolites. Many of the selected cultivars have a higher antioxidant content than Perigord, with Blue Boy having the lowest and Arp having double the antioxidant content of Perigord. The TPC measures phenolic compounds, including flavonoids and polyphenols with reductive capacity. The TPC seems to be variable in relation to the antioxidant content of each cultivar with some cultivars showing a high antioxidant content but a low TPC content, such as ARP. Varieties with higher TPC, notably Green Ginger, Vatican Blue and Barbecue, also performed well in the aroma public trials described later in figure 3.6.

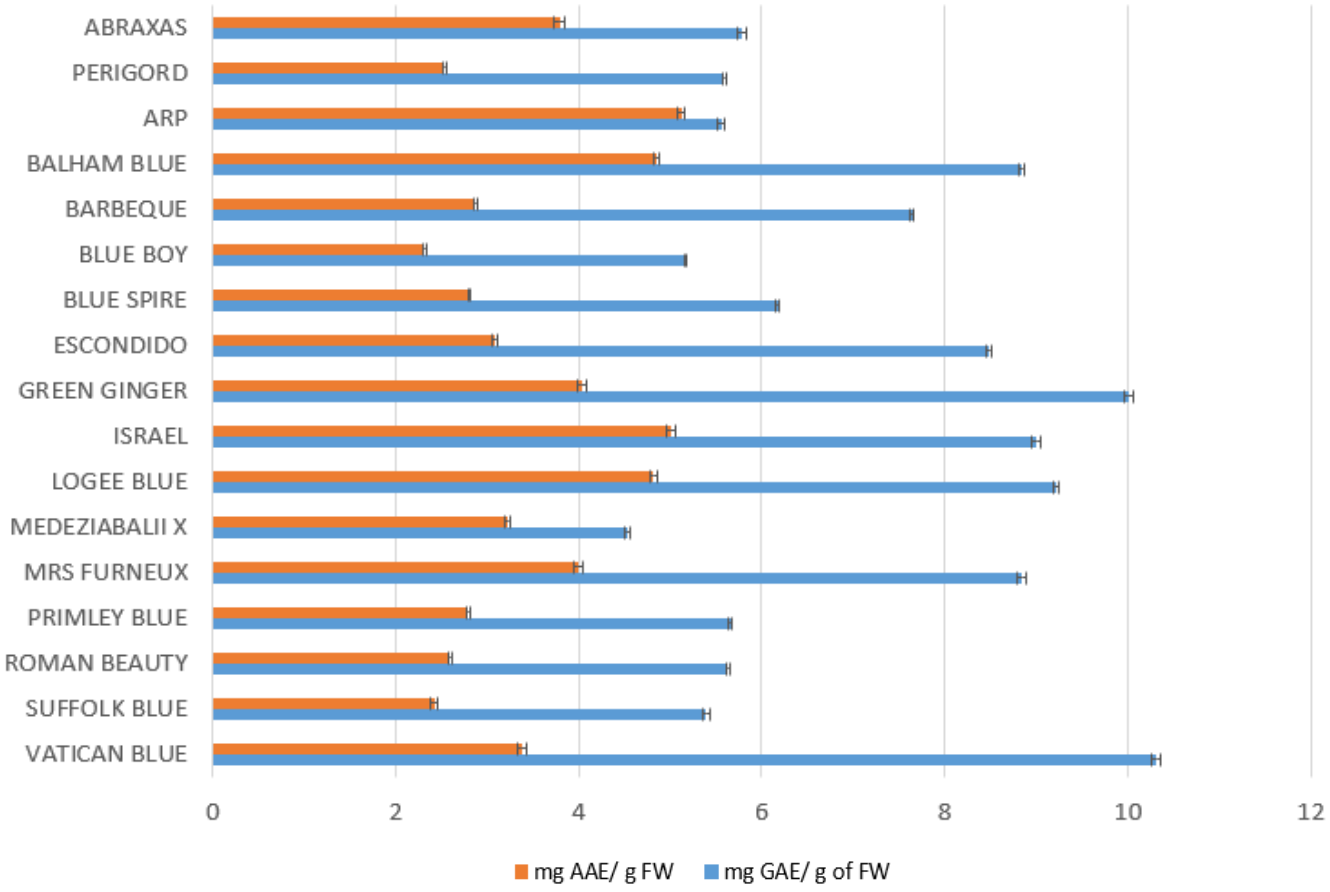


Figure 3. 3 Assesment of antioxidant and phenolic content of selected rosemary cutlviars.

Antioxidant and total phenolic content for the selected rosemary varieties. Rosemary shows a varietal difference in antioxidant and phenolic content. The selected varieties were compared to the current supermarket varieties, 'Perigord' and 'Abraxas' placed at the top of the chart. Error bars are SEM.

3.3.3 Volatile Profile of Rosemary Cultivars using GCMS

The volatile profiles of the selected rosemary cultivars have different constituents contributing to the differences in chemotypes (figure 3.4). Details and methodology of how the samples were taken for headspace GCMS can be found in section 2.10. Broadly, rosemary can be classified into three chemotypes based on the main constituents, these types consist of; 1,8 cineole (eucalyptol) type, camphor type, and myrcene type (Agriculture and Horticulture Development Board, 2018). For Perigord, the main constituents of the volatilome are eucalyptol followed by camphor. None of the other cultivars exhibit high quantities of eucalyptol in their volatilome, this is also present on the PCA analysis whereby no other cultivar groups close to Perigord (figure 3.4). The PCA analysis indicates that Abraxas, Blue boy, Roman Beauty, Vatican blue have similar volatilome, this would be due to high levels of camphor as their main volatile constituent (table 3.1). The cultivar Escondido is also grouped away from other cultivars, the main constituents of the volatilome is D-verbenone and β-pinene with low levels of camphor. The cultivar Logee Blue also had low levels of camphor, but other chemicals predominated in its volatilome, these were phellandrene, **β**-pinene, D-Limonene, D-verbenone and o-cymene (See table 3.1). Logee blue had a unique aromatic blend unlike other cultivars, and this is noticeable in its distinctive aroma. The cultivar Bolham blue has a similar aroma profile to Logee Blue, however it has high quantities of camphor which distinguishes it from Logee Blue in terms of descriptive aroma. Vatican Blue is the only cultivar to have high levels of myrcene, which is associated with aromas of spice and pepper. B-pinene is a volatile frequently found in the volatilome of rosemary, and in this analysis, it was present in high quantities in all cultivars. B-pinene is associated with aromas of pine and woody scents and α-pinene is an isomer with the same aromatic descriptors. Both cultivars Perigord and Escondido have the highest levels of α-pinene as well as β-pinene which gives aromatic descriptors for both cultivars of woody, piney and fresh scents. Cultivar Vatican blue was the only cultivar to have high levels of tricyclene, this chemical has significant importance to the antioxidative properties of rosemary essential oil.

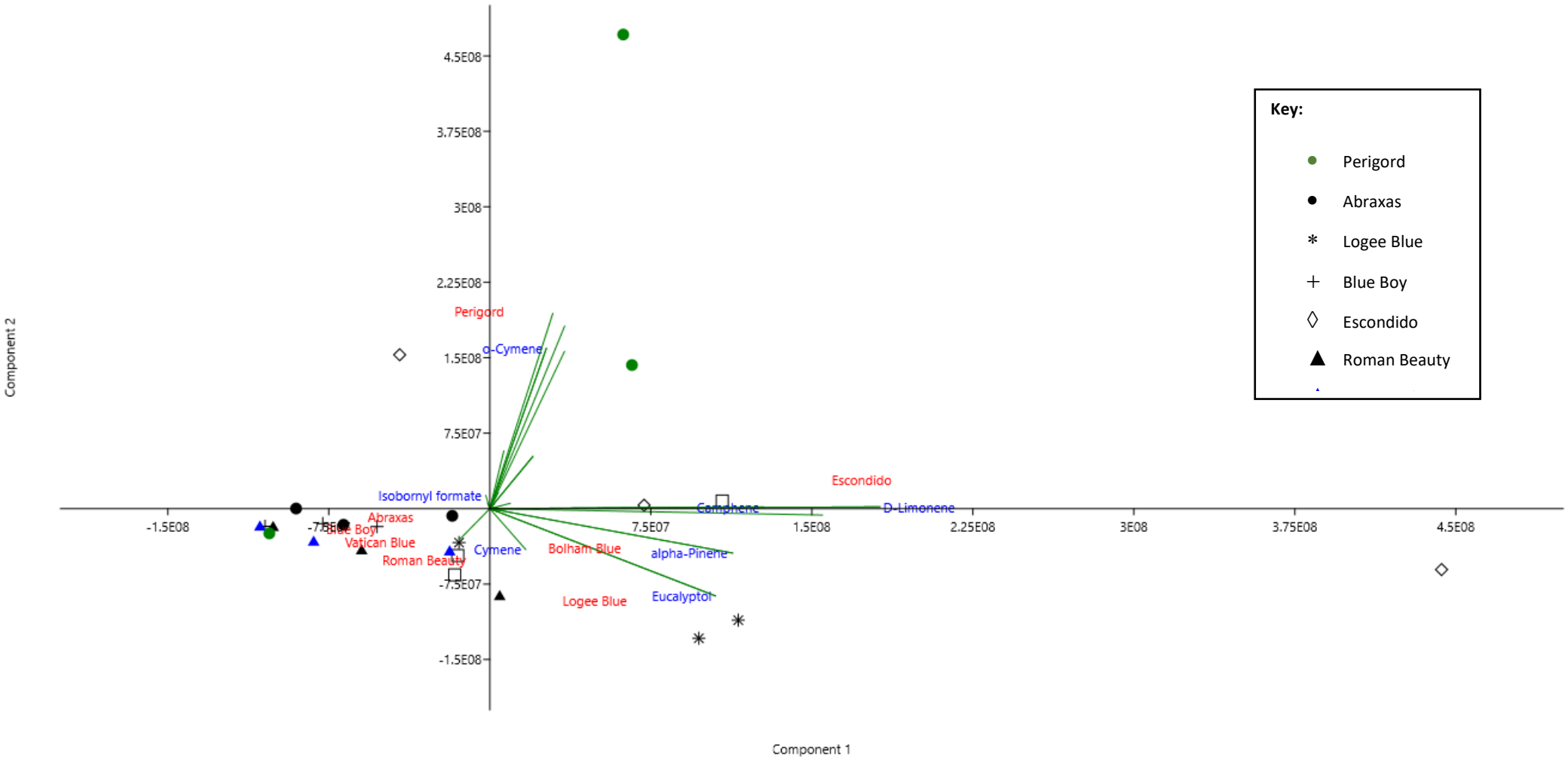


Figure 3. 4 PCA of the volatile composition of eight rosemary cultivars

PCA analysis bi-plot of all volatile chemicals emitted from in eight cultivars of Rosemary, detected by headspace GCMS. Symbols represent three biological replicates for each cultivar, the mean of which is represented by a red name label on the graph. The key indicates the corresponding symbols for each cultivar. Volatiles contributing to the most differences between cultivars are represented with blue text on the graph. Component 1 contributes 40.47 % and component 2 20.07%.

Table 3.1: Heatmap of volatile chemicals contributing the most difference to the volatile profiles of 8 rosemary cultivars (analysed with SIMPER analysis of the volatile reads from the GCMS output). Mean volatile emissions from each cultivar were recorded as PPM (part per million) and expressed as log values on the table.

Volatile name	Perigord	Abraxas	Blueboy	Bolham	Escondido	Logee Blue	Roman Beauty	Vatican Blue
Phellandrene	7.55	7.23	9.33	9.16	7.97	9.33	9.33	9.16
3-Carene	8.10	6.44	5.96	5.10	7.00	6.06	9.33	8.85
Acetic acid	7.39	7.27	6.84	7.23	7.87	7.56	7.44	6.81
α-Pinene	7.80	6.77	6.12	7.60	8.10	7.41	6.85	5.69
α-Terpineol	8.04	5.14	8.85	4.84	4.39	5.32	9.16	8.85
Aromatic	7.52	7.11	6.58	7.94	7.69	8.16	7.29	7.43
β-pinene	8.86	9.33	9.33	9.33	9.16	9.33	9.33	9.33
Camphene	6.80	7.22	6.17	7.56	7.82	8.13	7.77	7.26
Camphor isomer	8.85	9.33	9.33	8.85	5.49	4.72	9.16	9.16
carene	5.05	9.16	5.28	5.75	5.84	5.22	8.85	9.16
Cyclohexane	7.70	7.39	7.46	7.83	7.96	6.47	6.54	7.18
D-Limonene	9.16	8.85	9.33	9.33	8.85	9.33	9.33	9.33
D-pinocamphone	6.55	5.72	6.32	6.64	6.95	8.86	4.51	9.16
D-verbenone	9.33	9.16	9.16	9.16	9.33	9.33	9.16	9.16
Eucalyptol	9.16	6.66	6.93	6.68	7.53	6.42	6.21	6.15
Isoborneol	6.29	8.85	9.16	8.85	6.59	6.26	8.85	8.85
Myrcene	7.51	6.30	5.13	6.35	6.53	5.94	5.26	8.85
o-cymene	5.82	8.86	8.85	8.85	7.14	9.33	9.33	9.33
tricyclene	6.57	6.54	6.13	6.99	6.30	6.28	7.22	8.85

3.3.4 Gene expression of terpene synthases in several rosemary cultivars

The relative expression patterns of terpene synthases were different in each cultivar (figure 3.5), as was their volatile constituents (figure 3.4). Relative expression of each terpene synthase was calculated in comparison to the GAPDH housekeeping gene, therefore quantification of expression levels should not be compared between cultivars. Cultivar Perigord had high expression levels of linalool synthase and β-caryophyllene synthase was the second most expressed. Linalool synthase catalyses linalool which was present at intermediate levels in Perigord (log values of 7.16), cultivar Abraxas also had relatively high levels of expression of linalool synthase and linalool was present at intermediate levels in the volatiles (log value of 6.36). β-caryophyllene synthase is a sesquiterpene synthase that catalyses α-terpinene among other products. Other cultivars, namely Logee Blue and Escondido, had higher expression of this sesquiterpene synthase which may indicate a higher presence of sesquiterpenes in the essential oil. Bolham Blue had high expression levels of ocimene synthase which produces β-ocimene, β-myrcene and β-pinene which I consistent with its volatile profile containing high levels of β-pinene and o-cymene contributing to the overall aroma profile of Bolham Blue. Cultivars Logee Blue and Escondido had high antioxidative activity in their leaf extracts (figure 3.3), both cultivars had high expression levels of lupeol synthase, a triterpenoid synthase and the main product lupeol has antioxidative activity in the plant. Logee Blue and Bolham Blue had relative high expression levels of ocimene synthase responsible or the production of β-ocimene, β-pinene and myrcene. Both ocimene and cineole synthase can produce β-pinene, expression of both these terpene synthases are found at relatively high levels in two cultivars namely Bolham and Logee Blue. While other cultivars have base levels of gene expression in both or have higher expression in one of the genes, for example Blue Boy with cineole synthase as its most expressed gene. These results are indicative of a correlation between expression of terpene synthase gene expression levels and the quantity of emission of the associated biosynthesised volatile compound. Further analysis of this correlation was performed and is presented in chapter 8 section 8.2.5

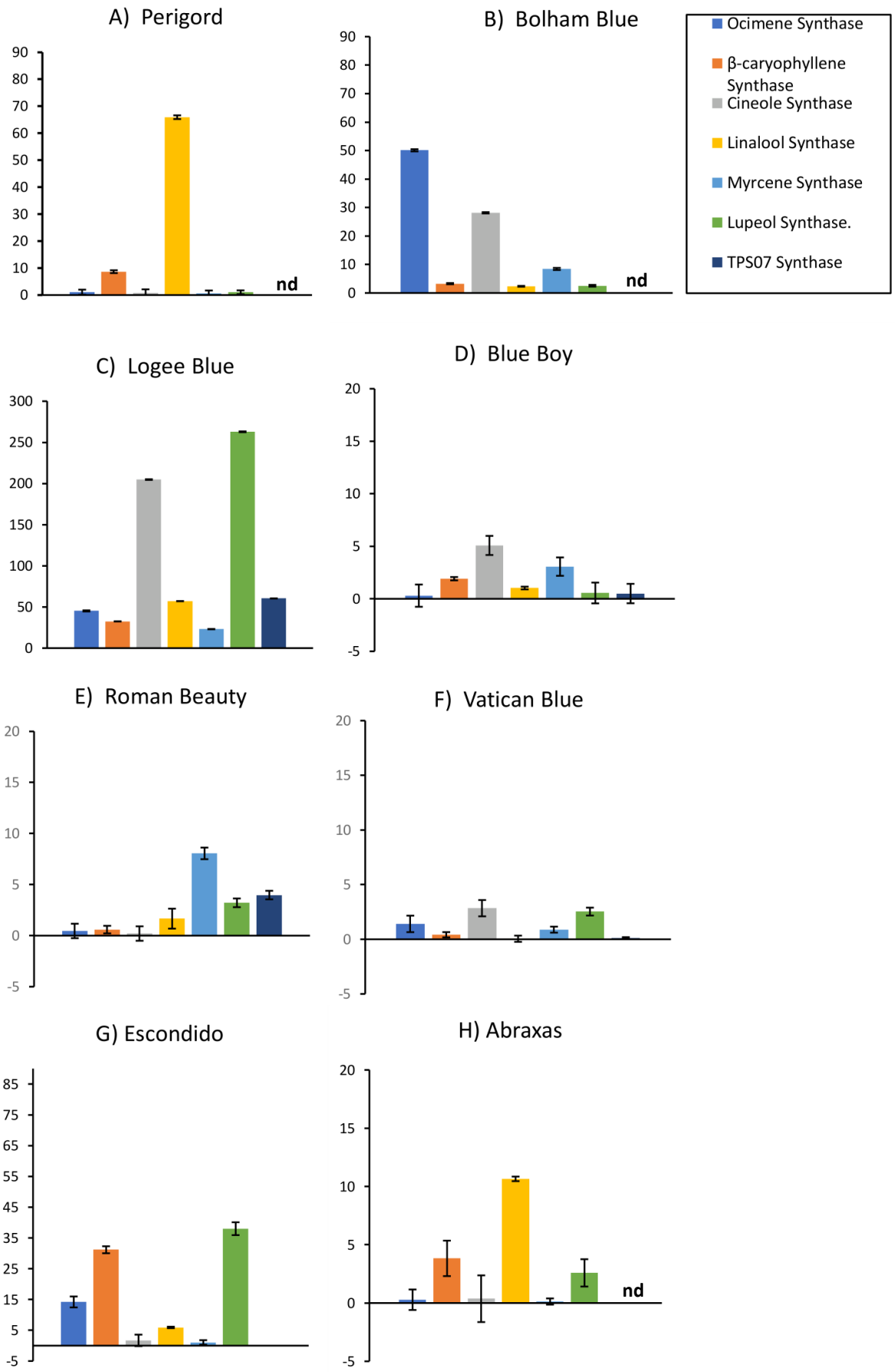


Figure 3. 5 Comparison of terpene synthase gene expression in eight rosemary cultivars.

Cultivar comparison of several terpene synthase genes in rosemary. Relative gene expression was calculated using GAPDH as the housekeeping gene. 'nd' represents no expression detected. Error bars are SEM.

3.3.5 Public Trials for the Preferred Rosemary Cultivar Based on Aroma

A consumer testing experiment was carried out to ask the public to vote for their preferred potted rosemary plant based on its aroma. These five varieties have noticeably different aromas, however due to financial restrictions and lead by further selections on morphological traits cultivars Barbecue and Starvodale could not be included in the aroma profiling with GCMS. For this experiment five varieties of rosemary were used, including the current supermarket variety, Perigord. The members of the public (there was no prior selection criteria, however the location of the trial in RHS Garden Wisley would limit the participants to gardeners and people interested in plants) were invited to smell the leaves of each plant and vote for their preferred rosemary aroma that they would use in home cooking. From this experiment, the variety Green Ginger was chosen by 42% of the public (represented by 923 total votes), followed by the Variety Vatican Blue with 25% of the public choosing this variety. Only 9% of the public preferred the aroma of the current supermarket variety, Perigord, over the other four cultivars. The aromatic descriptors (as defined on table 3, section 1.1.3) highlight the known aromatic differences between three cultivars. Since Green Ginger is the only cultivar with warm and spice aromas it is distinctive when compared to the other rosemary cultivars (table 3.2). Cultivars Starvodale and Barbecue did not have volatile profile analysis, or aromatic descriptors and are not included on table 3.2.

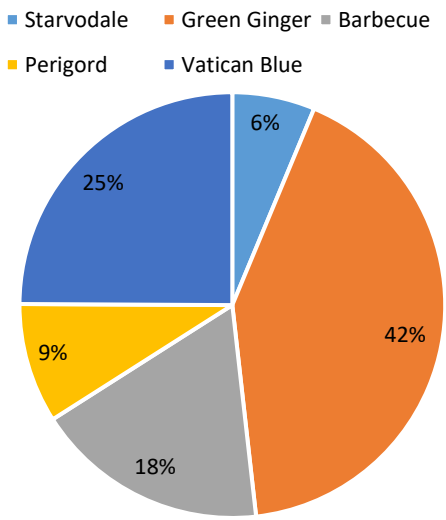


Figure 3.6 Public aroma trials of five rosemary cultivars

Public trials for five rosemary cultivars. The public voted for their preferred cultivar based on aroma alone. The variety Green Ginger had the most votes, the current supermarket variety had the second fewest votes. Total number of votes for male and female adults was 923.

Table 3.2 Aromatic descriptors of the main volatile constituents present in three rosemary cultivars Perigord, Green Ginger and Vatican Blue.

Cultivar name	Volatilome composition	Aromatic Descriptors
Perigord	β-pinene, Camphor, D-limonene, D-verbenone, Eucalyptol (1,8 Cineole)	Eucalyptol (1,8 cineole) type, with aromatic descriptors of pine, camphor, and eucalyptus. Other notes of citrus and orange zest.
Green Ginger	A-terpinene, β-pinene, Camphor, D-pinocamphone	Pine, woody, terpene, and herbal scent. Green ginger was also noted for ginger, spice, and warm aromas.
Vatican Blue	3-carene, β-pinene, Camphor, D-limonene, Myrcene, o-cymene, linalool	Myrcene type Vatican Blue has an herbaceous, woody, and resinous aroma. With the addition of citrus, and fresh pine aromas.

3.4 Discussion

3.4.1 Evaluation of the Selection process for Rosemary Cultivars

The selection of rosemary cultivars for horticultural production was based on the morphology, through visual appearance, desirable morphological traits and growth form. Selection was also based on the productivity of secondary metabolites, initially assessed through the antioxidant capacity (using the FRAP assay) and the phenolic content of plant extracts (assessed using the Folin- Ciocalteu assay for total phenolic content). These factors together guided the initial selection of 18 rosemary varieties (appendix 1). This selection was narrowed further based on performance in the secondary metabolite analysis, glasshouse performance and ease of propagation. The principal components analysis aided in the selection of cultivars with similar morphological characteristics to the briefing of desirable visual aspects as described in section 3.1, and similar to to the current supermarket varieties (Perigord and Abraxas). Varietal differences in antioxidant capacity and the phenolic content of leaf extracts forms an important part of the selection process. Rosemary varieties high in antioxidant and phenolic compounds may give a good indication of strong aroma and taste, as a higher production in secondary metabolites could also mean an increase in volatile and aromatic compounds. From this analysis, a further selection of cultivars can be chosen for volatile profiling using Gas chromatography/ mass spectrometry.

As the rosemary will be sold commercially in supermarkets, it is important to know what the public opinion is on the aroma and taste of different rosemary cultivars. The two cultivars with the most votes were Green Ginger and Vatican Blue. These two cultivars also have a high total phenolic content (as seen on figure 3.6), which may be a good indicator of that these cultivars have a high leaf content of monoterpenes, flavonoids and sesquiterpenes contributing to the volatile profile and aroma. The TPC assay may be a good initial test to

indicate high volatile content. The current supermarket variety Perigord, has a lower phenolic content compared to Green Ginger and Vatican Blue. The public response during the aroma trial was that the aroma of the current supermarket variety was weaker and received less votes. It is important to note that the supermarket variety Perigord was not bred specifically for aroma profile, it was bred because of other important horticulture factors as stated in the introduction section 3.1. The importance of consumer trials is to understand what the public look for when buying fresh herbs and will guide the selection process. There is also the chance to test novel varieties, such as green ginger, and see if the aroma and flavour would be appealing to the public. Cultivars producing different blends of volatile oils, assessed by GCMS, could also be subject to public trials to assess aromatic differences and public preferences. Volatile profiling with GCMS of selected cultivars can give a greater indication of the public's preferred aroma profiles. Enhanced environmental conditions to change volatile biosynthesis could be taste tested to see how growth conditions can make notable aromatic changes to one cultivar.

3.4.2 Rosemary cultivars provide a source of different chemotypes for industry

The eight rosemary cultivars assessed showed variation in their volatile constituents, contributing to differences in aroma and taste. The volatile β-pinene is a main constituent of rosemary essential oils and was present at high levels in all cultivars. This is due to its synthesis in the plants, β-pinene is synthesised by multiple terpene synthases independently. Ocimene synthase and cineole synthase are multi-product terpene synthases, and both produce β-pinene among other products (Roeder *et al.*, 2007). In the gene expression analysis, each cultivar expressed cineole synthase or ocimene synthase if not both, as in the case of Bolham Blue and Logee Blue. This could be the reason for high accumulations of β-pinene commonly found in the volatile profile of most rosemary cultivars. The presence of either ocimene synthase or cineole synthase genes could lead to production of β-pinene. However, of both genes are present in a particular cultivar that is not to say there will be increased production of β-pinene as this is limited by metabolic rates and presence of substrate for the reaction. The action of multiple terpene synthase in the genome could be contributing to this and amplifying the production of β-pinene as two or more separate terpene synthases are catalysing the same product. The detection of terpene synthase gene expression seems to indicate the presence of their products in the essential oil of the plant. However, this is not indicative of enzyme activity and further analysis is needed to link gene expression directly with their volatile compounds, here the conjecture is based on previous studies have been carried out to correlate synthase activity with terpene product (summarised on table 3 section 1.1.3) There is a need for genetic markers for aromatic quality in the plant breeding and production of herbs. After further assessment, there is a potential to use terpene synthases as genetic markers providing a fast-screening approach to classifying aromatic types. Overall, the use of gene markers to evaluate terpene gene expression has proven useful in this assessment of rosemary cultivar performance. This evaluation also has future potential to detect and monitor changes in terpene gene expression and in turn changes to the aroma.

The volatilome of the eight rosemary cultivars showed a large degree of biochemical diversity which has significance for industry. Rosemary essential oil is widely used by the food preservation industry for its beneficial effects as a natural antioxidant that can prevent colour deterioration and lipid oxidation (Irkin *et al.,*

2011). The demand for the use of natural preservatives rather than synthetic additives is increasing, and since rosemary is used by industry as a natural food preservative, high in antioxidants and with antimicrobial effects (Irkin *et al*., 2011), this forms a good basis to make improvements to rosemary cultivars. Tricyclene is a triterpenoid that has antioxidative properties but no aroma or taste. Tricyclene has been found to have antimicrobial and antioxidative effects and is present in herbs and other medicinal plants as part of the essential oil (Aleksic and Knezevic, 2014). From the volatile analysis, cultivar Vatican Blue showed high levels of tricyclene and the highest antioxidant content in the FRAP assay (figure 3.2). Tricyclene may be one of the acting antioxidants in the essential oil of Vatican Blue, contributing to the total antioxidant activity. Both methods of screening (using the antioxidant assays paired with the headspace GCMS) could be useful to industry to find potential cultivars for essential oil production. Lupeol is also an antioxidant and lupeol synthase had high levels of expression in Logee blue and Escondido coinciding with high antioxidative properties of their leaf extracts. Since lupeol is odourless their essential oil extracts can be beneficial to the food preservation industry. Lupeol synthase could be used as a genetic marker for chemotypes containing lupeol, other genetic markers would also need to be designed as there are more chemical constituents which contribute to high antioxidative potential. The food preservation industry requires that rosemary essential oil contain high levels of antioxidant activity but low levels of aromatics, as to not change the taste of the food being preserved. This contrasts with the horticultural industry, which has a requirement for aromatic cultivars. It is important to note the needs from plants by other industries, however this research will focus on aromatic production for horticultural rosemary growers which are desirable to the consumers.

Chapter 4

Investigation of Environmental conditions to Improve Rosemary Horticultural Production

Introduction to Chapter 4, 5 and 6

In the following three chapters different environmental conditions (biotic and abiotic) were investigated to evaluate their effect on rosemary from a physiological, morphological and genetic perspective. The environmental factors have been separated into three chapters and will be discussed separately. Some environmental conditions are already being implemented in glasshouses for the growth of herbs, such as UV-C and cold storage temperatures (Chapter 5). It is important to understand how rosemary plants perform with current practices, or under contrasting conditions, or with supplementary treatments in order to suggest improvements for the aroma and taste.

One of the aims is to understand the response of rosemary plants to changing environmental conditions, specifically on the volatile production and gene expression in the specialised metabolism. Light quality has been used to change the environment of the plant and changing light conditions is an environmental condition that can be easily implemented while maintaining other conditions constant. Plants also adapt quickly to changes in light, so the effect can be evaluated in a short time. For other herbs, such as basil changes in light quality has been shown to alter the morphology and production of secondary metabolites (Carvalho *et al.*, 2016a). Far-red light causes the shade response in plants, and the plant undergoes large changes through gene expression to adapt. Supplementary far-red lighting was used in this experiment to cause changes in gene expression in the plants and RNA sequencing performed on samples would show a greater span of the genome than in white light conditions alone. In Chapter 6 the use of supplementary far-red lighting to evaluate changes in rosemary gene expression and morphological responses will be discussed.

There has been a shift in the horticultural industry to use fewer chemical pesticides, fungicides, and fertilizers to grow plants. Abiotic treatments such as UV-C have been implemented by the horticultural industry for the prevention of powdery mildew (Janisiewicz *et al.*, 2016) as UV-C light inhibits fungal conidia germination (Pathak *et al.*, 2020). Biological pest controls and beneficial microbes have been implemented more in recent years to lessen the dependency on chemical fertilizers and pest control. In some cases, the biological equivalent does not perform as well, and there are many factors that need to be considered especially in the case of beneficial microbes. For Arbuscular mycorrhizal fungi (AMF) certain fungal species will associate with the roots of plants and the right combination is important to optimize the effect. Research has since addressed these issues, and research has been conducted to investigate possible plant-fungi combinations and the outcome is plant microbiomes specifically adapted to the crop for plant protection against diseases, to increase nutrient uptake, and reduce plant stress (Smolińska and Kowalska, 2018). Beneficial AMF and bacteria (such as those stated in table 1.4, section 1.3.4) have since been incorporated into many products for both the professional and home gardener markets as their success has been proven for growth enhancement and disease prevention. There are other benefits of using AMF that have only started to be investigated. In the

next chapter the use of AMF to improve plant performance in the absence of abiotic and biotic stressors and improve the final product in terms of visual aspects, aroma, and taste.

The following chapter 4 discusses the effect of arbuscular mycorrhizal fungi on the specialised metabolism, terpene synthase expression and morphology of six rosemary cultivars. The aim of this chapter is to evaluate the best application method of AMF in granule form to the root of rosemary and two methods were designed with the horticultural industry in mind for this. The method of application is important to the horticultural industry as it may involve extra processing steps in the glasshouse for the plants, some commercial growers may wish to limit changes to their current production line. For this an AMF application method was a mixture with the growing medium that could be done before filling the pots for planting. AMF has been beneficial in multiple crops for the enhancement of growth (Tarraf *et al.*, 2015; Begum *et al.*, 2021) . AMF has been shown to enhance the production of terpenoid products in other plants (Welling *et al.*, 2016; Seró *et al.*, 2019). This chapter evaluates the use of AMF to enhance volatile production and plant morphology for the horticultural production of rosemary.

There has been an interest in rosemary essential oil and research from a pharmaceutical perspective to evaluate the composition of essential oils used for food preservation. Other salvia species are of interest to the pharmaceutical sector as they have potential medicinal properties, so the genetics of these species has been studied more extensively than rosemary. Because of this, there are very few terpene synthase genes identified in rosemary in published research. It was necessary to design primers for terpene synthases in rosemary, and to achieve this the sequenced and annotated terpene synthases genes from the transcriptome assembly was used to design primers that would work on rosemary cultivars. Primers taken from previously published research did work for the rosemary cultivars in this work, furthermore there were a limited number of monoterpene synthase genes in published research that were specific to rosemary and that had primers designed to target them. The full transcriptome presented here allowed for specific primer design to target terpene synthases of interest. The transcriptomics also allowed for a detailed understanding of varietal differences in global gene expression, and more interestingly insight into the gravitropism response in Green Ginger (chapter 8). Since this transcriptomics was carried out prior to 2020, the rosemary genome and terpenoid genes had been published by Bornowski et al (2020) which showed terpenoid diversity in Lamiaceae and is a step forward to mining the biochemical MEP/MVA pathways (Bornowski *et al.*, 2020a).

The differences in aroma between rosemary varieties can to some extent be attributed to the expression of different terpene synthases. There are however two other factors that influence terpene synthase activity; firstly, although there was a detected difference in gene expression between varieties this may not have been translated to protein. Secondly, the different terpene synthases may also have different activities in different rosemary cultivars. Monoterpenes include a wide variety of compounds, each requiring a specific enzymatic step in order to be synthesised. These factors influencing terpene synthase activity summarised here are discussed in more detail in section 1.1.3. Evaluating the expression of genes involved in biosynthesis of the main VOCs in rosemary could be used to assess the aroma profile of a rosemary variety. qPCR will later be used to assess the expression levels of terpene synthases under different environmental conditions. Primers

designed for early synthesis enzymes in the MEP and MVA pathway will be used to identify the main production pathway for monoterpenes, diterpenes and sesquiterpenes in different rosemary varieties. The MEP pathway produces monoterpenes and diterpenes, and the MVA pathway produces sesquiterpenes. A more specific view of each terpene produced can be investigated using primers for the terpene synthases that produce volatile compounds such as eucalyptol, pinene and limonene. These three compounds found in volatile oils have a distinctive and characteristic scent and, when highly expressed, each can change the aroma of rosemary to produce a novel scent.

4. Addition of Arbuscular Mycorrhizal Fungi (AMF) to Enhance Aromatic Volatile Production

4.1 Introduction: AMF to enhance the aromatic volatile profile in rosemary cultivars

Rosemary displays a large variation in genetic traits, including variation in the aroma profile through selective breeding and creation of rosemary hybrids (Najar *et al.*, 2020). This natural variation is used as a tool for improving beneficial traits through cultivar selection. In the family *Lamiaceae*, there is a large chemo-diversity of terpene synthases, as studied in sweet basil *(Ocimum basilicum* L.), sweet marjoram (*Origanum majorana* L.), oregano (*Origanum vulgare* L.) and rosemary (*Salvia rosmarinus* Spenn.) (Bornowski *et al.*, 2020a). This diversity in terpene synthases provides the basis for breeding rosemary cultivars with desirable aromas by using gene markers for selected terpene synthases. The horticultural industry also seeks improvements to current rosemary cultivars on the market without the need for extensive breeding programmes. The following trials have been conducted to investigate the effects arbuscular mycorrhizal fungi (AMF) have on the volatile production in current horticultural rosemary cultivars.

The addition of AMF has been shown to improve the volatile production in rosemary (Tarraf *et al.*, 2015). Previous studies have generally investigated the effect of AMF bio-stimulants on rosemary growth and aroma while under environmental stressors, such as high salinity, drought, or nutrient deficiencies as these are the challenges that face field grown rosemary crops (Shenata *et al.*, 2019; Begum *et al.*, 2021). In glasshouse herbs, such as basil (*O. basilicum* L.), the addition of fungal bio-stimulants to the substrate mitigated the effects of salinity stress and improved the production of aromatic products in the plant (Saia *et al.*, 2021). However, another study conducted by Saia et al. (2021) investigated the effect of AMF bio-stimulants in plants that were not saline stressed. They found the antioxidant content in basil increased with AMF addition through increases in rosmarinic acid production (Saia *et al.*, 2021).

The addition of fertilizers (inorganic and organic) to the growth substrate of rosemary revealed a positive correlation between total terpene compounds and the nitrogen and phosphorus content in leaves (Bustamante *et al.*, 2020). It was suggested that the increased availability of nitrogen for terpene synthase activity, and the availability of phosphorus for precursors in the MEP pathway increased aromatic volatile production. Bustamante et al (2020) also reported that the fertilisation effects are a direct nitrogen trade-off between growth and the terpenoid pathways by which volatiles are synthesised. Therefore, increasing fertilization throughout the growth of the crop may not directly benefit the terpenoid pathway. Mycorrhizal

associations in rosemary have previously been found to enhance growth and antioxidant properties of the leaves through increases in carnosol, ferulic acid, asiatic acid and vanillin (Seró *et al.*, 2019). It is known that specific mycorrhizal and plant species associations improve growth in plants and can improve production of secondary metabolites (Emmanuel and Babalola, 2020). The specific mycorrhizae and plant species interactions are summarised on table 1.4 section 1.3.4. It is less well understood how AMF influence gene expression in specialised metabolism, and, in particular, terpene synthase expression.

One of the main challenges facing the horticultural industry is to find solutions that improve the growing conditions and the aroma profiles of the plants. In addition, there is a need in horticulture for environmentally conscious and sustainable solutions whereby there can be a reduced use of chemical sprays and fertilizers. Included in this, is the reduction of peat use in growing substrate, during this experiment peat was still in use but other media should be trialled, and it is predicted that AMF associations will form similarly in peat free soils (more detail on the type of peat soil used can be found in section 2.7). Mycorrhizal fungal associations with the plant can provide part of a solution to sustainable horticulture as they enhance metabolite production by increasing plant nutrient uptake. The aim of this investigation was to assess the effect of beneficial mycorrhizae on gene expression of terpene synthases for improved volatile production in horticultural rosemary. The effect of AMF on physiology and terpene synthase expression among different rosemary cultivars was investigated to better understand how cultivars respond to AMF in terms of growth and aroma production.

4.2 Methods

In this experiment, the cultivar Perigord was used to investigate whether AMF addition to the soil improved the aroma production in a variety with a less distinctive aroma profile than other rosemary cultivars. Details of experimental design can be found in section 2.7. The addition of AMF to the soil of the current supermarket cultivar, Perigord, was used to assess the change in aromatic content. This was achieved by performing trials at Vitacress involving potting on rosemary cuttings in peat soil with the addition of one of two treatments of AMF or a control of un-supplemented peat soil. The plants were left to grow in horticultural greenhouse conditions. There were two treatments for the addition of AMF to the soil; AMF treatment 1 was a mixture of AMF into the peat soil per pot of rosemary and AMF treatment 2 was a root plug application of AMF per pot of rosemary. The inoculated roots were stained and observed under a microscope to estimate percentage colonisation. The un-supplemented plants were kept apart from the AMF treatments to prevent cross contamination, they were also stained for fungal structures to verify absence of AMF colonisation in the roots. There were no AMF observed in the control plants when visually inspected under the microscope.

The plants were assessed for both morphological and physiological aspects. Firstly, the morphological aspect was assessed to investigate any changes to growth after AMF addition compared to growth in peat soil. The assessment included measurements of height and width and the weight of the shoot biomass. The weight of the shoot biomass was performed as an indicator of plant growth. Secondly, the antioxidant capacity and phenolic content was assessed with the addition of AMF by FRAP assay and Folin assay. The gene expression of seven terpene synthases was evaluated by qPCR with the addition of AMF to the growth media and in un-

supplemented growth media. The mixture of AMF into peat soil was further trialled on six rosemary cultivars and terpene gene expression was evaluated in each cultivar.

4.3 Results

4.3.1 AMF addition to the substrate increases phenolic content and alters gene expression of terpene synthases without altering the morphology of rosemary 'Perigord'

The fresh weight and dry weight of the plants was taken to assess any growth difference by measuring the total biomass of the stems and leaves of the plants. Graph A) shows the fresh weight and dry weight biomass measurement of plants treated with two applications of AMF: the AMF mixture into the soil of the potted rosemary (AMF treatment 1) or the root plug application method (AMF treatment 2). As can be seen there is no significant difference in the biomass between the control condition or the two treatments with AMF (Two-way ANOVA test p-value= 0.141, F value= 2.243). The Height and width data also show no significant difference in the height and width of AMF treated rosemary and the control condition (ANOVA p-value= 0.85).

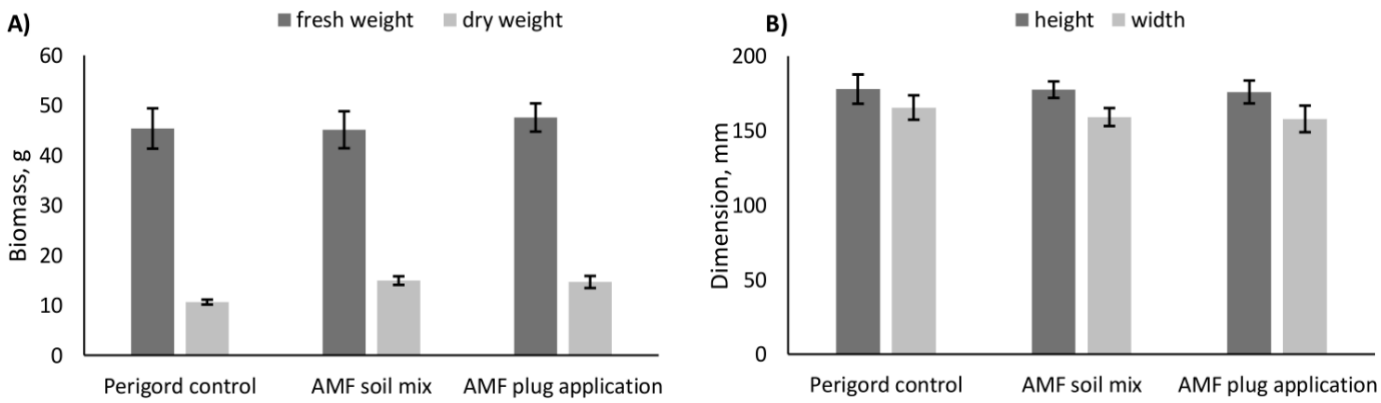


Figure 4. 1 Morphological assessment of Rosemary grown in soil with additional Arbuscular Mycorrhizal Fungi.

A) Fresh weight and dry weight of the stems of 6 plants of rosemary variety Perigord under two AMF treatments. The second AMF treatment was application of AMF surrounding the plant plug upon potting. The first treatment is an AMF mixture into the soil used for potting up the plugs. B) average height and width measurements of 5 plants from each group; control and two treatments of AMF. Error bars are SEM for each AMF condition.

In figure 4.2, part A) the percentage colonisation of 5 root samples from the two AMF treatments is shown. The mean percentage colonisation for the AMF mixed into the soil is 22.01%. While the mean colonisation of the AMF root plug application had a higher mean, the standard error of the mean is suggesting that this application method is more variable, and this was visible under the microscope with a more uneven distribution of vesicles in the roots [SEM = 8.14]. While the AMF mixture had vesicles more evenly distributed along the roots, and, therefore, had a lower standard error of the mean [SEM at 2.88] (even distribution of vesicles was seen with microscopical staining and determined by visual observation, Figure 4.2). This even distribution can be seen in image C) of figure 4.2, where AMF vesicles are consistently encountered along the root length. Statistical Student's T-test showed there was no significant difference between the percentage

colonisation of roots treated with AMF mixed into the soil and the roots treated with a root plug AMF application [Student t-test, p value=0.721837].

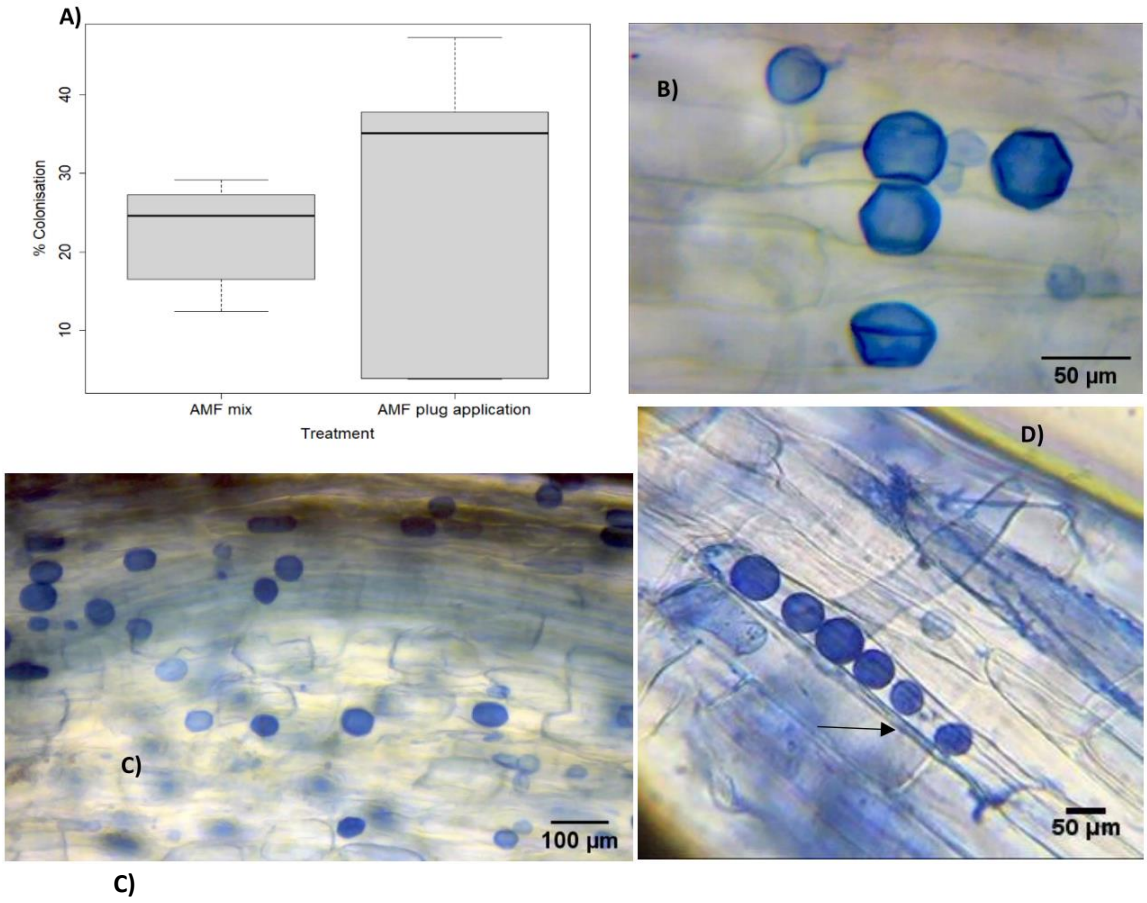


Figure 4. 2 Observations of Arbuscular mycorrhizal fungi colonization in the roots of Perigord Rosemary.

A) Percentage colonization of two AMF treatments; an AMF mixture added to the soil (orange) and an AMF application to the root plug (blue). B) Representative image of fungal staining of AMF intra-cellular vesicle with hyphae extension. Taken at 40 x magnification Scale bar =10 µm. C) Representative image of root staining for treatment AMF mix taken at 5 x magnification showing the abundance of vesicles in the root in a 1mm section. Scale bar = 100 µm. D) Representative root staining image taken at 20 x magnification shows AMF vesicles within one root cell with hyphae extension running the length of the cell. Scale bar= 50 µm. Root length of samples were 10cm, and 6 replicate slides were observed per treatment.

Figure 4.3 shows the preliminary assessment of secondary metabolite production in rosemary variety Perigord treated with Arbuscular mycorrhizal fungi. Figure 4.3 A) shows the phenolic content, and as can be seen, there is an increase in phenolic content for Perigord treated with the root plug application of AMF (AMF treatment 3) compared with the peat soil control. Statistical anaylsis of the phenolic content of Perigord treated with AMF no significant difference in the phenolic content [One way ANOVA p-value= 0.873 F value= 3.763]. Further investigation showed a significant difference in the phenolic content of Perigord treated with AMF root plug appication(AMF treatment 3) and the control of peat soil [Student t-test p-value= 0.01]. This suggests there is an increase in the phenolic content of rosemary leaves with the adittion of AMF directly to the plug of the cutting when potting on. This is a mean increase of 33%. Figure 4.3 B) shows the antioxiant content of Perigord with the two AMF treatments. There is no increase in antioxidant content with AMF addition to the soil. Statistical analysis shows there is no significant difference in the antioxidant content of rosemary variety Perigord after the AMF treatements [One way ANOVA p-value= 0.854 F value= 0.162].

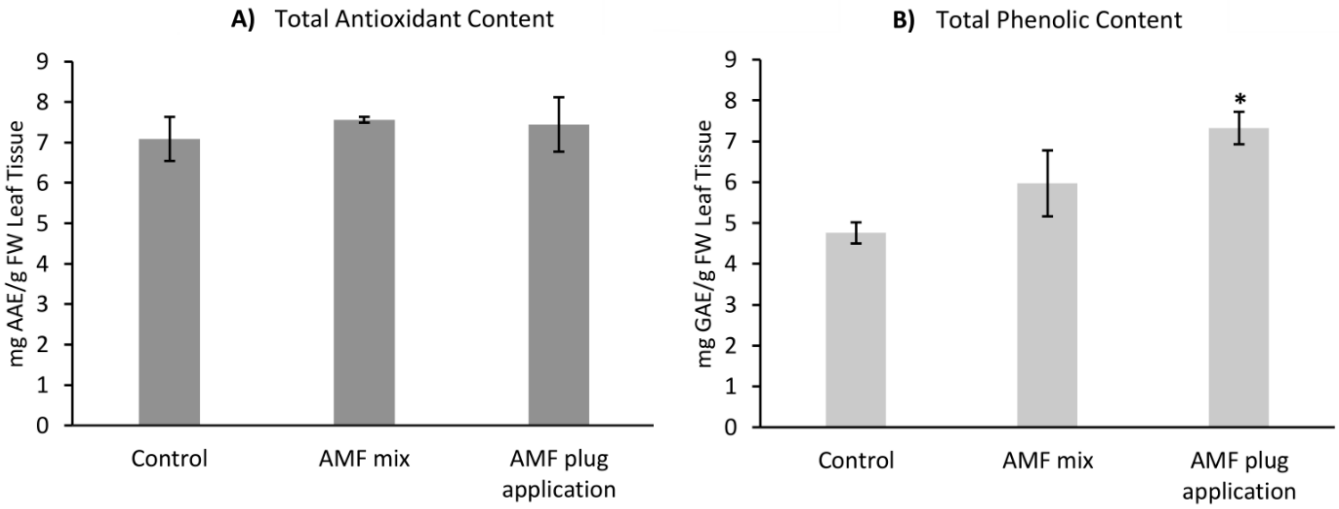


Figure 4. 3 Antioxidant and phenolic content of rosemary leaf extracts with two methods of AMF addition to the soil.

Secondary metabolite production in rosemary 'Perigord' treated with two Arbuscular mycorrhizal fungi AMF application methods. A. Antioxidant content expressed as ascorbic acid equivalent (AAE). B. Phenolic content expressed as gallic acid equivalent (GAE). Mean of treated plants with * above are significantly different to the control ($p<0.05$ based on the Tukey HSD-test). Error bars are SEM of each treatment group.

Terpene synthases play a key role in volatile and essential oil synthesis and their expression contributes to the aroma and taste of the plant. A range of terpene synthase genes were selected for analysis based on sequences previously identified in the rosemary genome sequence (Bornowski *et al.*, 2020b) which showed detectable transcripts when tested by qPCR. These represent a mix of mono- sesqui- and tri-terpene synthases responsible for terminal steps in synthesis of terpenes associated with rosemary volatiles and essential oil. Figure 4.4 below shows the gene expression of terpene synthases in both AMF treatments. The AMF root plug application showed upregulation of ocimine synthase, β-caryophyllene synthase and linalool synthase compared to control condition of peat soil. β-caryophyllene synthase showed a 30-fold increase in expression compared to Perigord grown in un-supplemented peat substrate (F= 151.3 p=<0.001). This synthase is responsible for the synthesis of several volatile terpenes, including gamma bisaboline, (E)-nerolidol and α-bisabolol. Linalool synthase also had increased expression levels, by 3-fold, in the plug application method ($F_{2,10}$=151.3 p=<0.01). Linalool synthase is mostly responsible for the synthesis of linalool, a monoterpenoid with a citrus and lavender aroma. Ocimene synthase showed a small increase of 1-fold in the plug application method, however this was not a significant difference compared to un-supplemented peat substrate ($F_{5,10}$=151.3, p=>0.05). This synthase is responsible for the synthesis of β-ocimine, terpinolene, β-myrcene, β-pinene all frequent constituents of rosemary volatiles and essential oils. The other three terpene synthases exhibited no change in expression with the AMF plug application method (Myrcene synthase, Lupeol synthase and Cineole synthase).

Meanwhile, for the AMF mixture into the substrate, there was an increased expression of β-caryophyllene synthase, by 7.93 fold, compared to the control plants ($F_{5,10}$=151.3, p=<0.001). However, this change in levels of expression were significantly lower than in the direct application method by a decrease of 0.24 fold in the AMF mixture ($F_{5,10}$=151.3, p<0.001). Other terpene synthases were downregulated with the addition of the AMF soil mixture. Cineole synthase showed a 0.98 fold decrease ($F_{5,10}$=151.3, p<0.001) compared to the control. Ocimene synthase and Myrcene synthase were both downregulated by 0.71 and 0.83 fold respectively ($F_{5,10}$=151.3, p<0.001). This upregulation of β-caryophyllene synthase followed by downregulation in other terpene synthases indicates that the aroma profile has been altered by the AMF addition. Since AMF root plug application showed downregulation of cineole synthase, lupeol synthase and ocimene synthase, with upregulation of three terpene synthases (linalool synthase, myrcene synthase and β-caryophyllene synthase), this change in gene expression in all terpene synthases may have an enhancing effect on the volatile profile rather than altered aroma profile.

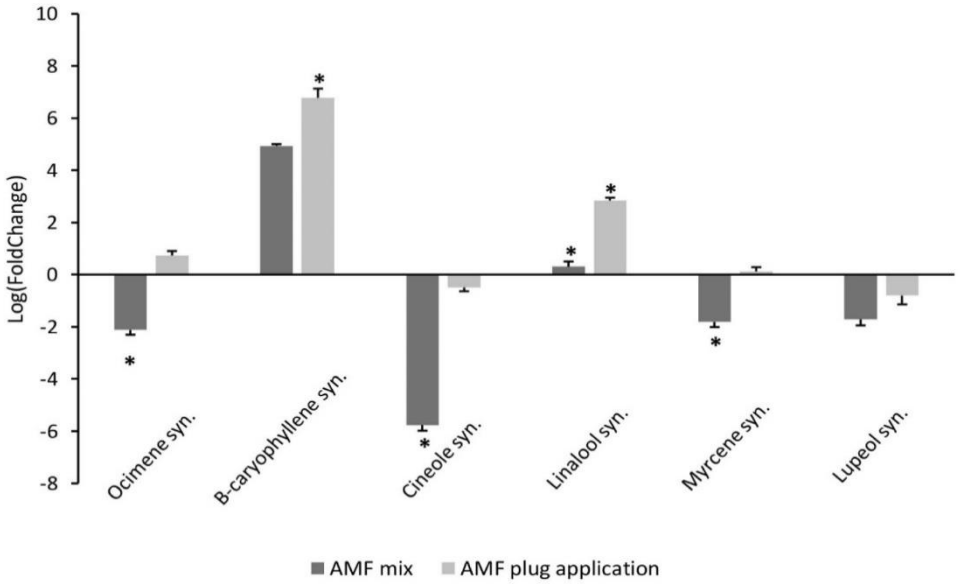


Figure 4. 4 Expression of seven Terpene synthase genes in the elaves of rosemary plants treated with two methods of AMFaddition to the soil. Change in gene expression of six terpene synthases in Rosemary 'Perigord' treated with different additions of AMF to the substrate. Fold change was calculated using 'Perigord' without AMF addition in peat substrate as control condition. Error bars are SEM of samples for each gene. * indicates a significant change versus untreated ($p<0.05$ based on the Tukey HSD-test).

4.3.2 AMF substrate mixture alters gene expression of terpene synthases in six rosemary cultivars

The effect of AMF addition to five additional rosemary cultivars was then assessed, while also characterising variation between the cultivars themselves. There were considerable differences in height and width between the various cultivars, 'Blueboy' and 'Vatican Blue' had the tallest plants and were wider than 'Perigord'. The shortest cultivar with a broader width was 'Bolham Blue' (Figure 4.5). The addition of AMF did not affect the height or width of these cultivars, however, apart from 'Logee Blue' which showed an increase in plant height by an average 34.3% ($F_{1,4} = 9.61$, $P < 0.05$). Secondary metabolite production was assessed by measuring antioxidant content as an indicator of increased activity of specialised metabolism and potentially the improved production of volatile compounds. The commercial cultivar 'Perigord' had the highest antioxidant content of all the cultivars when grown in control conditions of peat substrate with antioxidant levels on average two-fold those observed in other cultivars ($F_{1,5} = 35.11$, $P < 0.05$, Figure 4.6). All other cultivars showed a consistent level of antioxidants with no substantial differences to each other. Overall, there was no significant difference between the antioxidant content of rosemary cultivars grown in peat substrate and those grown with addition of AMF. A difference in antioxidant content may have been expected due to the role of AMF in mitigating plant abiotic stress, however the mode of action of mitigating plant stress may not be acting through the promotion of plant antioxidant biosynthesis. 'Logee Blue' treated with AMF showed 42.85% of the antioxidant content of control plants; however, this was not significant ($F_{1,5} = 35.11$, $P > 0.05$) based on the Tukey HSD-test.

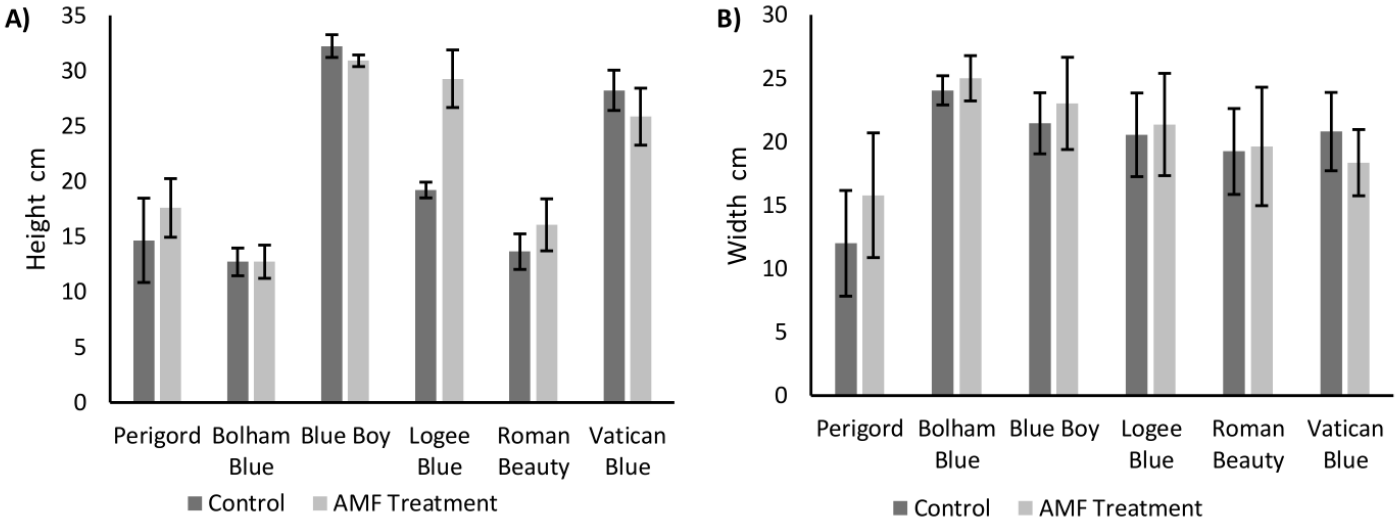


Figure 4. 5 Height and width measurements of the stems of potted Rosemary varieties treated with AMF.

The control condition of peat soil is used as a comparison of growth. A) is the average height of plants treated with AMF compared with control plants in peat soil, B) is the average width measurements of the corresponding plants. Logee Blue increased in height with the AMF treatment. Other cultivars showed no difference in growth with the addition of AMF. Error bars are SEM.

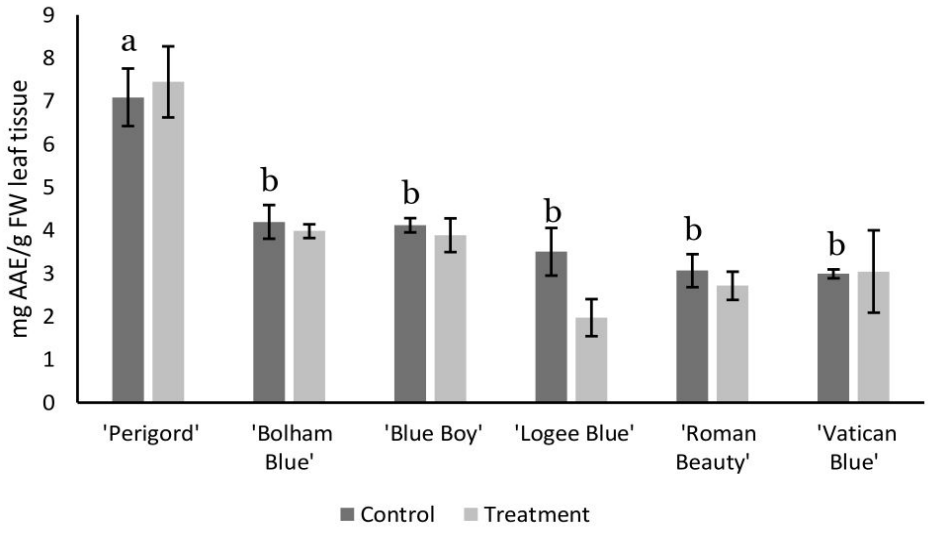


Figure 4. 6 Total Antioxidant content measured in ascorbic acid equivalents of rosemary leaf extracts from six cultivars with AMF addition to the soil.

Antioxidant content (ascorbic acid equivalent, AAE) of the leaves of six different rosemary cultivars grown in peat substrate or treated with the addition of AMF. Error bars are SEM. Means in control conditions not sharing any letter are significantly different ($p<0.05$ based on the Tukey HSD-test).

The total phenolic content was also measured in the six cultivars. Under control conditions, there were several small but significant differences between cultivars. 'Perigord' had the highest phenolic content, while the cultivar 'Bolham Blue' also had a significantly higher phenolic content than 'Blue Boy', 'Roman Beauty' or 'Vatican Blue' ($F_{1,5}$ = 2.21, P < 0.05, Figure 4.7). In addition, 'Perigord' showed a statistically significant (F1,5 = 2.21, P < 0.001) 1.5 fold increase in phenolic content when treated with AMF mix. However, the other rosemary cultivars showed no difference.

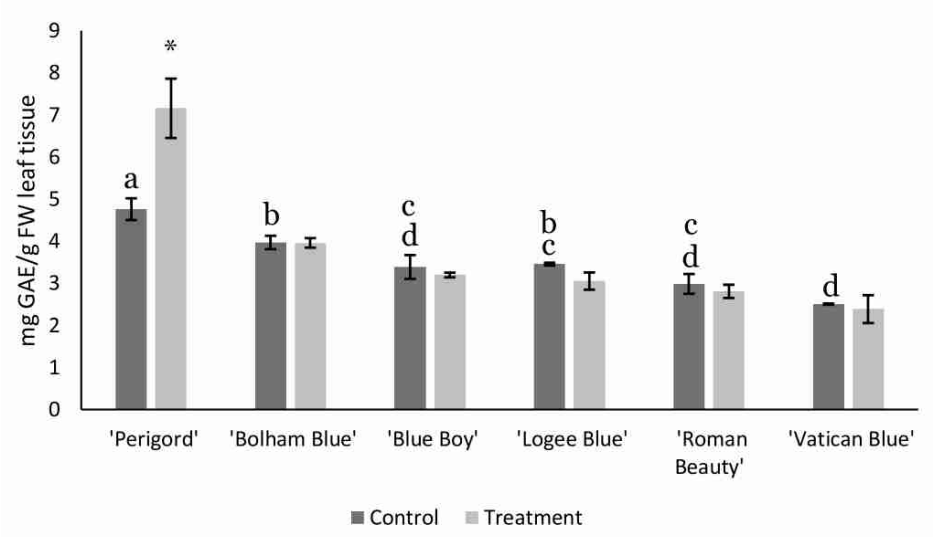


Figure 4. 7 Phenolic content of rosemary leaf extracts from six cultivars with AMF addition to the soil.

Total phenolic content (gallic acid equivalent, AAE) of 6 different rosemary cultivars grown in peat substrate containing AMF mixed in, compared with a control of peat substrate. Error bars are SEM. Means in control conditions not sharing any letter are significantly different; asterisk represents significant difference between treated and control; ($p<0.05$ based on the Tukey HSD-test).

The gene expression of seven terpene synthases was evaluated in the six cultivars of Rosemary with and without the addition of AMF. The gene expression of seven terpene synthases was evaluated in the six cultivars of rosemary with and without the addition of AMF. The plants grown in peat substrate showed different relative expression levels between cultivars (Figure 4.8). 'Logee Blue' showed the highest overall levels of terpene synthase gene expression, while 'Blue Boy' and 'Vatican Blue' showed relatively low levels of all synthases. However, each cultivar had a unique gene expression profile in the control conditions. Notably, 'Perigord' showed relatively high levels of Linalool synthase; 'Bolham Blue' showed relatively high levels of Ocimene and Cineole synthases; 'Logee Blue' showed relatively high levels of Cineole and Lupeol synthases, while Roman Beauty showed relatively high levels of Myrcene synthase. Logee blue showed the highest levels of terpene gene expression, with Lupeol synthase showing the statistically largest expression in all cultivars ($F_{5,24}$=171.19, $p<0.001$). The variation in gene expression between cultivars may be attributed to their different aroma profiles, as such the control conditions are considered as base reading for comparison of the AMF addition to the soil.

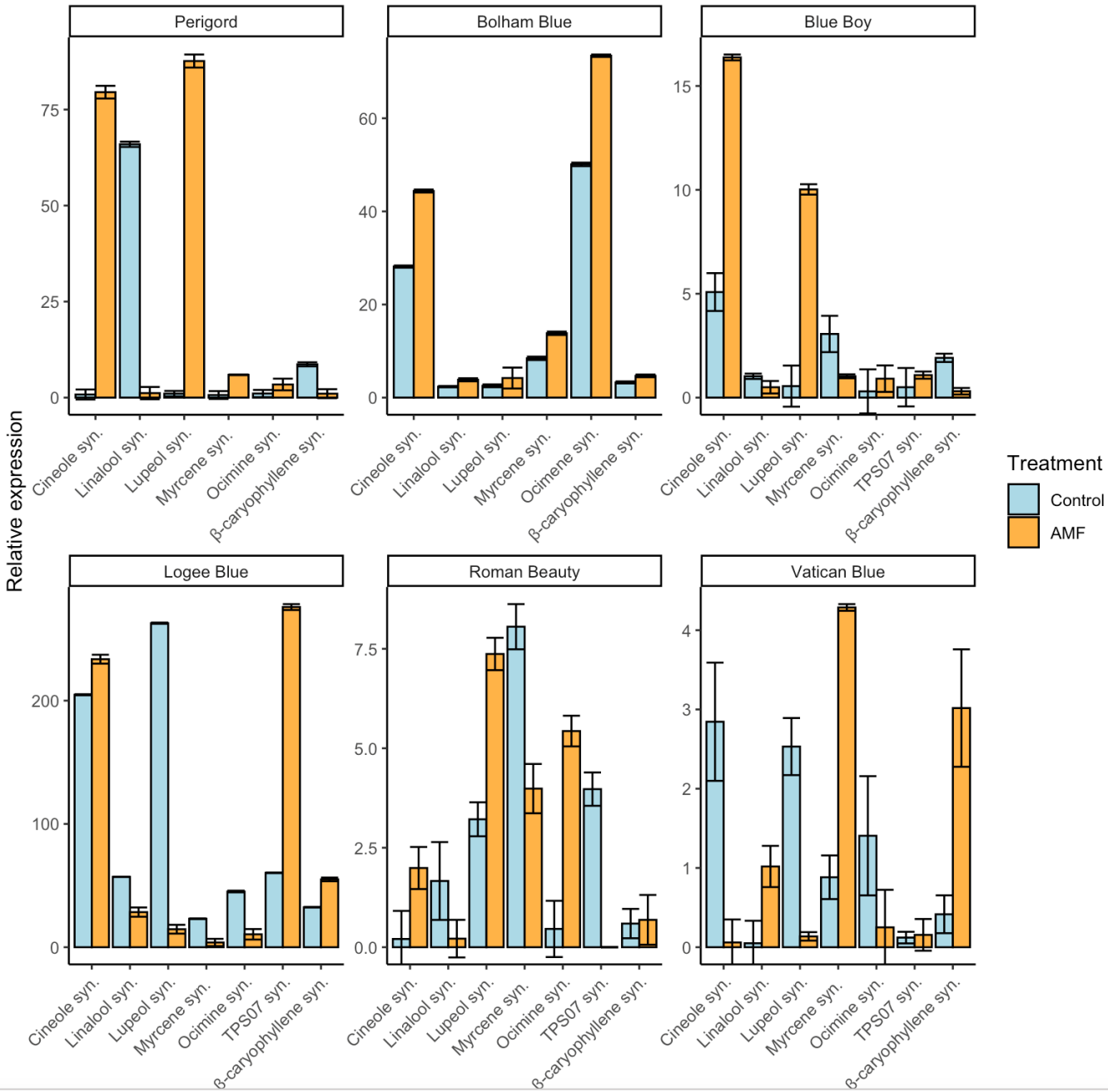


Figure 4. 8 Relative Terpene synthase gene expression in six rosemary cultivars with AMF addition to the soil.

Relative Expression levels of seven Terpene Synthases in different Rosemary cultivars grown with the addition of AMF to the substrate. The housekeeping gene GAPDH was used to calculate relative expression in each cultivar. Error bars are SEM.

The addition of the AMF mixture had varying effects on the expression of terpene synthases (Figure 4.9). 'Bolham Blue' showed small but significant increases in the expression of Ocimene, Cineole, Linalool and Myrcene synthases ($F_{5,24} = 171.19$, $P < 0.001$). 'Blueboy' showed a significant upregulation of Cineole and Lupeol synthases as well as an upregulation of Terpene synthase 7 but a downregulation in β-caryophyllene Linalool, and Myrcene synthases when treated with AMF, while 'Logee Blue' showed significant increases in β-caryophyllene synthase and Terpene synthase 7 but a downregulation in Myrcene, Linalool and Lupeol synthases ($F_{5,24} = 171.19$, $P < 0.001$). When 'Roman Beauty' was treated with AMF, the cultivar showed an upregulation of Ocimene, Cineole and Lupeol synthases but downregulation of Linalool synthase ($F_{5,24} = 171.19$, $P < 0.001$). 'Vatican Blue' showed significant upregulation of β- caryophyllene, Linalool and Myrcene synthases and downregulation of Ocimene, Cineole Lupeol synthases ($F_{5,24} = 171.19$, $P < 0.001$). In all, this suggests that selection of variety and addition of AMF offer opportunities for significant modulation of aroma and flavour in rosemary.

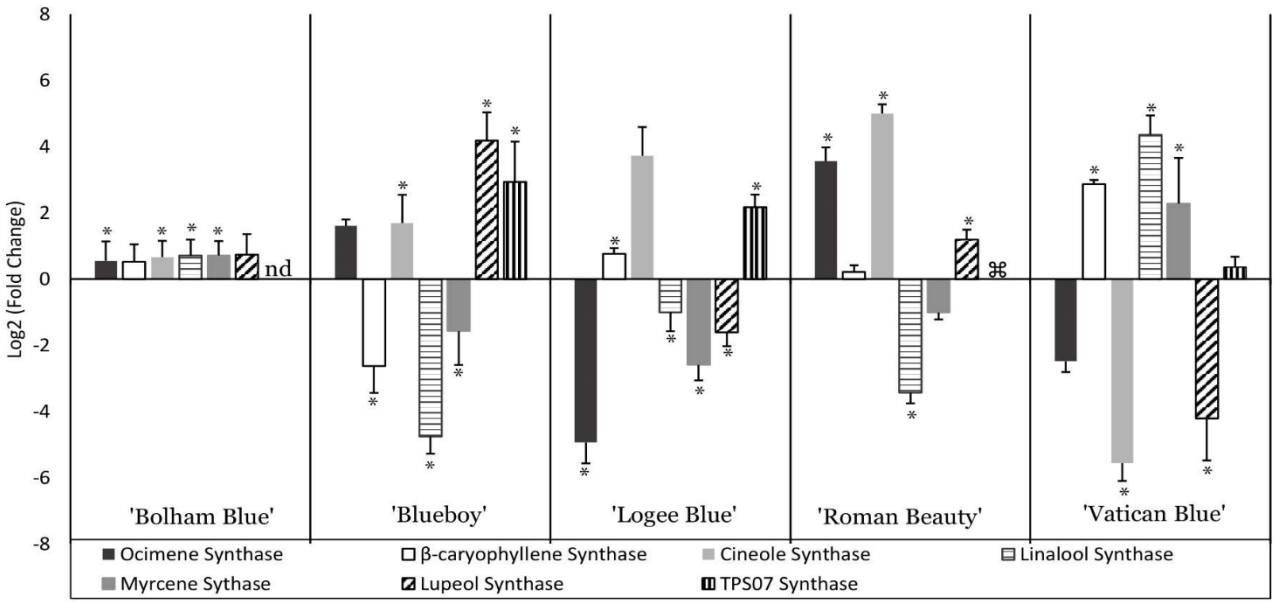


Figure 4. 9 Change in gene expression of seven terpene synthases in five cultivars treated with AMF mixed into the substrate.

Fold change was calculated by comparison of gene expression in each cultivar with controls in untreated peat substrate. * indicates a significant difference in gene expression between control and AMF conditions ($p < 0.01$ based on a Tukey HSD after ANOVA). nd: not detected in cultivar. ⌘: not detected following AMF treatment. Error bars are SEM.

4.4 Discussion

4.4.1 The Benefits of AMF addition to the soil of Rosemary plants

Both application methods showed an increase in total phenolics in the commercial cultivar, 'Perigord'. This could indicate that AMF stimulates the specialised metabolism including the MEP and MVA pathways for volatile production. The addition of arbuscular mycorrhizal fungi has been shown to boost the production of essential oils in rosemary cultivars (Seró *et al.*, 2019; Tarraf *et al.*, 2015). Increasing the content of phenolic chemicals likely includes those that are associated with aroma and taste. As consumers choose fresh herbs based on a strong aroma (findings on consumer preference in section 3.3.5), these changes to plant specialised metabolism would be beneficial. Preliminary tests from the AMF trials has shown that the addition of AMF to the soil can have a positive effect on the phenolic content and therefore aroma and taste for lesser performing rosemary varieties such as Perigord. However, for cultivars other than 'Perigord', the addition of AMF made no significant difference to the quantity of phenolic compounds in the leaf extracts in our assay. The change seen in the total phenolic content (TPC) of Perigrod may be due to the repsonse of the metabolic pahtways to substrate availablity that could be enhanced by AMF, which provides more nutrients to the plant; and when the increase in TPC of Perigord is compared with the no change in TPC observed in the other cultivars given the same AMF treament, it may be due to how each cultivar differs in gene expression along the specialised metabolic pathways. For varieties with more distinctive aromas (with more volatile compounds present in their aroma profile compared to Perigord table 3.1, section 3.3.3) such as Bolham Blue and Blue Boy the addition of AMF made no significant difference to the quantity of phenolic compounds in the leaf extracts.

The addition of arbuscular mycorrhizal fungi has been shown to boost the production of essential oils in rosemary cultivars. It is also important to note there was no morphological change after the AMF treatment for most of the rosemary cultivars, this would be due to the glasshouse conditions at Vitacress which are fully optimised for growth. Other studies which have shown that AMF addition can provide a considerable boost to plant growth have generally studied growth in stressed conditions where growth is not already optimised (Begum *et al.*, 2019; Begum *et al.*, 2021; Saia *et al.*, 2021; Shenata *et al.*, 2019). This lack of a morphological response to AMF addition is, however, beneficial for commercial cultivars, which already have a desirable morphology for horticultural production and so no change to its morphology with treatment is desirable. This suggests that AMF addition is desirable for rosemary producers who wish boost the specialised metabolism of the plants whilst retaining consistent morphological characteristics.

The *Glomus* species used in this AMF trial are vesicular mycorrhizal fungi with small hyphae. The vesicles are are terminal swellings of hyphae formed intracellularly and have a storage function while the arbuscules are formed of thinner hyphae also able to extend intracellularly and branch out in order to provide additional nutrients to the plant (Praveenkumar *et al.*, 2014). Colonisation of the rosemary roots as shown in figure 4.2 images B and D, they show AMF vesicles of *Glomus* species under high magnification. Observation of image B shows the short fungal hyphae extending from the vesicle within the root cell itself. Further evidence of fungal hyphae can be observed in image D figure 4.2, where a long hyphae runs the length of the cell and branches, indicated by the arrow. Evidence of hyphae formation is a good indicator of AMF providing key nutrients to the

plant for growth and synthesis of secondary metabolites. As the arbuscules are thin projections within the cell they were not as visible in these roots as whole roots were used without sectioning of cell layers. But the evidence of hyphae and the staining of blue structures within the cells of image C provides evidence for arbuscule presence and fungal-root cell interactions.

The colonisation rates were different between the AMF conditions. For the root plug application the more variable colonisation rate could be due to the application being in one are of the soil directly below the newly rooted cutting. Although it provided a boost to the secondary metabolite production due to a faster association woth the root compared with the AMF mixed into the soil and evenly distributed. In the soil mixture the the roots would need to grow and establish before finding the fungus within the soil and therefore the association may be later on in the development of the plant. This may work in a longer term but since the turn around for horticulturally produced rosemary is relatively short (between 50 and 80 days) the direct application may be more beneficial under these circumstances. Vesicular AMF have been shown to improve the establishment of cuttings in Hick's yew (*Taxus media*), a conifer which is also propagated by semi-hard wood cuttings. Other research eprfromed by Scagel *et al.,* 2003 found the speed of root establishment was increased with the addition of vesicular AMF indicating that mycorrhizal associations with young roots can significanlty boost root growth, AMF colonisation and of consequence increase nutrients avaiable to the plant in early stages of growth (Scagel *et al.*, 2003). As a consequence, this will increase nutrients available to the plant in the early stages of growth (Scagel *et al.*, 2003). Crucially, Scagel et al. (2003) demonstrated that roots established more quickly, and the fungal-root cell interaction happened earlier when AMF was added directly to roots of young cuttings. This supports the direct root plug application method of AMF as it shows that roots are establishing quicker and the fungal-root cell interaction is happening earlier under this treatment. It may also be beneficial to investigate the speed of rooting in cuttings with the addition of AMF into the rooting mixture.

After root staining, differences in colonisation of the roots were observed between the AMF application methods. The percentage root length colonisation was higher in the direct application method than using the AMF mix method. However, the AMF plug application method had a more variable colonisation rate (as seen in figure 4.2). The more uniform colonisation rates in plants treated with the AMF mixture will likely mean more consistent effects on the plants. Such consistency is desirable in pot grown herbs and would represent a further advantage of using the AMF mixture inoculation method (whereby the AMF is mixed with the initial propagation soil) over the plug application method in horticultural production.

4.4.2 AMF may be working to enhance gene expression of terpene synthases in rosemary cultivars

The gene expression analysis showed that there were considerable differences in terpene expression between control and AMF treated rosemary plants. It also showed that the cultivars had different patterns of terpene synthase expression without the AMF addition. 'Logee Blue' is reported to be a particularly aromatic cultivar compared to the others (The Sandy Mush Herb Nursery, 2004), which may be due to the high expression levels of seven terpene synthases observed in these plants. Responses to the AMF mixture also vary among cultivars. A general upregulation of terpene synthases was seen in 'Bolham Blue' which had increases in all six

of the terpene synthases detected. In other cultivars, responses varied, with large increases often seen in just one or two terpene synthases. Patterns of up and downregulation varied greatly between cultivars; though, there was some commonality between certain cultivars. For example, both 'Blue Boy' and 'Roman Beauty' both showed increases in Cineole and Lupeol synthases and downregulation of Linalool synthase. Likewise, both 'Logee Blue' and 'Vatican Blue' showed upregulation of β-caryophyllene synthase and downregulation of Lupeol synthase. Further analysis into relativeness between these cultivars may reveal similarities in terpene synthase gene expression may also have a genotypic effect by inheritance from similar parents, however this would be possible in future research and was not investigated here. For the grower, these results show that cultivar selection is relevant not only when selecting for increased aromatics but also when considering application of AMF. As rosemary cultivars have demonstrated here a wide variation in responses in terpene synthase gene expression, this implies that a similar variation might be expected in terms of aroma.

Prediction of details of aroma changes based purely on gene expression analysis is quite unreliable as the final aroma is a result of the blend of volatiles produced (Blerot *et al.*, 2018). However, some predictions based on individual pathway end products may be attempted. For 'Perigord', the AMF addition enhanced the gene expression of some key terpene synthases. β-caryophyllene synthase was upregulated, along with Linalool synthase. The upregulation of β-caryophyllene synthase could lead to an increase in synthesis of two sesquiterpene products, β-caryophyllene and α-humulene (Li *et al.*, 2017). These are associated with a peppery cloves smell, and woody, slightly bitter odour, respectively. The upregulation of Linalool synthase would also be likely to alter the aroma profile of 'Perigord' since Linalool is a main constituent of rosemary essential oil. It contributes to the aroma profile with an aroma of pine and floral notes. This may be a beneficial enhancement of the aroma profile of 'Perigord' as consumers may find this rosemary more fragrant. Ocimene synthase is responsible for the synthesis of β-ocimene, terpinolene, β-myrcene, and β-pinene, all constituents of rosemary volatiles and essential oils. This synthase was upregulated in 'Bolham Blue', 'Blue Boy' and 'Roman Beauty', indicating that AMF may be enhancing the quality of these aromatics in these cultivars. However, there were some notable decreases that should be considered. For example, 'Blue Boy' and 'Logee Blue' showed decreases in Myrcene synthase, with addition of AMF. This is responsible for the synthesis of myrcene, which has a peppery aroma (Blerot et al., 2018). Most frequently, addition of AMF led to an increase in expression of *Cineole synthase*, a phenomenon observed in cultivars 'Bolham Blue', 'Blueboy', 'Logee Blue' and 'Roman Beauty'. That would likely result in an increase in monoterpenes with strong woody, spicey, and floral scents.

Control of gene expression in metabolic pathways often involves complex regulatory systems. It is possible that specific changes in gene expression may be triggered by chemical communication between the AMF and the plant (Duc *et al.*, 2021). Equally, they may be triggered by changes in levels of an enzyme's substrate leading to increased enzyme production (Mandal *et al.*, 2015). In the case of AMF, such changes in substrate could result from improved availability of nutrients resulting in a change in the flux of metabolites through other connected biosynthetic pathways. For example, since sesquiterpenes are synthesised using the MVA pathway (Maffei, 2010), it is possible that AMF addition is altering secondary metabolite production at an

earlier stage of the MVA pathway. A possible mechanism for this is the AMF providing additional substrates and nutrients, such as phosphorus (Kobae, 2019), for the MVA pathway, thereby increasing the availability of the precursors, DMAPP and IPP, for terpene synthases. In support of this, a previous study has shown that phosphate fertilizers improve essential oil yield and linalool quantities in Lavender (Peçanha *et al.*, 2021). Therefore, AMF could be enhancing terpene synthesis through the additional nutrient uptake provided to the plant.

Chapter 5

5. The Impact of Current Practices on Volatile Production in Rosemary Cultivation

5.1 Introduction

Rosemary cultivation has seen many innovations to streamline production, increase growth rates and supply supermarkets year-round with fresh potted Rosemary. The effects of current practices of additional UV-C lighting were investigated for rosemary cultivar Abraxas. This cultivar Abraxas was used in this experiment as an alternative to Perigord which was not available due to supply issues. However, Abraxas has very similar morphological aspects (height/width cm of 'Perigord' = 1.22, 'Abraxas' =1.10 See Appendix 1. Table 1 for all morphological characteristics) and with a similar volatile profile (See Chapter 3, table 3.1) to Perigord and because of its similarities it is used in commercial settings interchangeably. UV-C light is being implemented more in glasshouse settings for the control of fungal pathogens and can significantly lower the use of fungicide sprays (Janisiewicz *et al.*, 2016). Low dosages of UV-C lighting can prevent fungal sporulation and is an effective control of powdery mildew without the use of fungicides. It is thought that UV-C exposure could increase phenolic content in the leaves and recent research has found that UV-c light is beneficial for the plant in terms of promoting accumulation of antioxidants and phenolic compounds (Urban *et al.*, 2016). In this section two lighting conditions were used at either a high exposure of UV-C, whereby the lighting passes over the plants twice in 24 hours, or a low dosage of UV-C in which the light only passes once in a 24-hour period. Both were assessed as it is common in horticulture to adjust the dosage within the safety parameters if there is concerns of powdery mildew. Cold storage is used by horticultural growers to preserve plants before shipment. Areas of glasshouse go unheated during the winter months and plants are kept in a non-active state to slow plant growth. Rosemary is kept in cold storage before distribution to supermarkets, here we evaluate the effect of cold storage and UV-C lighting of the specialised metabolism, volatile production and expression of terpene synthases.

5.2 Methods

Two replicate experiments were performed at the commercial herb grower Vitacress, UK under controlled glasshouse conditions. The control conditions consisted of supplemented natural daylight light without the use of UV-C light, with a 12 hour day/night schedule, and temperature conditions in the glasshouse of between 19-20 ^{o}C (see section 2.9 for light and temperature specifications). Further to the control conditions, Low and high dosages of supplementary UV-C light were applied during the night (UV-C light switched on at 3 am) to potted rosemary plants to evaluate the effect of UV-C on growth and the specialised metabolism. Low-dosage UV-C light was recorded at 6mJ/m^2 and high-dosage UV-C light was recorded as 11mJ/m^2. The effect of cold storage of rosemary was also evaluated in terms of specialised metabolic activity and aroma production, this involved short-term low temperature storage, of between 5 and 10 ^{o}C . Assessment of the specialised metabolism under all three experimental conditions by evaluating the antioxidant capacity of the leaves (by the FRAP assay), total phenolic content (by the Folin assay) of the leaves, details of the assays and replication in methodology section 2.1. The FRAP and Folin assays were chosen to give a preliminary assessment of changes

to the total antioxidant and phenolic content under experimental conditions. The volatile emissions of the plant were captured to assess volatile profile changes under experimental conditions and quantify the compounds found in the volatilome. Volatile emissions were captured by using headspace GCMS volatile analysis, methodology in section 2.10. Evaluation gene expression of seven terpene synthases was carried out by qPCR for each of the three conditions.

5.3 Results

5.3.1 Effect of cold storage and UV-C light on the phenolic production in Rosemary

The total and antioxidant and phenolic content was measured for the two conditions. Compared to the control condition (Rosemary variety Abraxas grown in glasshouse conditions), the addition of UV-C at high or low dosage did not impact the antioxidant or phenolic content of the leaves [Phenolic content: ANOVA Tukey's HSD, p-value > 0.05, Antioxidant content: ANOVA Tukey's HSD p-value > 0.05] (figure 5.1). This indicates the

UV-C lighting is not affecting the secondary metabolite production in the leaves. However, the cold treatment did have an impact on the antioxidant and phenolic content of the leaves [Phenolic content: ANOVA Dunn's post hoc, p-value < 0.01, Antioxidant content: ANOVA Dunn's post hoc, p-value < 0.05]. Cold storage may be influencing the specialised metabolism of the plants by slowing metabolism, with lower levels of secondary metabolites being produced in the leaves. Since there was no difference in UV-C exposure plants in the high exposure were used for the volatile analysis (due to financial constraints).

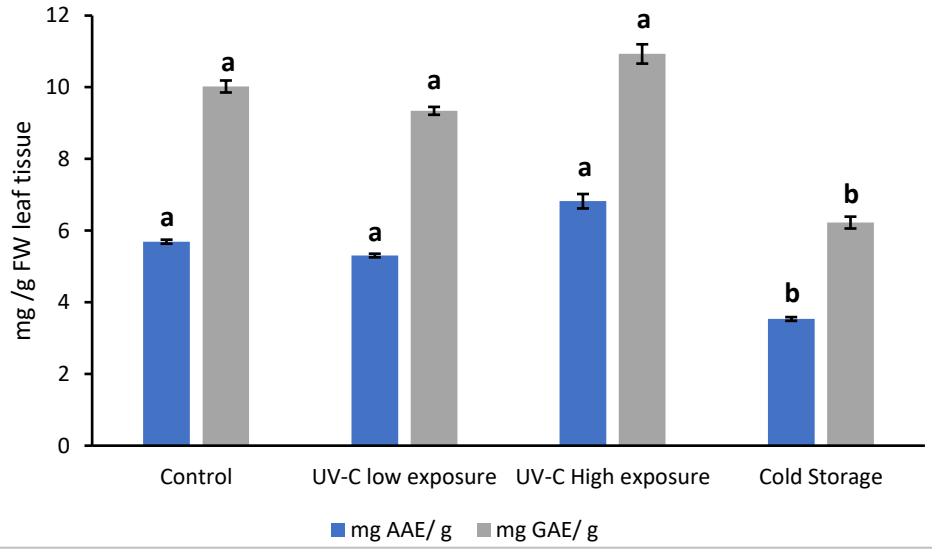


Figure 5. 1 Antioxidant and phenolic content of rosemary with suplementary UV-C light or Cold treatment.

Total Antioxidant content and phenolic content of leaves taken from Abraxas rosemary plants kept under both glasshouse treatments. UV-C high and low exposure showed no significant difference in antioxidant or phenolic content. The cold storage showed a significant decrease in antioxidant and phenolic content compared with the control condition. Error bars are SEM. Means in treatment conditions not sharing any letter are significantly different compared to the control ($p<0.05$ based on the Tukey HSD-test).

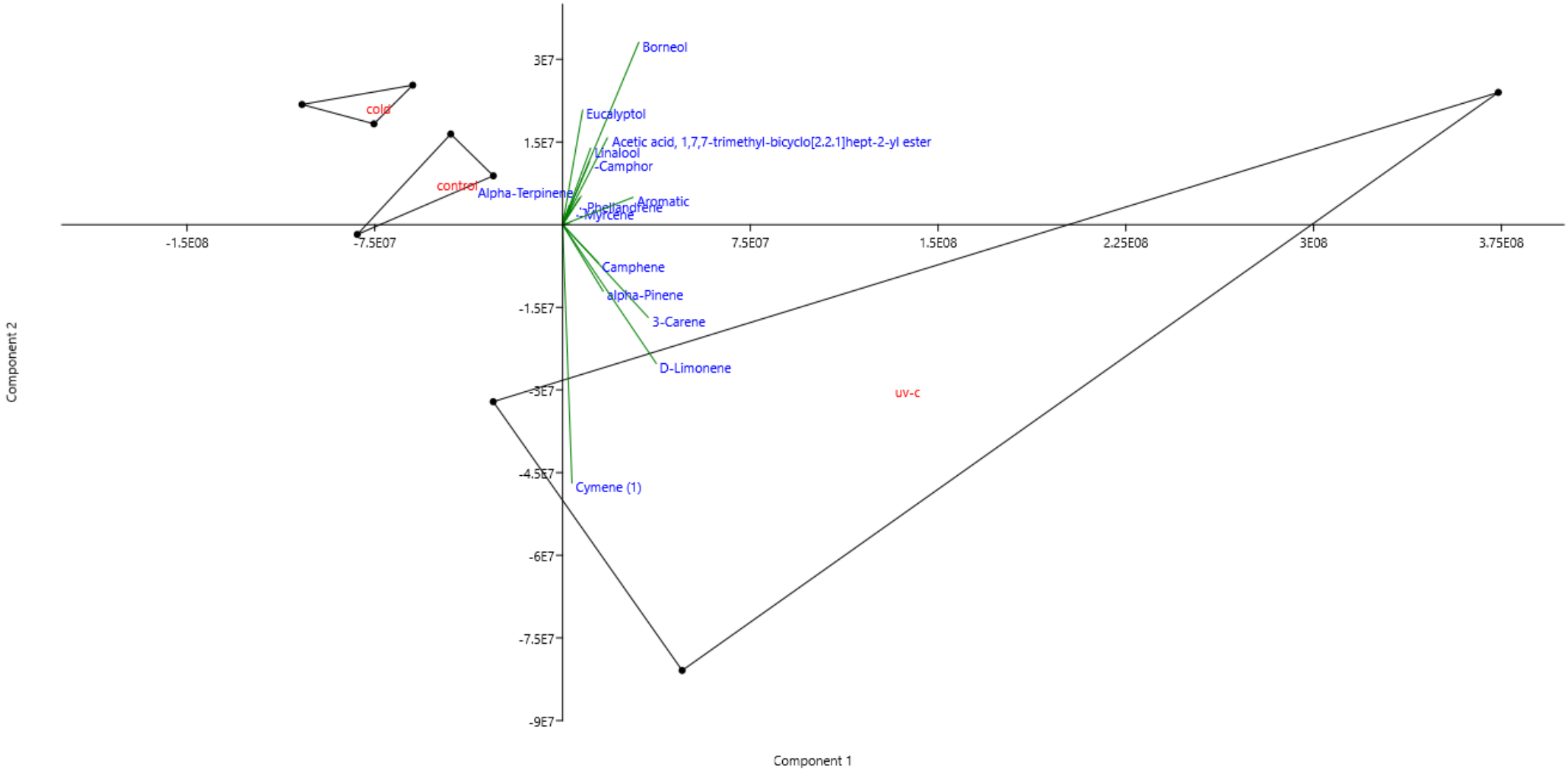


Figure 5. 2 Principal components analysis of volatile emissions from Rosemary cultivar Abraxas in control conditions, cold storage or with high dosage UV-C light application.

The volatile chemicals contributing to the most change is presented on the graph. Three replicates are represented by black dots and each group are labelled as ‘cold’, ‘control’ and ‘uv-c’.

Component 1 contributes 93.41 % and component 2 contributes 5.39 %.

Although the metabolite assays did not detect any chage, there were significant changes to the volatiles that were detected by headspace GCMS seen in figure 5.2. The change in the volatile profile through experimental conditions was selected by the SIMPER anaysis (figure 5.3), and was used to highlight volatiles with the most change between groups, two separate analyses were perfromed for each of the treatments; high dosage UV-C lighting and cold storage. Abraxas in ambient white light conditions has high levels of α-copaane, D-Limonene, Octanal, M-cymene α-pinene. On the PCA plot (figure 5.2) the control condition groups close with the cold storage conditions, yet in the SIMPER analysis there was 78% of volatiles that had a cumulative change between control and cold conditions greater than 50%. The PCA showed there was a clear difference between UV-C treated plants and the control plants, however there was a larger variation in response from the UV-C group. The high dosage UV-C showed increases in Octanal and a putative monoterpene 1. Octanal has aromas of citrus, herbal similar to orange peel. D-Limonene was also higher in the UV-C group, contributing another note of citrus with a lemon-like aroma. o-Cymene was higher in the UV-C treatment group, this volatile is commonly found in rosemary essential oil and o-cymene is responsible for terpy-like and woody scent. There were large changes in volatiles for rosemary in cold storage. There was decreases in the main constituents of rosemary volatilome D-limonene, camphene, myrcene, and α-pinene (figure 5.3). There were also increases in o-cymene, caryophyllene and phellandrene under cold storage indicating a change in the aroma of the plants in cold storage.

Volatile name	Mean log(FC) Abraxas with supplementary UV-C light	Mean log (FC) Abraxas in Cold Storage
alpha-Pinene	-0.34	-0.29
Camphene	-0.32	-0.34
Caryophyllene	0.00	0.30
Cubebene isomer	-0.18	-0.30
Cymene	-0.48	-0.13
Cymene isomer	-0.18	0.00
D-Limonene	-0.51	-0.26
Eucalyptol	-0.24	0.16
Linalool	-0.76	-0.07
Monoterpene 1	-0.48	0.30
Myrcene	-0.29	-0.35
Octanal	-0.48	0.30
o-Cymene	-0.43	3.70
Phellandrene	-0.71	-0.37

Figure 5. 3 Heatmap of log mean Fold change levels of volatiles in reference to control conditions in rosemary treated with supplementary high dosage UV-C lighting and cold storage conditions, in comparison to rosemary in white light conditions and ambient temperature of 21°C.

Red represents high levels of the volatile detected; low levels are represented in green. SIMPER analysis was used to select the volatiles showing the most change after treatment, compared to the control and are shown here.

The gene expression of seven terpene synthases was investigated in both UV-C light conditions and for plants kept in cold storage. There were differences in patterns of terpene synthase gene expression between the treatment groups (figure 5.4). The Log2 (fold change) was calculated for both conditions using Abraxas under controlled light conditions (night/day schedule of 12 hours) and regulated temperature conditions of 20°C during the day and 19°C at night as a control. For the rosemary plants treated with low dosage UV-C lighting, there were four terpene synthases (β-caryophyllene synthase, cineole synthase, linalool synthase and ocimene synthase) which changed from the control condition. Significant changes can be seen for cineole synthase and myrcene synthase in both UV-C exposure levels. A large increase in expression of cineole synthase was seen in the low dosage UV-C treatment. Cineole synthase is responsible for the synthesis of β-pinene, a major

constituent of the rosemary volatile fraction. A smaller increase was seen in the high dosage UV-C light. An upregulation of ocimene synthase and lupeol synthase was seen in the plants under both low and high dosage UV-C light and could indicate an increased production of b-ocimene, a-farnesene and b-amyrin, lupeol respectively. Myrcene synthase had an increase in expression in plants treated with both low and high dosage UV-C light. For rosemary plants kept in cold storage, there were increases in ocimene synthase, cineole synthase, myrcene synthase and lupeol synthase. However, there was a significant decrease in expression of linalool synthase in cold storage, this could be a cold stress response.

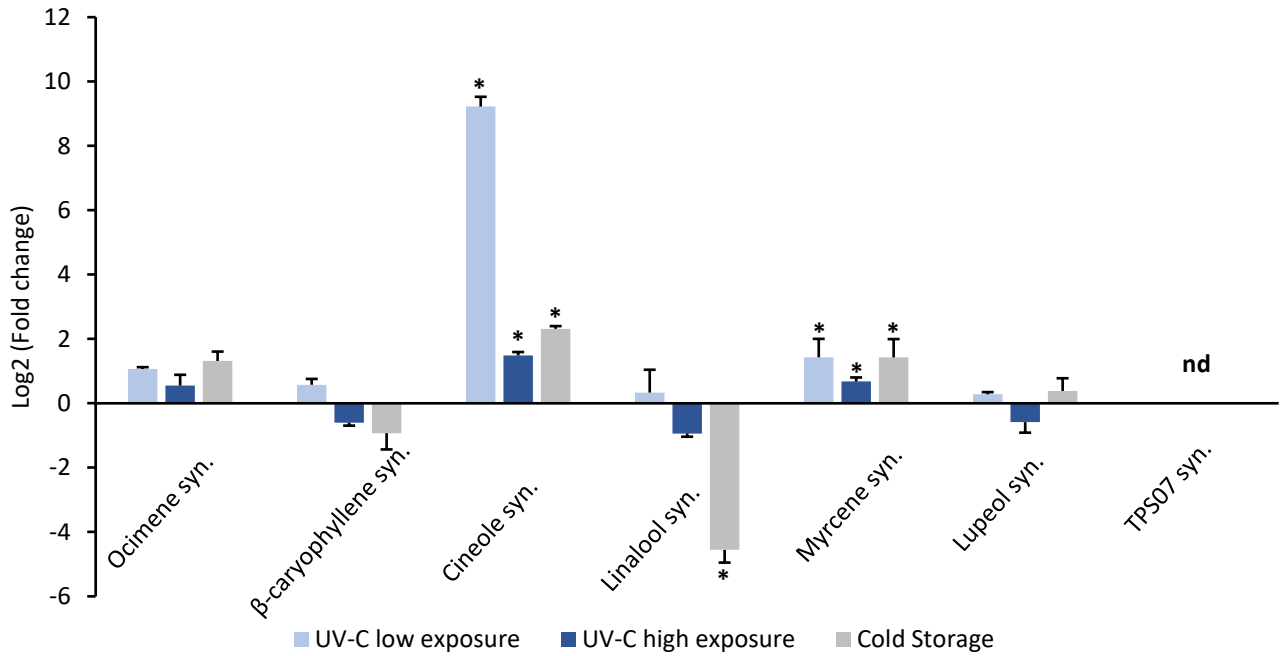


Figure 5. 4 Expression of terpene synthases in rosemary with supplementary UV-C light or cold treatment.

Terpene synthase gene expression, as Log2 (Fold Change) compared with the control of white light conditions and ambient temperature, in Rosemary cultivar Abraxas treated with two current horticulture practices. UV-C at high and low dosage showed differences in terpene synthase expression. Cold storage also showed changes in gene expression. Error bars are SEM. * represents significant difference in gene expression compared to control conditions.

5.4 Discussion

5.4.1 The Impact of current Horticultural practices on Volatile Organic Compound production

Current practices in horticulture have evolved to allow faster production, minimise infection from plant pathogens and supply fresh herbs year round even out of growing season. The UV-C lighting was originally used to prevent powdery midew infection, but recent studies have shown UV-C lights can benefit secondary metabolite productuction in different agricultural crops (F. Nigro *et al.*, 2000; Urban *et al.*, 2016))Recent research treating Basil with UV-C lighting in 10 minute exposure increased phenolic content of the leaves and increased rosmarinic acid concentration by 2.3 fold greater than the control (Nazir *et al.*, 2020). Short-bust application of UV lighting may be beneficial to herb growers and could improve the aroma and taste of the leaves. The effect on UV-C lighting reported here showed no significant difference to the antioxidant or phenolic content, however there was a difference in terpene synthase gene expression and subsequently the volatile emissions.

The composition of volatiles changed in the UV-C high exposure rosemary plants. There were beneficial increases in volatiles with strong aromas, such as D-limonene, α-pinene and m-cymene (figure 5.3). These are frequently found in rosemary volatilome and their increased production could be beneficial to the overall aroma of the plant. Some volatiles showed decreases in emission such as ocimene and caryophyllene. Ocimene has a medium strength aroma of citrus and wood and caryophyllene has a spiced, clove and nutty scent. The decrease in these two volatiles could indicate a change in the aroma and taste, and the decrease in caryophyllene could well be detected as it is a prominent aroma. The decrease in caryophyllene observed in in the volatilome of plants treated with high dosage UV-C light (figure 5.3) could be a result of changes in the gene expression of β-caryophyllene synthase, which decreased in expression as detected by qPCR (figure 5.4). One of the main products biosynthesised by β-caryophyllene synthase is carypohyllene (section 1.1.4, table 3) however in this case further protein function analyisis, such as protein microarray, is required to screen for synthase activity and products.

Ocimine synthase and cineole synthase were both upregulated with the UV-C lighting treatment. Ocimine synthase produces monoterpenes, of which pinene and β-ocimine are the main products which contribute to the aroma of rosemary and α-pinene had increased in emissions as seen in the volatile analysis. Therefore, it's upregulation would be beneficial to the overall aroma of rosemary. The upregulation of myrcene synthase in both UV-C exposures may have resulted in increases in cymene, but other products of this synthase remained unchanged in the UV-C lighting group (myrcene and o-cymene). In terms of gene expression, there were some differences between the low and high exposure UV-C light. There was decreased expression of lupeol synthase and linalool synthase in the high exposure group but these were increased in the low level UV-C exposure. High levels of UV-C light can trigger stress responses in the plants, with signalling triggering biochemical pathways that lead to the production on antioxidative and photoquenching chemicals. When plants are under high light intensities such as UV light it promotes the formation of reactive oxygen species (ROS) which can cause DNA damage. In response to ROS, the plant produces antioxidative chemicals through regulation of phenlypropanoid and flavonoid pathways (Müller-Xing *et al.*, 2014). Phenyl-propanoids and flavonoids are

derrived from the Shikimate pathway and PEP substrates may be redirected to this pathway during high light stress to produce the antioxidants necessary. The dosage of UV-C light must be considered in order to have a positive effect on the metabolism of volatiles without causig plant stress.

Cold storage is used for rosemary as it is a perennial able to adapt to cold conditions. The use of cold storage allows for rosemary supply to supermarkets year round. Rosemary can adapt to cold storage and is chilling resistant. During cold storage there was a significant drop in antioxidant and phenolic content. Plants have evolved mechanisms to adapt to cold temperatures, as long as the process involves a level of acclimatisation by prior exposure to low temperaturess. Acclimatisation involves physiological and biochemical adaptations in the plant including the synthesis of soluble sugars, proline and cold-resistance proteins (Ding *et al.*, 2019). The volatile analysis shows a decrease in volatiles contributing to the aroma of rosemary, including monoterpenes α-pinene, D-limonene, 1,8 cineole (eucalyptol) and myrcene. A large increase in o-Cymene is evident in cold storage plants. Changes in cymene isomer concentrations have been seen in research into arctic plants and plants cultivated at low temperatures. *Dracocephalum palmatum* Stephan ex Willd. is an arctic plant with medicinal uses and when cultivated at low temperatures (1 °C) it stimulated the production of phenolic compounds such as cymene (Olennikov *et al.*, 2017). This may be part of the cold tolerance response in plants as cymene, as it's derrivative thymol, has antioxidative properties which may help to mitigate cold stress through scavanging of reactive oxygen species (ROS) protecting plants from oxidative damage (Salehi *et al.,* 2018) However, more research is neeed to investigate the role of monocyclic monoterpenes such as cymene in response to abiotic stress in plants.

There was upregulation of some terpene synthases under cold storage, this is an interesting result as the metabolite assays and volatile analysis would suggest a slowing down of specialised metabolism. In contrast to the UV-C light conditions β-caryophyllene synthase had decreased in expression during cold storage. Linalool synthase had also decreased the most during cold storage. Large decreases in expression of these two synthases were not seen in conjuction with significant increases of other synthases. This may not be of benefit to the aroma and taste, and may further evidence the slowing of metabolism during cold storage. Lupeol synthase is one of the terpene synthases with increased expression in plants kept in cold storage. There is evidence to suggest that lupeol synthase plays a role in mitigating cold stress (Majumder *et al.*, 2020). Lupeol synthase is derived from oxidosqualene cyclases, that can cyclize epoxy-dihydrosqualine into lupeol (Majumder *et al.*, 2020). This is part of photo-oxidative stress response, required for the thermal dispersion of excess light energy (Reinhold *et al.*, 2008) and the by-product of this even is filtered through to triterpenoid biosynthesis resulting in volatile production and emission. Lupeol synthase may be one of the triterpenoid synthases accting to synthesise lupeol from the precurosr epoxy-dihydrosqualine and lupeol can then form part of the plant emissions. Under cold stress, the photosystem of plants works more slowly and when this is combined with high light intensities it results in oxidative stress. If the rosemary plants in the experiment are stressed by cold storage this could lower the metabolic rate, slow the production of specialised metabolism which could then impact the aromatic productivity of the plant.

Chapter 6

6. Transcriptome analysis of two Rosemary Cultivars

6.1 Introduction

At the time of analysis, there was no published genome for rosemary. Therefore, a transcriptome was required in order to evaluate differential gene expression in rosemary and elucidate sequences for terpene synthases. RNA sequencing from an experiment to test the effect of cultivar and of supplementary far-red light treatment on the rosemary transcriptome was performed and the reads were used for *de novo* transcriptome assembly (experimental design see section 2.2) . Reads from both conditions (control and supplementary far-red light treatment) were used to give greater depth to the transcriptome assembly, giving a better representation of gene expression. Two cultivars of rosemary were used in the experiment (namely, Perigord and Green Ginger), and both were sequenced to compare varietal differences in gene expression. The workflow in figure 6.1, based on the trinity method for de novo assembly (Grabherr *et al.*, 2011), was used to assess the differential gene expression between two light conditions. This is a preliminary assessment of gene expression against the assembled transcriptome for Green Ginger and to test the quality of the assembled transcript against collected sample data.

6.2 Methods

The experiment involved perturbing the biosynthetic pathways of rosemary with supplementary far-red light and evaluate differences in gene expression through RNA sequencing. Supplementary far-red light was applied

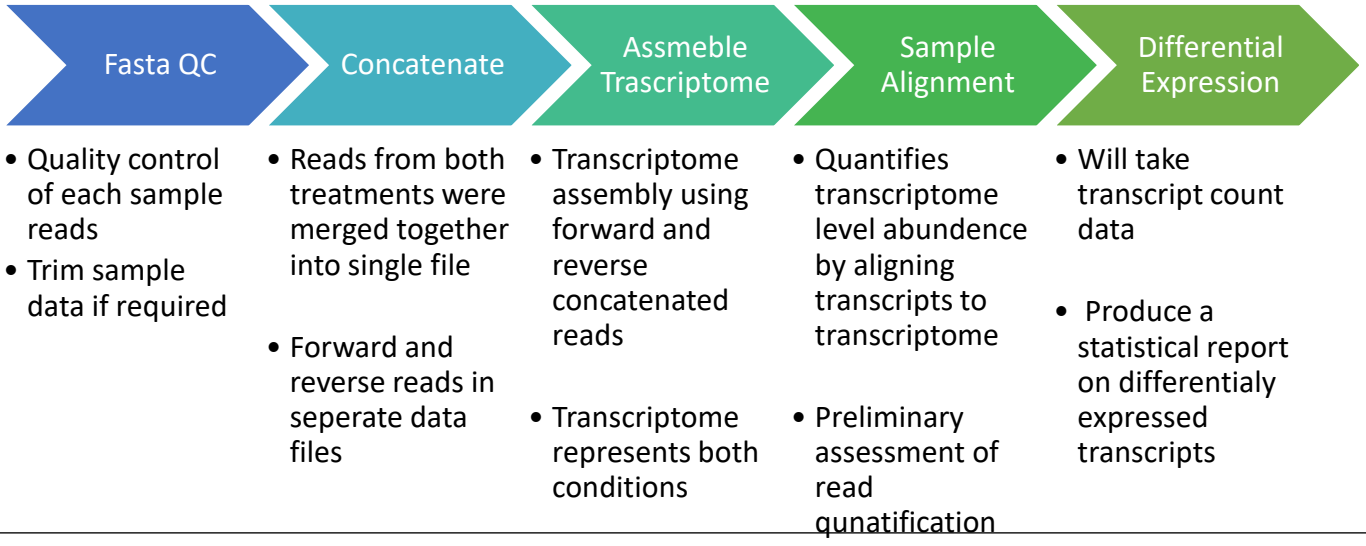


Figure 6.1 Workflow for processing RNA sequencing reads and analysis of differential gene expression.

Workflow based on trinity de novo assembly (Grabherr *et al.,* 2011). All sample data was quality controlled before merging data into single input files. Forward and reverse reads were used for de novo transcriptome assembly. Data samples from each condition were aligned separately to the transcriptome then transcript counts form both conditions were assessed to produce a preliminary stat report on differential gene expression.

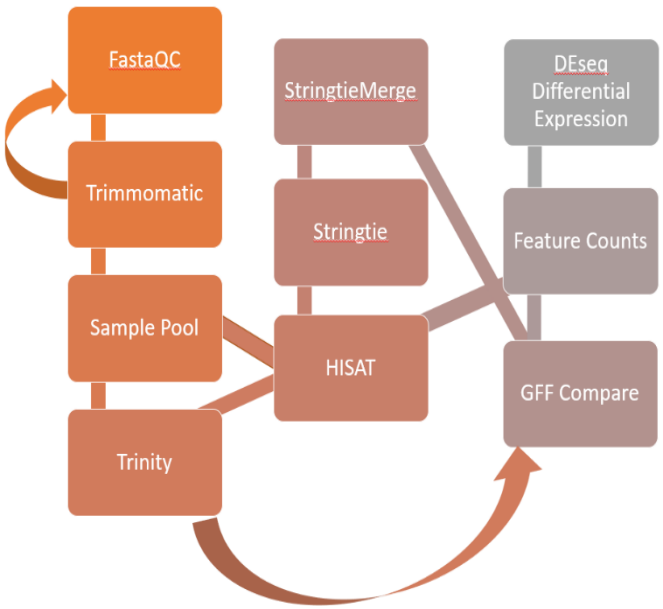


Figure 6. 2 In depth workflow on Galaxy server for the assembly and analysis of differential expression of the Illumina RNA seq reads.

The sample reads were quality checked and trimmed before de novo assembly of the transcriptome using TRINITY (Grabherr *et al.,* 2011). Replicate samples were aligned to the *de novo* transcriptome assembly using HISAT (Kim *et al.,* 2015) and STRINGTIE (Pertea *et al.,* 2015). The expression levels of each gene in each replicate were counted using Feature Counts, this input was then collated together into DESEQ (Love *et al*., 2014) to produce comparison tables of differential gene expression. Genes from DESEQ were then annotated against a well referenced *Arabidopsis* proteome.

to plants in a growth cabinet under controlled temperature and white light conditions. The details of the temperature in the cabinets and the lighting conditions for the control and far-red light treatment can be found in Materials and Methods, section 2.2. The control plants were also placed in a growth cabinet with only white light. RNA was extracted from three replicate plants in white light or supplementary far-red light conditions and sequenced on illumina NextSeq500 Sequencer producing around 30 M reads per sample (for methodology see sections 2.3 and 2.4). After sequencing, reads were assembled into a transcriptome using Galaxy server EU.

Illumina-sequencing was used to sequence the RNA extracted from the leaves of two rosemary varieties in the supplementary far-red lighting experiment described below. The bioinformatics tool Galaxy Analyse was used to analyse the Illumina-seq reads. The workflow in figure 6.1 above was used to assemble the reads into the transcriptome using TRINITY Assembly software (Grabherr *et al.*, 2011). The samples from white and far-red light treated rosemary and two varieties of rosemary were used to produce a full transcriptome representative of all conditions. The replicate samples were used to compare the effect of supplementary far-red lighting on gene expression between two varieties of rosemary using DESEQ against the de-novo Trinity transcriptome assembly.

The purpose for the DESEQ is to compare differential expression of genes between samples. It does this by estimating variance-mean dependence in gene count data from the sequencing and tests for differential expression based on a model using the negative binomial distribution. DESEQ2 shows similarity and dissimilarity of gene expression between samples using a sample-to-sample distance matrix. The aim was to compare the two treatment conditions and the two varieties independently to highlight differences in gene expression (as seen in Figure 6.2). This produced a starting point for downstream analysis looking into the effect of supplementary far-red light on terpene gene expression in two varieties of rosemary, Green Ginger and the supermarket variety Perigord. The hypothesis being the differential gene expression of volatile genes between two rosemary varieties with different aroma profiles. The DESEQ heatmaps on figure 6.3 below

illustrates the clear difference in gene expression patterns between the varieties of rosemary. The samples from Perigord, even those treated with far-red supplementary lighting have grouped together away from the Green Ginger samples. This grouping indicates a genotypic difference between the two cultivars, and that the two different genotypes are having a greater influence on sample clustering than the influence of the far-red lighting treatment. In order to illustrate differences between control lighting and the supplementary lighting, further DESEQ analyses were performed with sub-grouping of data for the two different varieties (as seen in figures 6.3 and 6.4). The result of this analysis shows differences between gene expression patterns under far-red lighting against control white lighting, as seen by the grouping of samples in cabinet A (white light) and the samples in cabinet B (with supplementary far-red light). In order to ascertain what the changes are, the DESEQ must be annotated against a well-studied genome. The heatmaps in figures 6.3 and 6.4 also cluster the genes and samples based on their similarity and the degree of similarity is represented by the dendrogram.

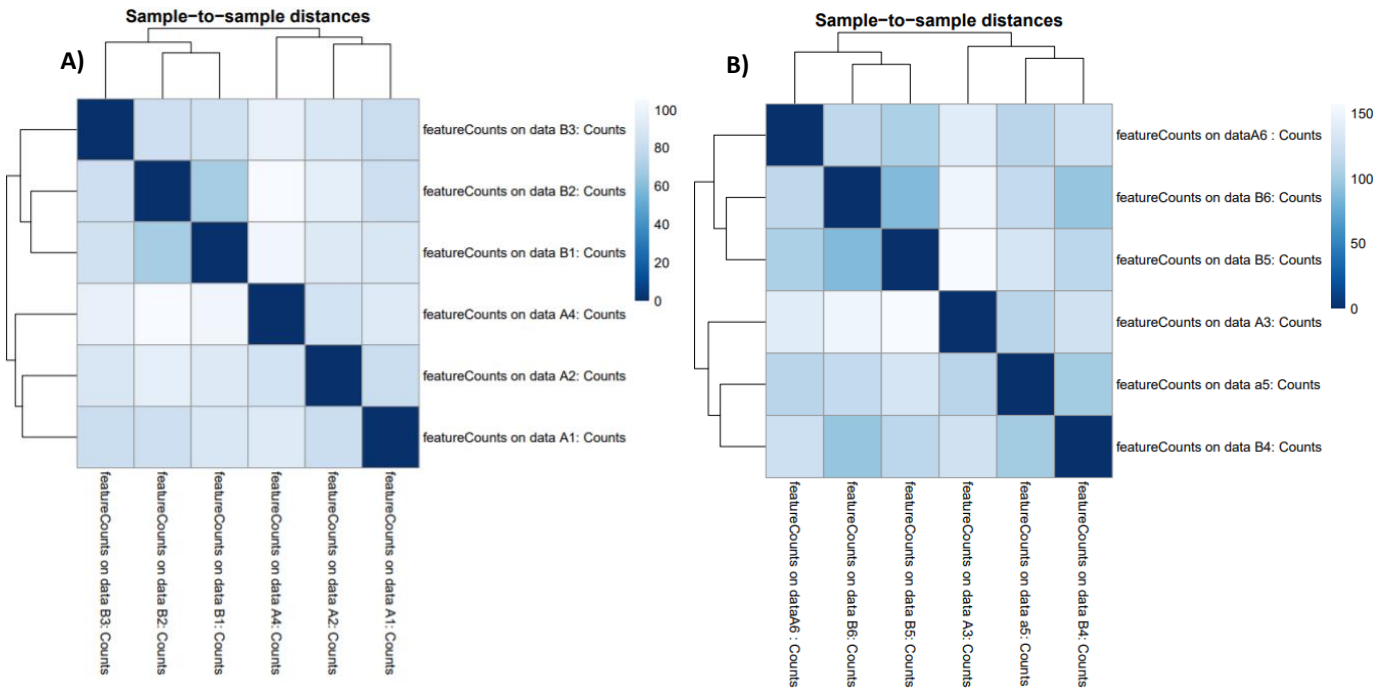


Figure 6.3 3DESEQ heatmaps plot of sample-to-sample distance matrix expressed as a hierarchical tree illustrating the differential gene expression between control white light conditions and the treatment of far-red supplementary lighting for two varieties of Rosemary.

A) Perigord control samples (A1, A2, A4) are closely grouped meaning they are expressing similar genes, there is grouping of the far-red treated plants (B1, B2, B3) with B1 and B2 showing the highest similarity. **B)** Green Ginger does show less clear similarity between samples from the same treatment, but shows some clear differential expression between, A3 and B5, B5 and A6, B4 and A6. Scale= 0 means samples have similar expression of genes, value higher than 100 means samples have differential expression.

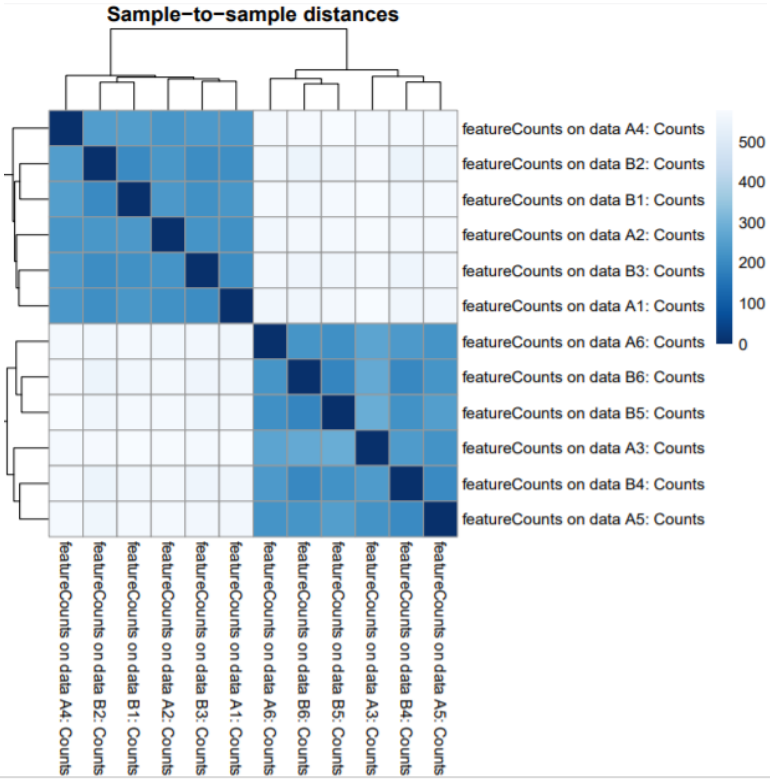


Figure 6. 4 DESEQ2 heatmap plot of sample-to-sample distance matrix expressed as a hierarchical tree of all samples, confirming differences in gene expression between varieties of rosemary.

Samples A1, A2, A4 are Perigord under white light treatment, compared with A3, A5, A6 which are Green Ginger under white lighting. The heatmap also shows a difference in gene expression in rosemary plants treated with supplementary far red lighting (A samples are grown in white light and B samples are treated with far-red supplementary lighting). Scale= 0 means samples have similar expression of genes, value higher than 100 means samples have differential expression.

6.3 Results

6.3.1 Assembly and Annotation of the Rosemary transcriptome with TRINITY

A full annotation of the *de novo* transcriptome was undertaken. TRINITY *de novo* assembly was used for the full assembly and then the annotation of the transcriptome was performed against the *Arabidopsis* proteome using Blastx software. The software package was installed locally for the annotation of large FASTA files, such as the *de novo* transcriptome. The annotated transcriptome was then used to assess the upregulated and downregulated differential gene expression of the compared conditions from DESEQ. The *Arabidopsis thaliana* proteome was used as it is well characterised, and each protein has an accession number on the well referenced database, called UNIPROT, that can be used to identify protein function and for further downstream analysis. UNIPROT is a collection of sequences and annotations for over 120 million proteins across different branches of living organisms (Consortium, 2019). The proteome can be described as the set of proteins thought to be expressed by an organism. UNIPROT provides proteomes for organisms with completely sequenced genomes. Currently, there is no model organism that is closely related to rosemary that can be used as a base genome and provide enough detail of the identified protein to predict its function and expression in rosemary. Therefore, *Arabidopsis* was a better choice for the annotation. The *Arabidopsis* proteome (Luis Zapata *et al.*, 2016) was used instead of the genome as it provides protein codes and since Blastx was used for this analysis it provided a direct protein to protein comparison for better alignment than, for example, using an *Arabidopsis* genome such as TAIR10. This is because BLASTx translates the RNA sequences into protein sequences and TAIR10 is a genome is reference used for DNA sequences. This would not be compatible, so the use of a proteome with direct protein sequence comparison is a better fit for the RNA sequence data. The reference proteome used was version proteome ID UP0000006548. Arabidopsis was chosen because it is far better and more systematically annotated than closer related species to rosemary. There are other species that are closer related to rosemary, however their sequences are either an incomplete annotation or the annotation is poorly supported by evidence. The Arabidopsis proteome is the only proteome published that has enough annotation for gene ontology analysis, gene ontology analysis also requires selection of a single proteome. Using the Arabidopsis proteome for this analysis does include the drawbacks of not identifying rosemary-specific stages in the metabolic pathways.

The main aim is to understand the regulation of volatile production in rosemary cultivars. Within that aim, was to find key genes of interest that can be used as quality markers for beneficial traits of rosemary. These quality markers are for beneficial traits for improved growth, aroma and taste. By looking at gene expression under different growth conditions, genes of interest can be identified and potentially used as quality markers for improved traits. Therefore, primers have been designed based on the identified key genes, primarily for improved aroma and taste by targeting volatile genes, and qPCR will be performed on rosemary plants under different environmental conditions to also assess if altered environmental factors can boost volatile production (primer design in methodology section 2.8 and table 2.1). Primer design was based on the RNA sequence annotation and targeted genes identified through expression analysis and pathway mapping (see section 7.3.2 for analysis of expression along specialised metabolic pathways). The advantage of this approach

is that qPCR primers will be designed specifically to target rosemary terpene synthases. The primers are designed from the de novo illumina sequencing and will be used in qPCR for quantitative expression analysis. There are limitations associated with this method, although the terpene synthase is being used as a marker this does not directly translate to aroma production and further analysis and functional characterization of terpene synthase would be required to validate the aromatic terpenoid substrates that are synthesised. To mitigate this, the terpene synthases selected for this analysis are known in literature to synthesise terpenoids with aromatic descriptors (see section 1.1.4, table 3 for terpene synthase genes and their synthesised products with aromatic descriptions).

6.4 Discussion

6.4.1 The Annotated Rosemary Transcriptome elucidated Terpene Synthase Genes for Quality Markers

The in-depth RNA sequencing and sequence annotation allowed for the identification of several terpene synthase genes. It was important to assess the expression levels as terpene synthases are involved in the final enzymatic steps towards volatile organic compound synthesis in the MEP and MVA pathways and therefore a good indicator of volatile production. The terpene synthase primers were designed from the assembled sequences and are specific to the rosemary terpene synthases (as described in section 2.8).

This offers terpene synthase sequences for rosemary that, to our knowledge, have not been published before and the depth of sequencing performed allows for assessment of primary and specialised metabolism allowing for investigation of multiple areas of the rosemary genome. A recent study of the genomes of four Lamiaceae species, including rosemary, identified ortholog families in the MEP and MVA pathways (Bornowski *et al.*, 2020a). Their aim was to characterise terpenoid diversity in the Lamiaceae family and provide a resource for further investigation of chemo diversity in herbs. Although they used a different method of sequencing, shotgun RNA sequencing, which resulted in a smaller assembled genome size. What they also reported was a clustering of gene orthologs in the MEP and MVA pathways for rosemary. They found gene orthologs in the TPSa family group and the TPSb family groups, from *Arabidopsis*. These Ortho groups included terpene synthases commonly found in rosemary 1,8 cineole synthase and linalool synthase, β-caryophyllene synthase. This confirms our finding of these terpene synthases in rosemary.

rosemary terpene synthases are responsible for multiple volatile products, as seen in methods section 2.8, table 2.1 . This is an important and complex aspect of terpene synthases, and this was taken into consideration when selecting terpene synthases as quality markers for enhanced aroma. Since the trinity assembly was annotated using the *Arabidopsis* proteome, it is likely not all terpene synthases present in rosemary were annotated. This is due to the species distance between *Arabidopsis* and rosemary. The sequences annotated were for the more abundant terpene synthases in rosemary and can be used to assess expression among cultivars as these are generally expressed in most varieties of rosemary at different levels. With further investigation of the volatile profile of rosemary, the terpene synthase selection here was narrowed down to a

few quality gene markers that may be able to inform on the performance of different cultivars and performance in environmental conditions. The seven terpene synthase genes selected as markers of aroma profile quality in rosemary are used in this research and are evaluated on their performance as genetic markers as well as monitoring gene expression in different environmental conditions by qPCR.

Chapter 7

7. Gravitropic responses in Green Ginger under supplemetary far-red light

7.1 Introduction

Gravitropism is the orientation of shoot and root growth by the plant through gravity sensing. Shoot growth is orientated against the gravity vector toward light, conversely root growth is orientated with the gravity vector away from light and into the soil. There are known interactions between the gravity sensing pathways in plants and the light transduction pathways, due to the similarities in signal transduction and response, yet each pathways have genes and steps which are unique to each (Correll and Kiss, 2002). The crosstalk between these pathways means that light can influence the gravitropic responses, and trigger growth responses such as hypocotyl elongation, internode elongation and other plant re-orientations is search of light. In most plant taxa red light effectively induces gravitropic responses, triggering either positive or negative gravitropic movements depending on the plant taxon. The gravitropic response to red/ far-red light is regulated by phytochromes (Gaiser and Lomax, 1993; Behringer and Lomax, 1999). There is little research on the gravitropic responses in rosemary. In this chapter the induced gravity responses by far-red light are investigated in two rosemary cultivars with a focus on the responses of the semi-upright, cultivar Green Ginger. The RHS describe Green Ginger as an upright cultivar, and a similar description was described by Vitacress (available at https://seasonherbs.co.uk/products/green-ginger-rosemary, accessed: 10-11-2023) however Green Ginger is clearly less upright when compared to other upright cultivars, such as Perigord, due to Green ginger having more branching at the lower levels that spread almost horizontally whereby the Red : Far-red light ratio would be high. This results in young plants in pots yet to achieve a bushy status of a mature plant having a semi-upright appearance. It is chosen in the following experiment as a semi-upright cultivar to investigate the morphological responses to supplementary far-red light.

7.2 Methods

Three plant replicates of rosemary cultivar Green Ginger were placed in a growth cabinet and treated with supplementary far-red light (light intensity of 50 µmol m-2 s-1 and a red: far red ratio of 5.1, further details in methods section 2.2), a separate growth cabinet was used with white light as a control (light intensity of 50 µmol m-2 s-1 and a red: far red ratio of 0.2). After five days the three replicate plants were assessed for physiological responses to supplementary far-red light. In the essential oil analysis carried out in chapter 8, involving 5 days treatment, resulted in pronounced changes in gravitropism in the Green Ginger cultivar which was further investigated here. RNA was extracted from the leaves of the plants in both lighting conditions and RNA sequencing was performed. After transcriptome assembly of the reads and sequence annotation, function classification of gene expression was performed using MapMan software by Gabi and Julich, David Bioinformatics Resources (Huang, *et al.*, 2009).

7.3 Results

7.3.1 Morphological changes observed in Cultivar Green Ginger under supplementary far red lighting

During the experiment, a difference in the morphology of the semi-upright variety Green Ginger was observed under the far-red light treatment. The lateral shoots in semi-upright forms of rosemary had a horizontal orientation under white light conditions. Under the far-red light treatment with an altered R:FR ratio caused reorientation of the shoots to grow vertically in semi-upright forms, as seen in figure 7.1 for Green Ginger rosemary. There is a statistically significant different change in height/width for Green Ginger [student T-test on height/width data, p=0.00156] Whereas Perigord showed an increase in height, there was no significant difference in height/width. The internode lengths for both cultivars were longer in the far red-light treatment by 28.57% increase in height for Perigord and a 47.05% increase for Green Ginger. The reorientation of the stems under far red light gave the semi-upright plant the appearance of an upright plant, which is a desirable trait in the production of horticulturally produced rosemary.

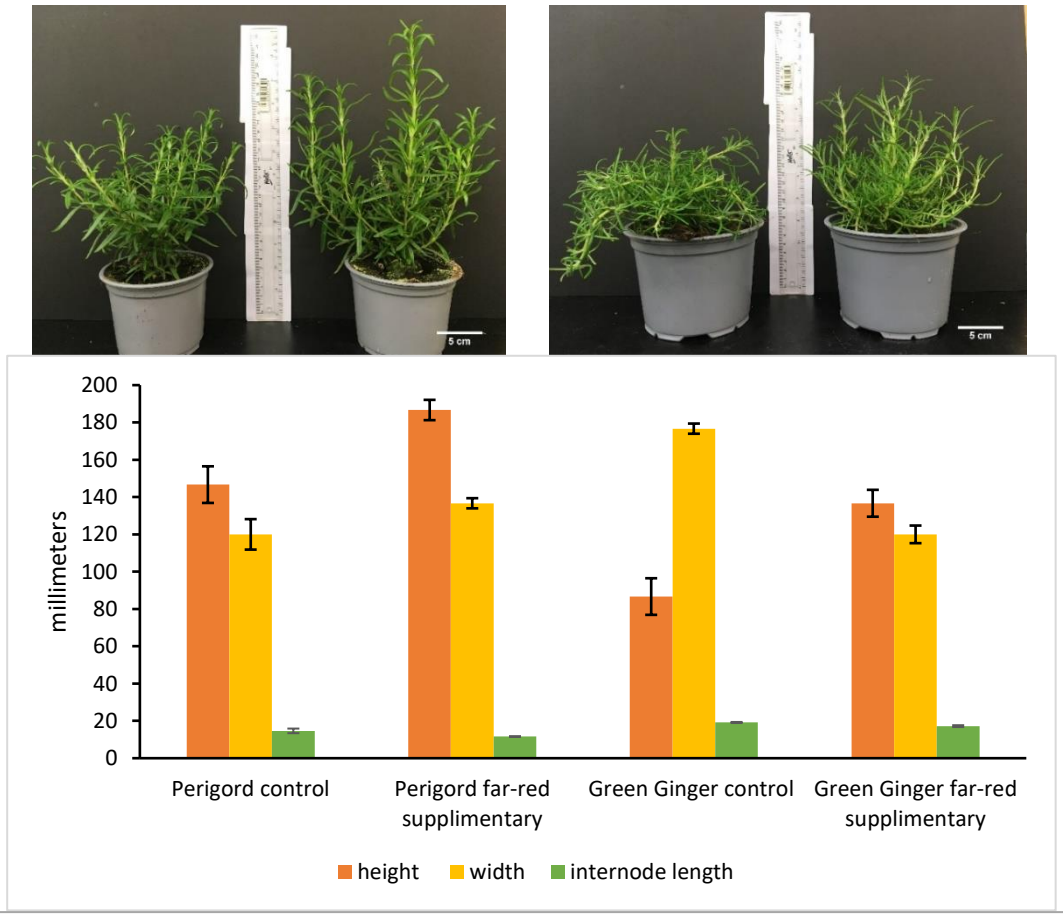


Figure 7. 1 Growth measurements of two rosemary cultivars grown in supplementary far-red light.

Height, width, and internode measurements from two rosemary varieties under high R:FR ratio (white light) conditions, (control) and a low R:FR ratio conditions (white plus supplementary far red light). Error bars represent standard error of the mean. Images: top left is Perigord, and top right is Green Ginger, where the left plant in both images in the control and the right plant is the supplementary far red-light treatment, scale bar= 5 cm.

The gravitropic response seen in Green Ginger under far-red light was investigated further using the differential expression analysis. Annotated transcripts and their logfold change gene expression were analysed on PageMan, which divides into gene categories based on cellular function. The PageMan analysis (Usadel *et al*., 2006) (Figure 7.2) showed an overrepresentation of gene groups of the hormone metabolism, as seen on heatmap 7.2 A), specifically in auxin and gibberellin metabolism groups. These two hormones are the main regulators of directional and elongation growth and gravity sensing, as studied in barley leaf sheaths by Ross *et al*., (2006). Auxin and Gibberellin are being upregulated in Green Ginger and Perigord under supplementary far-red light (figure 7.2 A) Green ginger under white light conditions shows overrepresentation of brassinosteroid metabolism and gibberellin metabolism gene groups but does not show upregulation of all the gibberellin regulation that green ginger plants treated with far-red light showed. Figure 7.2 B) shows cell wall metabolism. Green Ginger treated with supplementary far-red light shows gene overrepresentation of three key areas of cell wall modification: cell wall degradation, synthesis of pectate lyases and polgalacturonases, and cell wall modification. Pectate lyase and polygalaturonases are important for degradation of cell wall pectin, which then allows for cell wall expansion. These three areas of cell wall metabolism are important to the expansion of the cell wall to allow for elongation growth. For Perigord, supplementary far-red light caused downregulation of cell wall reversibly glycosylated proteins (RGPs). These proteins are involved in transportation and incorporation of arabinose and xylose into the cell wall.

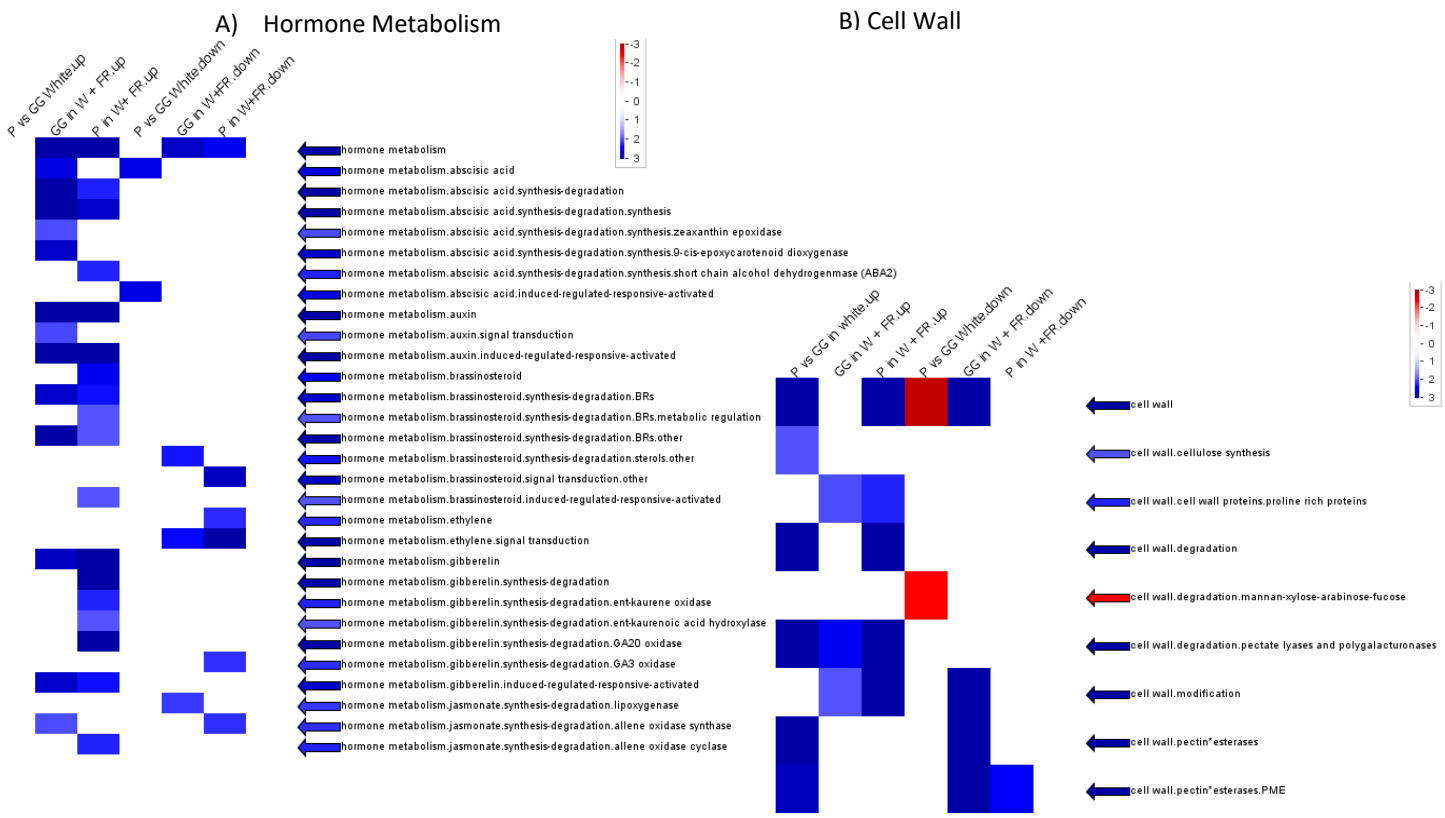


Figure 7. 2 heatmaps of gene expression in Perigord and Green Ginger under control conditions of white light used as a base comparison against Perigord and Green ginger grown in supplementary far-red light.

A) Hormone Metabolism showing overrepresentation of Auxin and gibberellin metabolism for Green Ginger and Perigord rosemary varieties under supplementary far-red lighting. B) Cell wall metabolism, showing overrepresentation of cell wall cellulose synthesis gene groups, cell wall degradation and cell wall modification in Green Ginger and Perigord under far-red light. Colours: Red= underrepresented gene categories and Blue= Overrepresented gene categories.

7.3.2 Evaluation of gene expression in the specialised metabolic pathways for Perigord and Green Ginger

After the *de novo* rosemary transcriptome was fully annotated against the *Arabidopsis thaliana* proteome the resulting annotated rosemary transcriptome was used as a reference to retrieve gene identification for the DESEQ differential expression analysis. As multiple differential expression analyses were performed, having one rosemary reference transcriptome made it possible to compare multiple metabolic responses from the plants in the experiment on supplementary far-red lighting. The gene IDs retrieved from the DESEQ were organised using MAPMAN software (Huang *et al.,* 2009), to group genes into cellular functions and separate out relevant pathways for volatile synthetic pathways and view pathways involved in morphological changes.

As an initial assessment, MAPMAN provided a few key genes within the terpenoid pathways, MEP and MVA pathways for volatile synthesis. MAPMAN also provided gene groupings for key genes involved in shade avoidance including auxin signalling pathway Aux/IAA, the gibberellin signalling pathway and the light

signalling pathways in order to understand the morphological changes occurring under supplementary far-red lighting as seen in figure 7.3. Using PAGEMAN analysis, the primary and specialised metabolism was investigated under different lighting conditions. PAGEMAN provided an overview of metabolic responses to far-red light, showing overrepresentation of gene categories in the specialised metabolism.

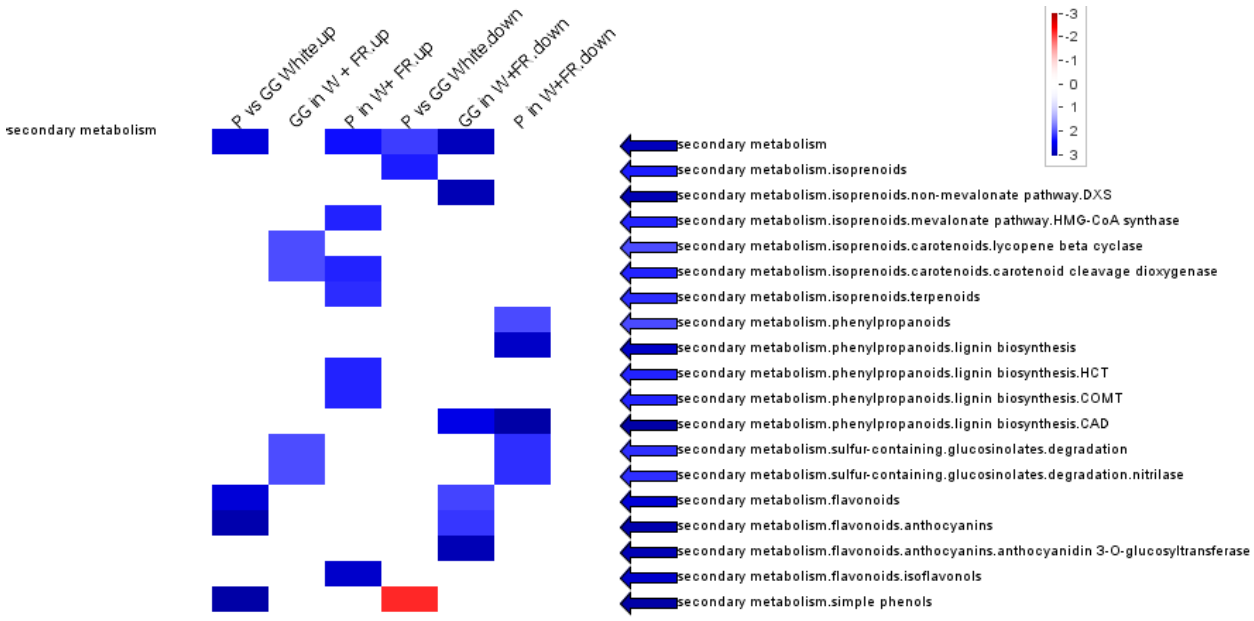


Figure 7. 3 Heatmap of over-represented and under-represented biological processes associated with changes in gene expression of specialised metabolism in two Rosemary cultivars.

Perigord and Green ginger were treated with normal white light or white light (WL) supplemented with Far-red Light (W+FR). Logfold changes were calculated, making the following comparisons: Perigord vs Green Ginger in White light, Green Ginger in White light vs Far-red Light and Perigord in White light vs Far-red light. Gene expression in both cultivars under white light used as baseline for comparison. Up and down representation shown. Colour code: Red= underrepresented categories and Blue= overrepresented categories. Heatmap produced using MAPMAN software (Huang *et al*., 2009).

7.4 Discussion

7.4.1 Analysis of Gene Expression in Green Ginger undergoing Morphological changes observed under Supplementary Far-red Light

The light experiment was designed to assess the effect of supplementary far-red lighting on secondary metabolite production and differential gene expression, specifically in the terpene biosynthetic pathways. The far-red lighting altered shoot orientation in the variety Green Ginger, this can be explained by gravitropic responses. The *lazy-2* mutant tomato causes conditional reversal of shoot gravitropism by light, which has been shown to be mediated by phytochromes in the gravitropic signal transduction pathway.

lazy-2 tomato mature plants exhibit a prostrate growth form, known as inhibited negative gravitropism, under white light conditions. When seedlings of *lazy-2* tomato are grown in the dark they exhibit normal shoot negative gravitropic response, this response was also observed under far red-light conditions (Gaiser and Lomax, 1993). The change from the wildtype response indicates that far-red light treatments alter the growth form of the plant. Under far-red lighting the plant believes it is in dark conditions, and phototropic responses are limited. This then allows for gravitropic responses to re-orientate the seedlings to seek-out light. Therefore, the observed effect of the supplementary far-red light treatment on the semi-upright form of rosemary, Green Ginger cultivar, can be explained by the effect of gravitropism causing re-orientation of the shoots from low growing to upright form, as observed in the images of figure 7.1. The effect of the supplementary far red-light treatment on the cultivar Perigord can be attributed by the effect of shade avoidance, in the presence of far red-light the plant phytochrome photoreceptor system does not transmit growth inhibiting signals, allowing the elongation growth to take over and begin growth in search of light. From the gene expression analysis, key genes in the auxin-pathway and gibberellin pathways for elongation growth response were upregulated including an upregulation of the *Lazy-1* gene in Green Ginger under supplementary far-red light. The transcription factor PIF3 is upregulated in far-red light, which activates auxin-responsive genes involved in shade avoidance and elongational growth such as YUC8, TAA1 and PIF5 (Guilfoyle and Hagen, 2007). In addition to this, cell wall modification was being upregulated under far-red light. This is important to allow cell expansion and therefore directional growth. The Pageman analysis (figure 7.2) showed multiple areas of cell wall metabolism and hormone metabolism which are known pathways in directional control of plant growth. This analysis hinted at relevant pathways in gravitropic response and a re-organisation of the photosynthetic apparatus. Further analysis with a closer relative to Rosemary that is well annotated would be a better choice to provide a closer match in genes and prevent narrowing of the initial sequence list based on non-alignment with *Arabidopsis*.

Recent research has been conducted to understand how the *Lazy* genes function to influence gravitropism (Yoshihara and Spalding, 2020). The findings suggest that mutations to region II of two conserved amino acids of the *AtLazy* gene can switch shoot gravitropism from negative gravitropism with upright growth, to positive gravitropism resulting in a weeping or prostrate phenotype. This is a new revelation, which could explain the small differences in amino acids for *LAZY1* can influence the auxin gradient and cause positive gravitropism or prostrate growth. They rescued *AtLazy1 Arabidopsis* plants with transgenically modified *Lazy* and showed a

decrease in branch angle and an upright growth of florescence. It may be interesting to investigate and align the Rosemary *Lazy* gene to the *AtLazy1* and transgenic *patLazy1* sequences to establish similarities and differences in the coding region of L92A/I94A. If so, the observed semi-upright phenotype in Green Ginger cultivar can be attributed to a mutation in region II of the *Lazy* protein.

Red and far-red light have been found to influence plant morphology and oil production in rosemary (Mulas *et al.*, 2006). The use of end of day far-red light treatments over the course of a month changed the composition of essential oil and increased oil production in rosemary. In the far-red light experiment above, the treatment had the overall effect of decreasing leaf antioxidant content for Green Ginger. With continued exposure to supplementary far-red lighting, it is possible that Perigord would also show a reduction in antioxidant content as Green Ginger did. This may be consistent with the morphological changes that occurred in response to the far-red lighting, which were more pronounced in Green Ginger than in the supermarket variety suggesting that green ginger may be generally more sensitive to shade. The change to an upright growth form is a beneficial trait for Green Ginger, but as there was a reduction in secondary metabolite production and terpene synthase expression meaning there may be a need to alter the supplementary lighting.

Chapter 8

8. Volatile pathways analysis by RNA sequencing of two rosemary cultivars with supplementary far-red light

8.1 Introduction

RNA sequencing is a powerful tool to elucidate global changes in gene expression. The transcriptome of two rosemary cultivars was constructed to evaluate global changes in volatile synthesis and at this point has not been looked at before. This experiment uses the same experimental design as the previous chapter 7 with the two rosemary cultivars selected for treatment with supplementary far-red light because of financial restrictions. In this experiment FR light was used as a non-invasive way to perturb the volatile pathways, and to understand their response when environmental conditions change. This also gives a greater range to the assembled transcriptome which includes genes expressed under far-red light conditions. A better understanding of how the volatile pathways respond will also form part of the knowledge that may allow for targeted interventions to improve aroma and taste. Plants from both cultivars were placed in two growth cabinets: one with white light and the other with white light plus supplementary far red light. Leaf tissue samples were taken after five days of treatment for RNA extraction to later observe differences between gene expression of the volatile biosynthetic pathways and to also perform antioxidant assays.

In chapter 3 the volatilome of several rosemary cultivars was assessed. In this chapter a detailed analysis of two different rosemary chemotypes was evaluated in depth, including gene expression of the terpenoid pathway genes known to regulate the production of aromatic volatiles. The gene expression of several terpene synthases was evaluated using both RNA sequencing and qPCR techniques. Headspace GCMS was used to evaluate the volatile organic compounds emitted from rosemary cultivars Perigord and Green Ginger. Research seems to suggest that terpene synthases are multi-substrate and multi-product, in this chapter the correlation between the gene expression of terpene synthases and the volatile emissions was analysed and assessed based on the known catalytic function of the terpene synthases. In most cases, terpene synthases correlated positively (some negatively) with multiple volatile compounds.

There is research to suggest far-red light signals in the plant alters the terpene profile. Rodriguez-Concepcion et al (2004) observed that mutants for certain photoreceptors, such as phytochrome B, can also have downregulation of enzymes in the isoprenoid pathways. They tracked phytochrome gene expression and gene expression along the MEP pathway during seedling emergence of *Arabidopsis thaliana* in darkness. It was found that phytochromes have a role in mediating the activity of the MEP and MVA pathways and will activate transferase genes that shuttle substrates to other isoprenoid pathways for synthesis of hormones such as gibberellins (Rodriguez-Concepcion *et al.*, 2004). The crosstalk between the MEP and MVA pathways may mean that substrate availability can be moved from one pathway to the other in coordination with light responses (further detail section 1.1.3). In this chapter the response of the MEP and MVA pathway under white light and supplementary far- red light conditions will be evaluated in terms of changes along the

pathways. The transcriptome analysis is accompanied with headspace GC-MS of the volatilome and secondary metabolite production.

8.1.2 Methods

RNA sequencing analysis was performed on two cultivars under white light conditions (control) and white with supplementary far-red lighting (treatment) to compare gene expression of genes along these pathways and to evaluate expression of terpene synthases involved in volatile synthesis (for methods see section 2.2). White light was used as a control and to investigate cultivar differences in expression patterns of the terpene biosynthetic pathways. Post analysis of the annotated RNA sequences (performed on two cultivars Perigord and Green Ginger) was performed using R Studio Software and the abundance of transcripts expressed as FPKM (Fragments per kilobase of transcript per million mapped fragments). To validate RNA sequencing results, qPCR was also conducted in cultivars Perigord and Green Ginger under the experimental and control conditions. Moreover, qPCR analysis was also performed to examine expression in cultivar Abraxas, a third cultivar commonly supplied by the grower, Vitacress. To evaluate the specialised metabolism, the antioxidant and total phenolic content was measured to assess difference between cultivars Perigord and Green Ginger under control and treatment. The volatile composition of the essential oil was analysed by headspace GCMS to observe any effects from the treatment on the two cultivars. The correlation analysis was performed on the two rosemary cultivars, Perigord and Green Ginger to investigate possible correlation between terpene synthase gene expression and the aroma profile.

8. Results

8.2.1 Varietal differential expression of Volatile pathways

As described above, different rosemary cultivars have different aroma profiles. The underlying genes responsible in part for this phenomenon are the terpene synthases that synthesise volatile organic chemicals. Upregulation of a certain terpene synthase could contribute to a change in the aroma profile and does not necessarily mean a boost to volatile production. In terms of aroma enhancement for one variety of rosemary this could actually mean altering upregulation of key terpene genes in order to synthesise more volatile chemicals that are associated with desired aroma. Such as cineole synthase; its major volatile product is 1,8-cineole but this terpene synthase can also produce b-myrcene, sabinene, limonene, a-terpineol, a-pinene and b-pinene in small quantities compared to its production of 1,8-cineole. Therefore, when comparing terpene

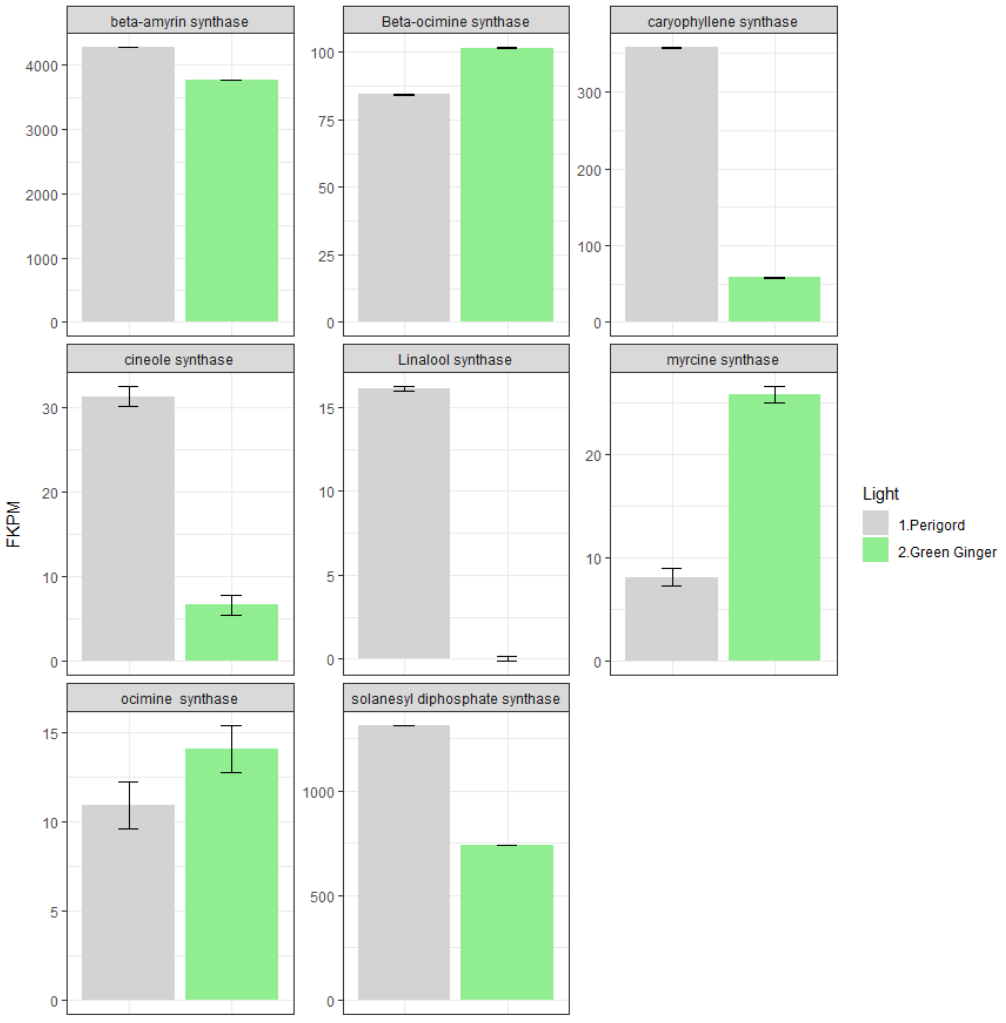


Figure 8. 1 Relative gene expression of terpene sythnases in two rosemary cultivars grown in white light.

RNA seq relative gene expression for key terpene synthases in two varieties of rosemary under white light conditions. The varietal differences in expression levels of these genes is apparent, highlighting the differences in terpene synthase expression contributing to the difference in aroma profiles. Mean FKPM calculated from three RNA sequenced leaf tissue samples per variety, per treatment. Error bars are SEM.

synthase expression between varieties of rosemary in relation to the aroma profile it is important to note the potential of each terpene synthase to produce more than one product.

In figure 8.1 the difference in terpene synthase expression between the two varieties of rosemary in control conditions of white lighting is shown. As a starting point, RNA sequencing data has been used to illustrate differences in varietal terpene synthase expression from *de novo* transcriptome assembly in Rosemary. The variety Perigord, the current supermarket variety, shows upregulated expression of caryophyllene synthase which leads to the production of limonene, terpinolene, β-pinene, gamma-terpinene, α-terpinene. With some terpene synthases being expressed in small amounts in comparison to others, it may be more relevant to look at those highly expressed synthases in Rosemary. In figure 8.1 above these terpene synthases which are highly expressed are β-amyrin synthase and caryophyllene synthase, both of which are expressed more in Perigord. These show greater levels of gene expression and the greater upregulation in one variety may even contribute greatly to the difference in aroma profiles between these two varieties. Yet, small changes in terpene synthase expression should not go unnoticed as these can contribute to the aroma profile through volatile chemicals with strong scent at low concentrations (such as α-pinene which has a high odour strength but is produced in relatively small abundance in Rosemary). From the *de novo* assembly of the rosemary transcriptome, primers have been designed to target these terpene synthase genes and monitor their expression levels in other high performing varieties of rosemary and under growth conditions thought to enhance the production of volatile aromatic compounds (methods see section 2.8 table 2.1).

Figure 8.2 shows the gene expression of key genes in the MVA and MEP terpenoid synthetic pathways. It is relevant to monitor gene expression in these pathways as it can identify which pathway, if any, is more favoured by a particular rosemary cultivar for the synthesis of terpenoids. As stated above, MEP pathway is usually favoured to produce mono-, di- and tetra- terpenes and the MVA is used more for the synthesis of sesquiterpenes and triterpenes. Although both pathways are capable of producing monoterpenes through the production of IPP (the backbone of terpenoids). Rate-limiting steps along the pathways could lead to over or under production of substrates for terpene biosynthesis. The synthase DXPS1 (deoxyxylulose phosphate synthase) catalyses the first step along the MEP pathway for the production of IPP and has been found to be rate-limiting through the use of *Arabidopsis* DXPS1 over- and under- expression mutants MVD from the MVA pathway and GCPE from the MEP pathway are at possible rate limiting steps in the pathway responsible for synthesising IPP(see section 1.1.3). While both are expressed more by Perigord it is GCPE which shows a much higher gene expression by a 1.559 fold-change.

For MVD in the MVA pathway Perigord expresses this gene more than in Green Ginger. Therefore, it is possible that Perigord is favouring the production of terpenoid backbone IPP through the MEP pathway and the higher production of this substrate could attribute to a greater production in volatile organic compounds. Further along the MEP pathway is the production of GPP (Geranyl pyrophosphate). GPP is more specifically used for the production of monoterpenoids and GPP is synthesised by FPS1 and FPS2 as part of the MVA pathway. These genes are expressed at low levels in both rosemary varieties and FPS1 is more significantly expressed in Perigord. This could indicate that the composition of Perigord's aroma profile constitutes more monoterpenes

while Green Ginger may show a variety of more complex volatiles such as sesquiterpenes. Further investigation by GCMS and functional gene analysis would be required to evidence the difference in the aromatic profiles of these two cultivars, which due to financial constraints could not be undertaken in this work. FPS genes are also intertwined with the MEP pathway, they are not exclusively part of the MVA pathway. They are further down this cytosol pathway. Therefore, the MEP pathway also feeds products for the conversion of GPP and geranyl synthase. Reductase and transferase form part of the link here between the two pathways for GPP. Geranyl synthase is expressed more highly in Green Ginger (at 1.765 fold-change expression), possibly leading to the synthesis of monoterpenoids. Geranyl transferase (expressed at a 1.432 fold-change) and geranyl reductase (expressed at a 1.538 fold-change) are also expressed at higher levels in Green Ginger, but these are further downstream of the pathways and form terpenoid-quinone compound.

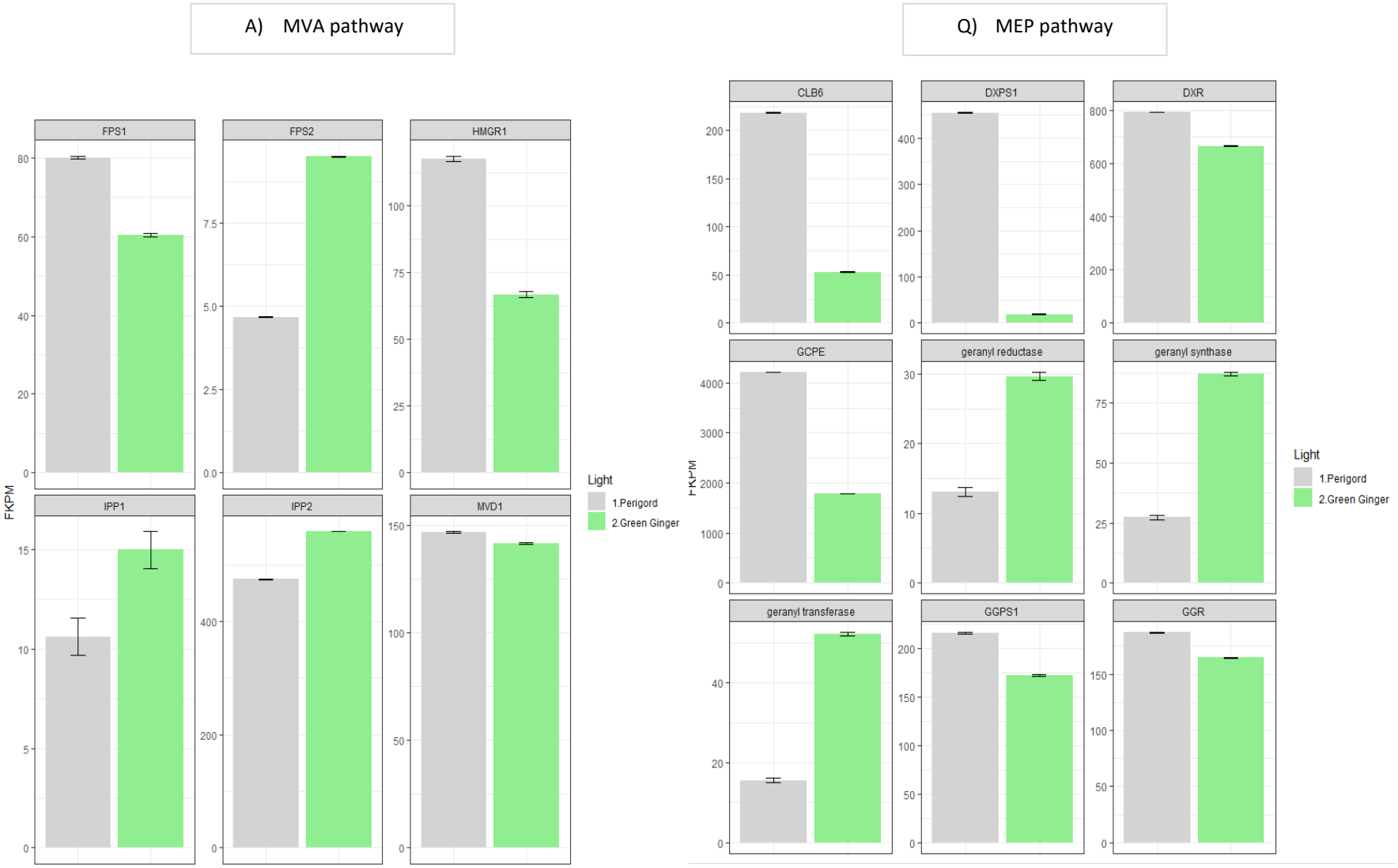


Figure 8. 2 Comparison of relative gene expression of MEP and MVA pathway genes in two rosemary cultivars.

RNA seq relative gene expression for key genes from the MEP and MVA pathways responsible for the synthesis of terpenoids and monoterpenes that form the aroma profile in two varieties of rosemary: Perigord and Green Ginger. A) Gene names for the MVA pathway FPPS1 (farnesyl diphosphate synthase 1) FPS2 (farnesyl diphosphate synthase 2) HMGR1 (hydroxy methylglutaryl coa reductase 1) IPP1 (isopentenyl pyrophosphate 1) IPP2 (isopentenyl pyrophosphate 2) MVD1 (mevalonate diphosphate decarboxylase 1). B) Gene names for the MEP pathway CLB6 (hydroxymethylbutyl diphosphate synthase) DXPS1 (deoxyxylulose phosphate synthase) DXR (deoxyxylulosephosphate reductoisomerase) GCPE ((hydroxymethylbutenyl diphosphate synthase) GCPS1 (geranylgeranyl pyrophosphate synthase 1) GGR (geranylgeranyl reductase). Mean FPKM calculated from three RNA sequenced leaf tissue samples per variety, per treatment. Error bars are SEM.

Gene expression of terpene synthases was investigated in cultivars of rosemary to evaluate cultivar differences in gene expression. The seven terpene synthases were selected from the RNA seq and according to the literature they are common terpene synthases found in Rosemary (see section 1.1.3) contributing the synthesis of volatile compounds and can be used as gene markers of terpene production in Rosemary (methods see 2.4 table 2.1).

The gene expression of seven terpene synthases in different rosemary cultivars is shown in figure 8.3. The cultivars are compared to Perigord, to calculate log2foldchange and to assess cultivars performance in terms of gene expression. As seen for some terpene synthases, the differences between cultivars can vary. For example, cineole synthase and lupeol synthase which show different expression level and are even downregulated in some varieties. β-caryophyllene synthase is upregulated in all three cultivars, but at different levels of expression compared to Perigord and Green Ginger shows a 7.8 log2 fold change in expression of this gene. β-caryophyllene synthase is responsible for the synthesis of gamma bisabolene, (E)-nerolidol and α-bisabolol. These three compounds are classified as sesquiterpenes and are being synthesised at high levels in Green Ginger. Green Ginger and Logee Blue both have a similar upregulation of ocimene synthase compared to Perigord. Ocimene synthase synthesises volatile compounds typically found in Rosemary volatile fraction, such as; b-ocimine, terpinolene, b-myrcene and β-pinene. The differences in gene expression seen here can be attributed to varietal differences among rosemary.

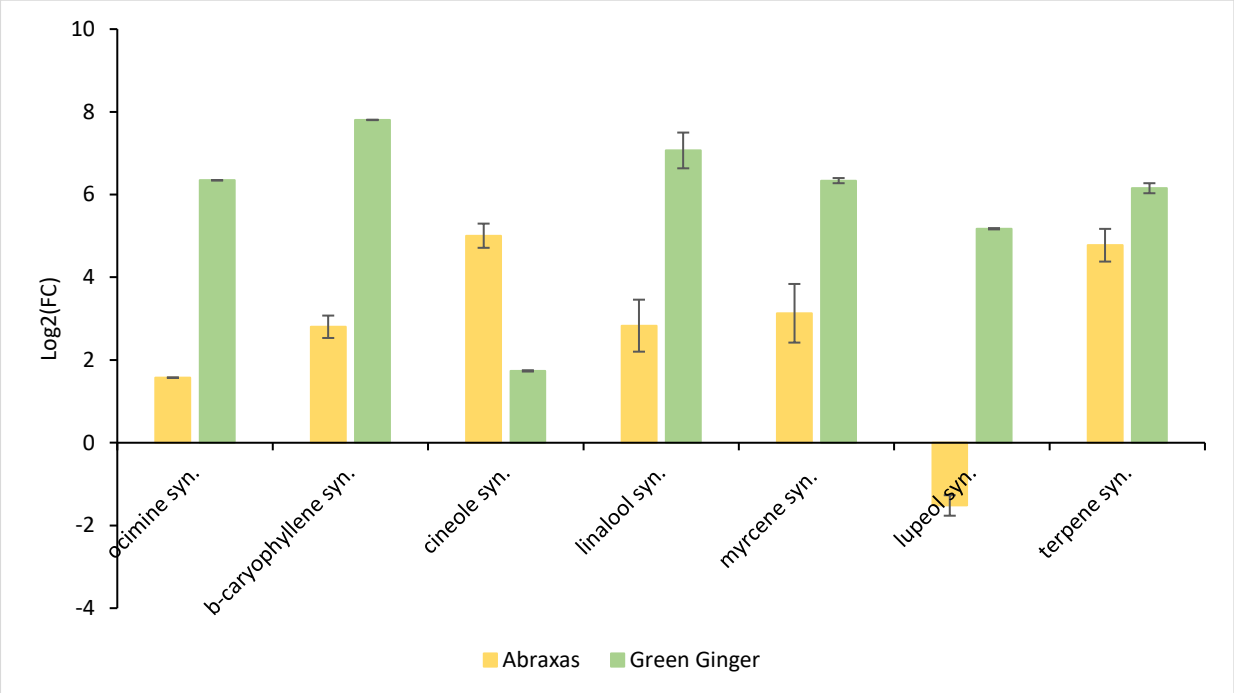


Figure 8. 3 Gene expression of seven terpene synthases in two rosemary cultivars compared to expression levels in Perigord.

Log2(Fold Change) calculated using Perigord as a control comparison and GAPDH used as housekeeping gene. Error bars = SEM.

8.2.2 Changes in secondary metabolite production under supplementary far-red lighting

There was a significant difference in both the antioxidant content and the phenolic content between the two cultivars (ANOVA p value= < 0.01). The Phenolic content in Perigord is higher than in Green Ginger, however the antioxidant content in lower in Perigord compared to Green Ginger (figure 8.4).

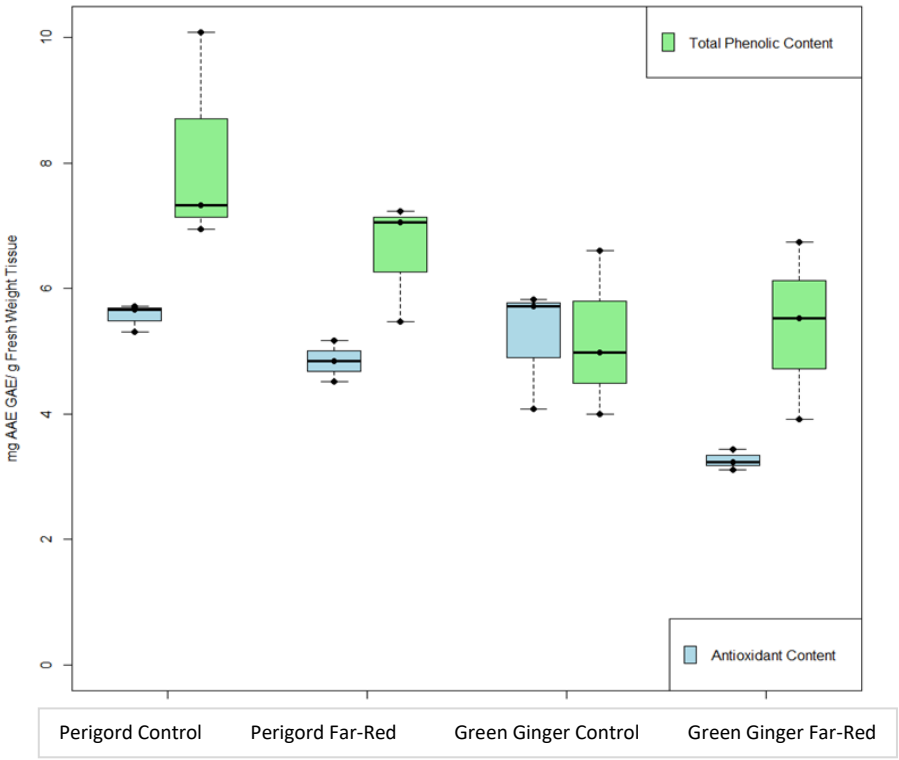


Figure 8. 4 Antioxidant content and total phenolic content for two varieties of rosemary grown in white light (control) and under supplementary far-red lighting.

The antioxidant content was measured by FRAP assay and expressed as mg of AAE/g of FW leaf tissue. Total phenolic content was measured by the Folin assay and expressed as mg of GAE/g of FW leaf tissue. The rosemary varieties are Perigord, one of the current supermarket varieties and Green Ginger. Antioxidant content is shown in blue in mg Ascorbic acid equivalents/g fresh weight leaf tissue, Phenolic content is shown in green in mg Gallic acid equivalents/g fresh weight leaf tissue.

The supplementary far-red lighting showed no significant change in the antioxidant content or total phenolic content for Perigord after five days of treatment. Under both light conditions the total phenolic content showed no significant change in either rosemary variety (ANOVA p value= <0.05). The antioxidant content for Perigord also showed no significant change under the far-red lighting (ANOVA Dunn's post-hoc p value= 0.281). However, for the variety Green Ginger, the antioxidant content of the leaves reduced under supplementary far red lighting (ANOVA Dunn's post-hoc p value= 0.02). In summary, the far-red light treatment decreased the antioxidant potential of the plants.

8.2.3 Supplementary Far-red light altered the chemical composition of the volatilome

The volatile composition is different in each cultivar, and changes in the volatiles are even more pronounced with far-red lighting. A first-step analysis was performed using SIMPER (based on the Bray-Curtiss dissimilarity matrix, (Clarke, 1993)) which showed the volatiles that contribute the most difference between groups, with both cultivars and treatments included in the analysis. In control conditions, cumulative difference of 74% seen

between Perigord and Green Ginger was contributed by cultivar differences. This indicates the cultivars alone had a different blend of volatile chemicals emitted. In both Perigord and Green Ginger 77% of the volatiles had a significant change in quantity when treated with far red lighting, indicating far red light was adding to the difference observed. Since there are many volatile chemicals that form part of the rosemary volatilome all analysed volatiles in both cultivars and treatments were used for the principal component analysis (PCA). The key volatiles contributing to the most change within treatment groups are highlighted in the PCA bi-plot below (Figure 8.5). The PCA was produced using Past3 software, component 1 contributes to 59.46 % and Component 2 contributes 22.86 %. Three samples from each condition are plotted, and from the analysis we see grouping of the samples based on cultivar differences in control conditions. Plants in the treatment group from both cultivars group more closely on the PCA.

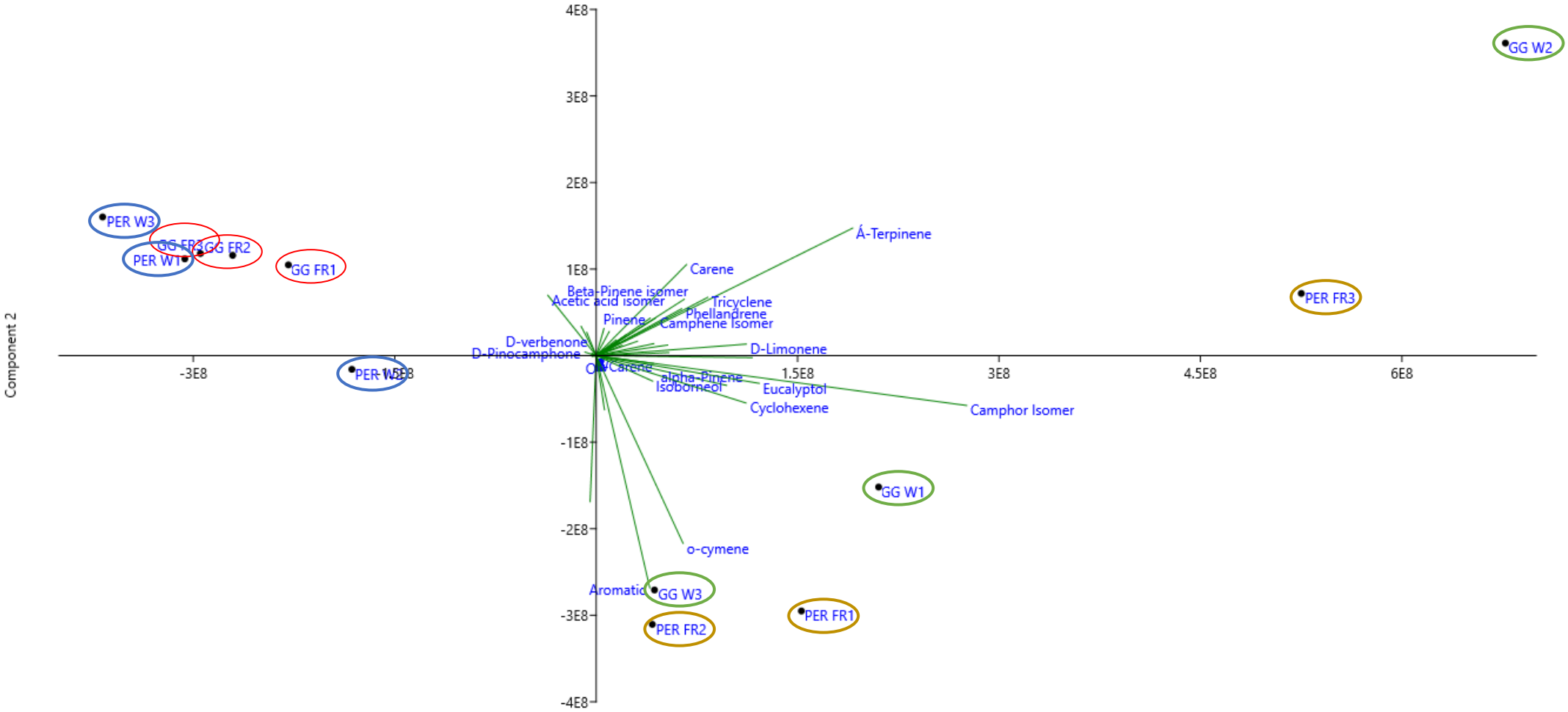


Figure 8. 5 Principal components analysis of the volatilome in two rosemary cultivars (Perigord and Green Ginger) treated with supplementary far-red light.

The volatiles contributing the greatest change to the volatilome are shown on the graph. PER W1 to PER W3 denotes three biological replicates of Perigord grown under white light.GG W1 to GG W3 are Green Ginger plants also grown in white lighting. PER FR1 to PER F3 are the three biological replicates for Perigord treated with supplementary far-red light. GG FR1 to GG FR3 are replicates of Green Ginger grown with supplementary far red light. Component 1 contributes to 59.46 % and Component 2 contributes 22.86 %.

Table 8.1: SIMPER analysis of 18 aromatic volatile monoterpenes that contribute a high percentage of dissimilarity between cultivars Perigord and Green Ginger in white light conditions. The mean quantity of volatiles emitted from both cultivars is included as PPM (parts per million).

Volatile name	***Contribution to similarity***	*Contribution %*	*Mean Green Ginger in white light*	*Mean Perigord in white light*
3-Carene	0.04256	0.0815	72.4	104
Acetic acid	0.06289	0.1204	48.5	138
Á-Terpinene	0.7329	1.403	941	2150
Camphene	0.4927	0.9434	852	38.4
Camphor isomer	0.2025	0.3878	446	103
Carene	0.8108	1.552	813	2150
Cyclohexane	0.364	0.6971	22.9	716
D-Limonene	0.09451	0.181	303	151
D-pinocamphone	0.822	1.574	1430	22.3
D-verbenone	0.02044	0.03914	23.4	47.8
Eucalyptol	0.1024	0.196	207	32.3
Isoborneol	0.7146	1.368	1460	719
o-cymene	0.06723	0.1287	211	120
Phellandrene	0.7812	1.496	88	1430
Pinene	0.7182	1.375	1470	719
Tricyclene	0.4735	0.9066	789	4.02
α-pinene	0.08096	0.155	176	36.9
ß-pinene	0.4379	0.8386	1430	2150

Table 8.2: SIMPER analysis of 18 aromatic volatile monoterpenes that contribute a high percentage of dissimilarity between Perigord treated with white light and supplementary far red light. The mean quantity of both light treatment groups is included as PPM (parts per million).

Volatile name	***Contribution to similarity***	***Contribution %***	***Mean Perigord white light***	***Mean Perigord far red light***
3-Carene	0.046	0.09845	104	38.8
Acetic acid	0.06074	0.13	138	47.2
Á-Terpinene	0.3528	0.7552	2150	1540
Camphene Isomer	0.01296	0.02773	1.27	23.8
Camphor Isomer	0.2115	0.4527	103	472
Carene	0.4327	0.9262	2150	1430
Cyclohexene	0.07528	0.1611	52.2	180
D-Limonene	0.07786	0.1666	151	266
D-pinocamphone	0.7844	1.679	22.3	1430
D-verbenone	0.02058	0.04405	47.8	18.8
Eucalyptol	0.08635	0.1848	32.3	186
Isoborneol	0.05927	0.1269	123	150
o-cymene	0.08721	0.1867	120	280
Phellandrene	0.6657	1.425	1430	752
Pinene	0.5418	1.16	719	744
Tricyclene	0.4736	1.014	4.02	792
α-Pinene	0.09342	0.1999	36.9	202
ß-pinene	0.3883	0.8312	2150	1470

Table 8.3: SIMPER analysis of 18 aromatic volatile monoterpenes that contribute a high percentage of dissimilarity between Green Ginger treated with white light and supplementary far red light. The mean quantity of both light treatment groups is included as PPM (parts per million).

Volatile name	*Contribution to similarity*	*Contribution %*	*Mean Green Ginger white light*	*Mean Green Ginger far red light*
3-Carene	0.01865	0.03395	72.4	93.4
Acetic acid	0.05979	0.1088	48.5	146
Á-Terpinene	0.7754	1.411	941	2150
Camphene Isomer	0.03042	0.05536	51.7	4.55
Camphor Isomer	0.2131	0.3878	446	107
Carene	0.7315	1.331	813	1450
Cyclohexene	0.4431	0.8064	222	766
D-Limonene	0.06907	0.1257	303	201
D-pinocamphone	0.8702	1.584	1430	10.9
D-verbenone	0.01599	0.02911	23.4	50.1
Eucalyptol	0.1038	0.189	207	41.1
Isoborneol	0.03788	0.06895	158	96.7
o-cymene	0.09977	0.1816	211	45.7
Phellandrene	0.05202	0.09468	88	17.7
Pinene	0.8867	1.614	1470	6.23
Tricyclene	0.4981	0.9066	789	8.55
α-Pinene	0.0543	0.09883	176	89.5
β-pinene	0.4285	0.7798	1490	2150

Volatile name	Mean Log(FC) Perigord with supplementary far-red light	Mean Log(FC) Green Ginger with supplementary far-red light
3-Carene	-0.43	0.11
acetic acid	-0.47	0.48
α-Pinene	0.74	-0.29
Aromatic	0.00	0.47
Á-Terpinene	-0.14	0.36
β-pinene	-0.17	0.16
Camphene Isomer	1.27	-1.06
Camphor Isomer	0.66	-0.62
Carene	-0.18	0.25
Cyclohexene	0.54	0.54
D-Limonene	0.25	-0.18
D-pinocamphone	1.81	-2.12
D-verbenone	-0.41	0.33
Eucalyptol	0.76	-0.70
Isoborneol	0.09	-0.21
o-cymene	0.37	-0.66
Phellandrene	-0.28	-0.70
Pinene	0.01	-2.37
Tricyclene	2.29	-1.97

Figure 8. 6 Heatmap of volatiles present in the volatilome of two rosemary cultivars Perigord and Green Ginger treated with supplementary far red lighting.

The mean log fold change values are calculated from the values in white light. Colours represent positive and negative changes in log(FC) values: Green shows positive values, Yellow no change, Red shows negative values.

Samples of Perigord grown in white light had three significant volatiles that were different from Green Ginger in white light. These volatiles are D-pinocamphone, β-pinene isomer, an acetic acid isomer and D-verbenone. β-pinene, D-verbenone and D-pinocamphone are aromatic monoterpenes typically present in the volatilome of rosemary. The acetic acid isomer (full name acetic acid 1 7 7-trimethyl-bicyclo 2.2.1 hept-2-yl ester) is also typically found in rosemary and has the same chemical structure as bornyl acetate, a bicyclic monoterpene with a camphor and herbal aroma. When plants of Perigord were grown in far red lighting the volatile composition of Perigord changed. Volatiles most present in Perigord treated with far red light were o-cymene, α-pinene, camphene and Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)- (also known as camphor isomer). A-terpinene had a mean log FC of 9.19 in Perigord treated with far red lighting. There was a small decrease in A-terpinene between white light and supplementary far red light for Perigord, with a mean log FC value of -0.14. A-terpinene has aroma descriptors of woody, pine, and thymol. Since A-Terpinene is present in large quantities in the volatilome of Perigord, a decrease in the emission could have an overall impact on the aroma. The SIMPER analysis (Table 8.2) shows the mean quantities of 17 monoterpenes in Perigord, with the supplementary far red light increasing the quantities of several volatile monoterpenes; D-pinocamphone, o-cymene, eucalyptol, D-Limonene and Pinene show large increases with supplementary far red light. Supplementary far red light was a good tool to perturb the plants volatile emissions, as there was a large percentage of volatile changing in Perigord when grown with far red lighting. With far red light there were also decreases in 3-Carene, acetic acid, β-pinene, Carene, D-verbenone and Phellandrene. These changes in the Perigord volatilome are shown on figure 8.6.

There are many differences in the volatile emissions of Perigord and Green Ginger cultivars. Green ginger had higher emission levels of D-pinocamphone, Isoborneol, Tricyclene and Pinene than Perigord. Other volatile chemicals were lower in Green Ginger than in Perigord, these include cyclohexene, phellandrene, carene, β-pinene and A-terpinene. When Green Ginger was placed in white light the eucalyptol content was higher than in Perigord, and this can be seen from the SIMPER analysis, table 8.1. These differences between cultivars highlight the variety of chemical volatiles present in rosemary and supplementary far-red light further amplified the changes in the volatilome. The volatile composition of Green Ginger in white light was similar to the volatile composition of Perigord in far red light, with cyclohexene and carene increasing in both cultivars with supplementary far red light (Tables 8.2 and table 8.3). When Green ginger is treated with supplementary far-red light the volatile composition changes. GG FR samples group close to Perigord under white light samples, showing similarities in volatiles present in the Green Ginger far-red samples to Perigord in white light (figure 8.5). Green Ginger in supplementary far red light showed decreases in eucalyptol, camphene, o-cymene, α-pinene (and other pinene isomers), D-pinocamphone, D-limonene and phellandrene. There were increases in β-pinene, carene, cyclohexene, and D-verbenone for Green Ginger treated with supplementary far red light (figure 8.6).

8.2.4 Expression along the terpenoid metabolic pathway influences the volatilome in two rosemary cultivars

Terpene synthase gene expression is different between the cultivars Perigord and Green Ginger (figure 8.7). There are also differences in the expression of genes involved in the MEP and MVA pathways between the cultivars (figure 8.8 and figure 8.9). Terpene synthase genes were identified in the two Rosemary cultivars, and their expression levels under different light conditions were calculated. The expression of terpene synthases under supplementary far-red light can be seen in figure 8.7. The expression of these synthases was compared to white light conditions as a control, to investigate if supplementary far-red lighting can enhance the expression of genes involved in the production of volatile organic compounds in rosemary and ultimately possibly alter the aroma and taste. Even between the two varieties different terpene synthases are found, which reflects the different aroma profiles of these two rosemary varieties. For Perigord the expression of most terpene synthases significantly increased under far-red lighting except for cineole synthase and β-caryophyllene synthase (figure 8.7 A). β-ocimene synthase had an increase in expression under supplementary far-red light and is responsible for the synthesis of monoterpenes, with β-myrcene and α-farnesene being its main synthesised compounds. Expression of terpene synthases was also examined in Green Ginger grown in with light or with supplementary far-red lighting. The upregulation of β-amyrin synthase, ocimene synthase and terpene synthase is noticeably greater under supplementary far-red light. The expression of these terpene synthases under far-red light is more variable in Green Ginger indicating a change in volatile compounds being produced and possibly a change in the aroma profile.

In figure 8.8 the expression of key genes in the MVA pathway are shown for both rosemary varieties treated with supplementary far-red lighting. The MVA pathway is responsible for the synthesis of complex terpenoids such as sesquiterpenes. The graph shows variability in gene expression of these genes in the MVA pathway, but it is important to note the expression levels of MVD, as this protein is responsible for the synthesis of IPP. The expression of MVD increases slightly for Perigord under far-red lighting. For Green Ginger the expression of MVD remains the same under far-red lighting, shown by the mean expression of MVD in white light for Green Ginger remaining at the same level. As can be seen the expression of FPS1 and FPS2 in Green ginger has increased under supplementary far-red lighting. This could indicate Green Ginger favours production of terpenes through the MVA pathway when treated with far-red lighting.

The MEP pathway is shown in 9.9. The expression of GCPE has increased in both varieties when treated with far-red lighting. For Perigord there is a high increase in gene expression of GCPE, which is involved in the synthesis of IPP at the second to last step of IPP production. However, geranyl synthase expression is lower in both varieties treated with far-red light. For Green Ginger the expression levels of GCPE also increases under far-red lighting but there is a decrease in geranyl synthase. Geranyl reductase and geranyl transferase have decreased expression levels under supplementary far-red lighting in Perigord. Green ginger shows an increase in geranyl transferase but a decrease in geranyl reductase. This gives an indication of changes to the MEP pathway for the production of terpenoids in both varieties under supplementary far-red lighting. Figures 8.10 and 9.11 show the relative responsiveness of genes along the MEP and MVA pathways in Perigord and Green

Ginger under supplementary far-red lighting. The map allows a direct comparison of gene expression along the pathways towards terpene biosynthesis. Figure 8.10 shows Perigord and Green ginger individually. Both cultivars show remarkably similar changes in gene expression in response to supplementary FR. Both show increased gene expression in both the MEP and MVA pathway, but there are some differences. For example, Perigord uniquely shows an increase in expression of genes associated with monoterpenoid biosynthesis. When these maps are overlaid, in figure 8.11, it shows that Perigord (in yellow) has the greater change in gene expression in both the MEP and MVA pathways when treated with far-red light compared to Green Ginger. This indicates the volatile pathways in Perigord have undergone a greater responsiveness to far red light.

A) Rosemary Variety Perigord

B) Rosemary Variety Green Ginger

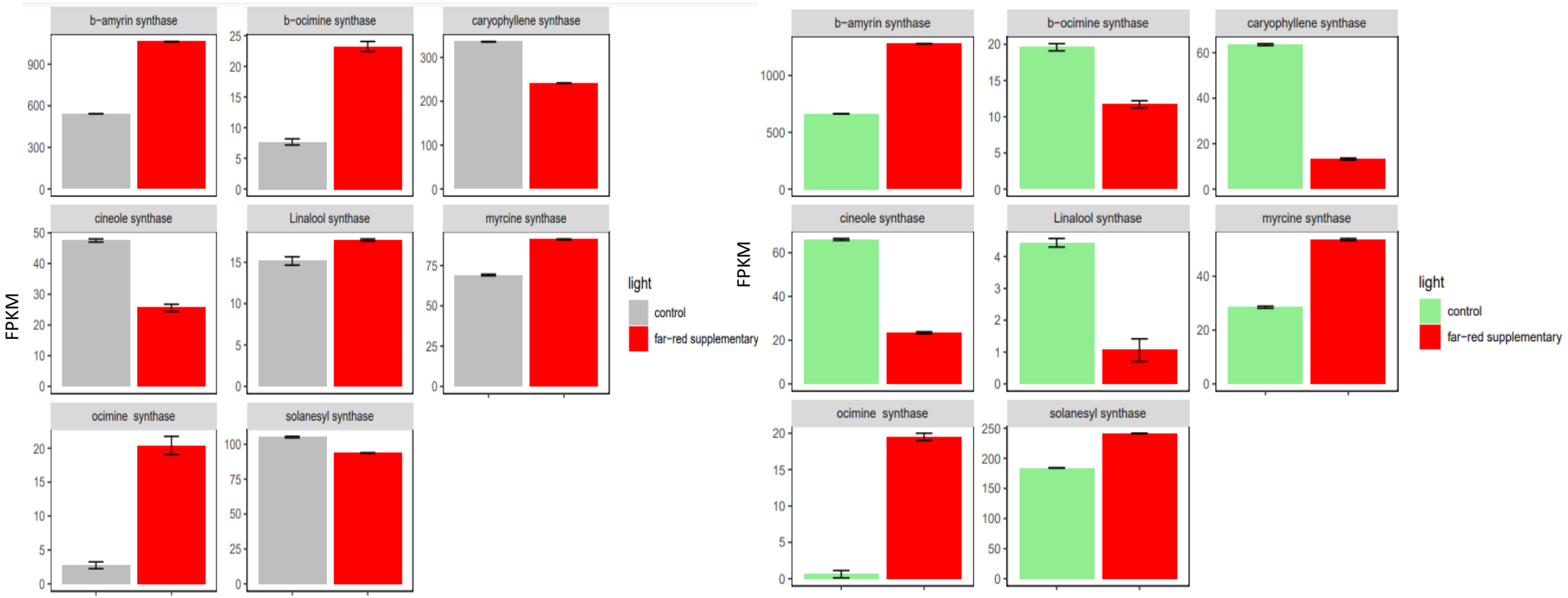


Figure 8. 7 Gene expression of Terpene Synthase genes from RNA sequence analysis of the two varieties of Rosemary treated with supplementary Far-red lighting.

A) Basemean gene expression of terpene synthases in the rosemary variety Perigord under control of white lighting and supplementary far-red lighting. B) Basemean gene expression of terpene synthases in variety Green Ginger under control lighting and with supplementary far-red lighting. Basemean calculated from three RNA sequenced leaf tissue samples per variety, per treatment. Error bars are SEM.

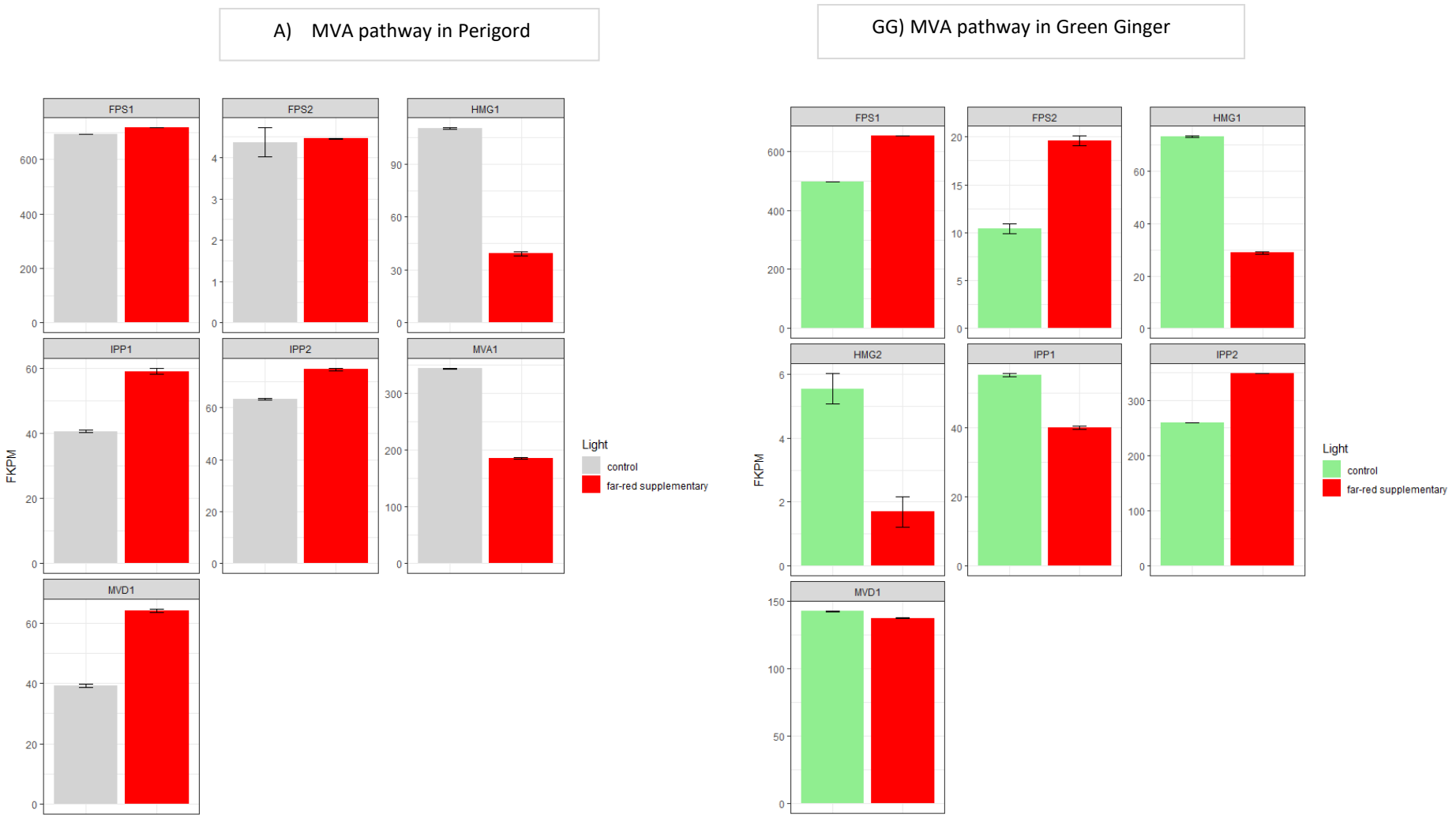


Figure 8.8 Relative gene expression of key genes in the MVA pathway in two Rosemary cultivars treated with supplementary far-red lighting.

A) Gene expression of key genes along the MVA pathway in Perigord, FPPS1 (farnesyl diphosphate synthase 1) FPS2 (farnesyl diphosphate synthase 2) HMGR1 (hydroxy methylglutaryl coa reductase 1) IPP1 (isopentenyl pyrophosphate 1) IPP2 (isopentenyl pyrophosphate 2), MVA1 (acetyl-CoA C-acetyltransferase) MVD1 (mevalonate diphosphate decarboxylase 1. B) Gene expression of Key genes along the MVA pathway in Green Ginger. Gene names: FPPS1 (farnesyl diphosphate synthase 1) FPS2 (farnesyl diphosphate synthase 2) HMGR1 (hydroxy methylglutaryl coa reductase 1) IPP1 (isopentenyl pyrophosphate 1) IPP2 (isopentenyl pyrophosphate 2), MVA1 (acetyl-CoA C-acetyltransferase) MVD1 (mevalonate diphosphate decarboxylase 1. Basemean in FKPM calculated from three RNA sequenced leaf tissue samples per variety, per treatment. Error bars are SEM.

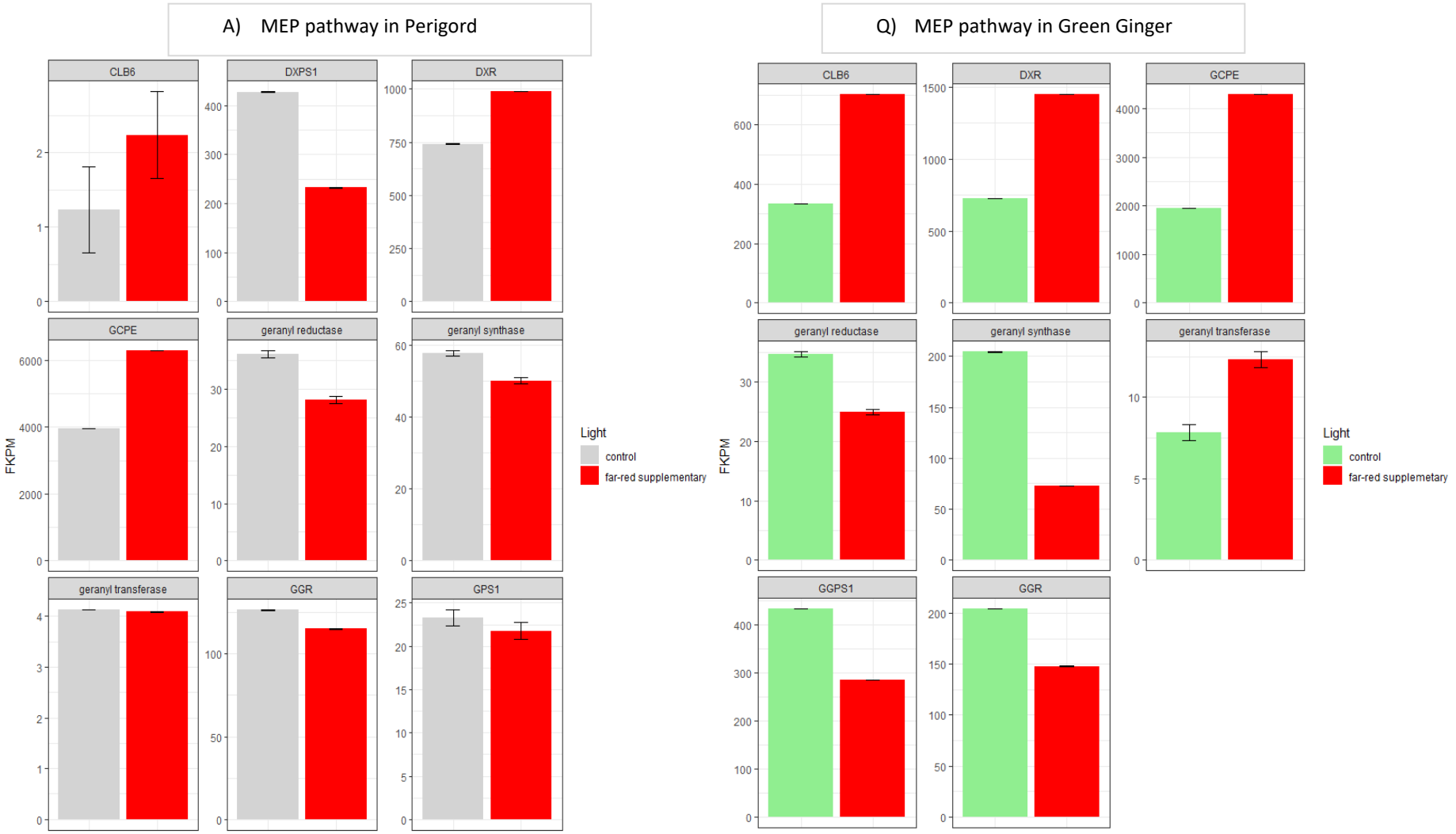
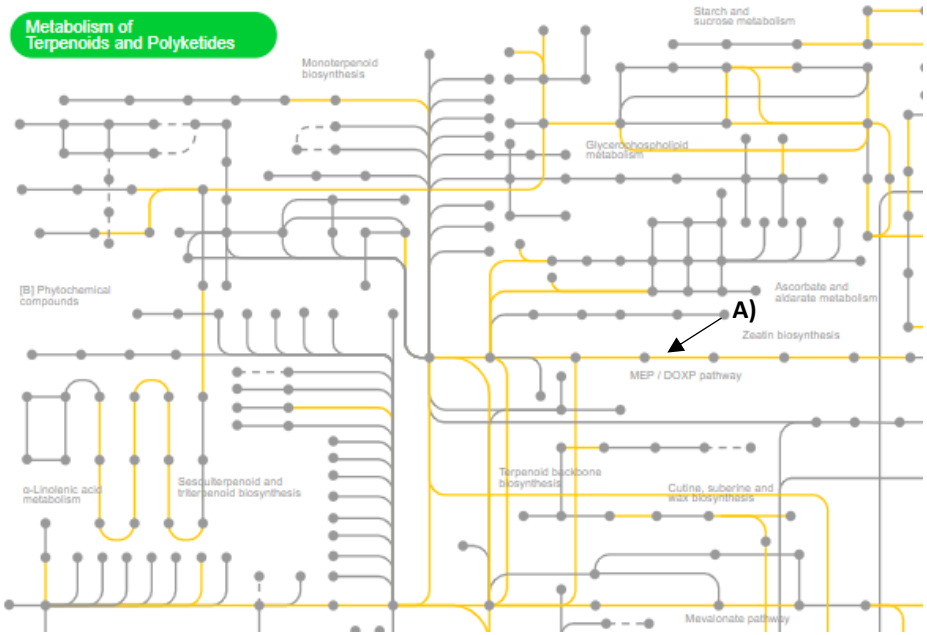


Figure 8. 9 Relative gene expression in FKPM of key genes in the MEP pathway for two rosemary cultivars treated with supplementary far-red light.

A) MEP pathway genes in Perigord. B) Gene expression of Key genes along the MEP pathway in Green Ginger. Gene names CLB6 (hydroxymethylbutyl diphosphate synthase) DXR (deoxyxylulosephosphate reductoisomerase) GCPE ((hydroxymethylbutenyl diphosphate synthase) GCPS1 (geranylgeranyl pyrophosphate synthase 1) GGR (geranylgeranyl reductase). Basemean in FKPM calculated from three RNA sequenced leaf tissue samples per variety, per treatment. Error bars are SEM.

1.Perigord



2.Green Ginger

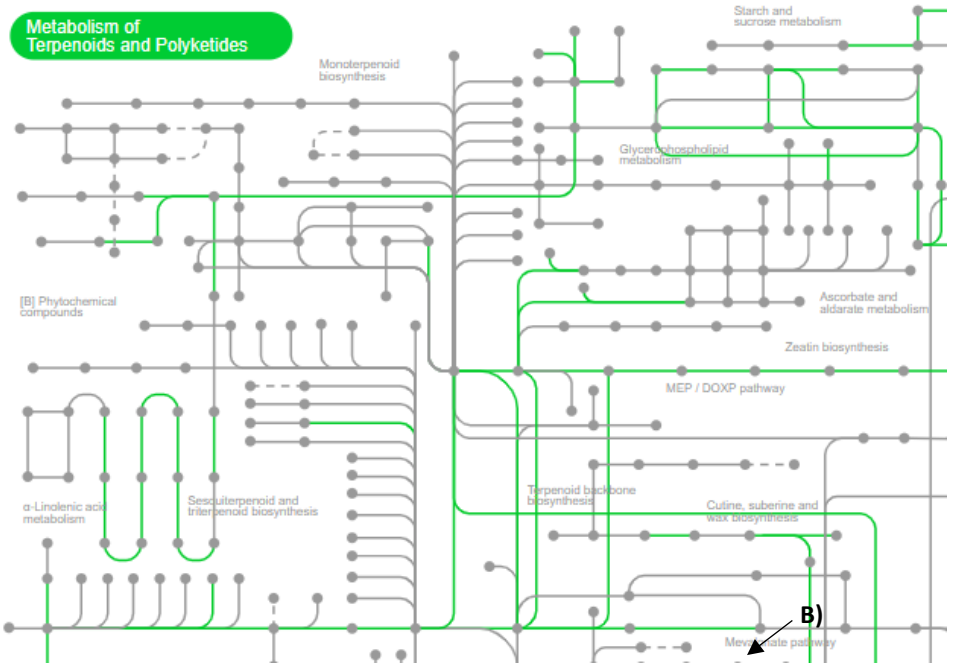


Figure 8.10 Relative responsiveness of terpenoid metabolic pathway under supplementary far-red lighting.

Metabolic pathway showing the relative responsiveness to supplementary far-red lighting of terpenoid pathway genes in two varieties of rosemary under supplementary far-red light conditions, for Perigord (image 1) and for Green Ginger (image 2). Arrow A) shows the MEP pathway and arrow B) shows the MVA pathway. Image 1, Yellow shows genes expressed in Perigord in the MEP and MVA pathways. Image 2, Green shows gene expressed in Green Ginger in the MEP and MVA pathways. Gene expression data from RNA sequence analysis, filtered to include top differentially expressed genes >0.5 log2 fold change. Map made on iPath interactive pathway explorer, software created by [*Letunic et al.,* 2008].

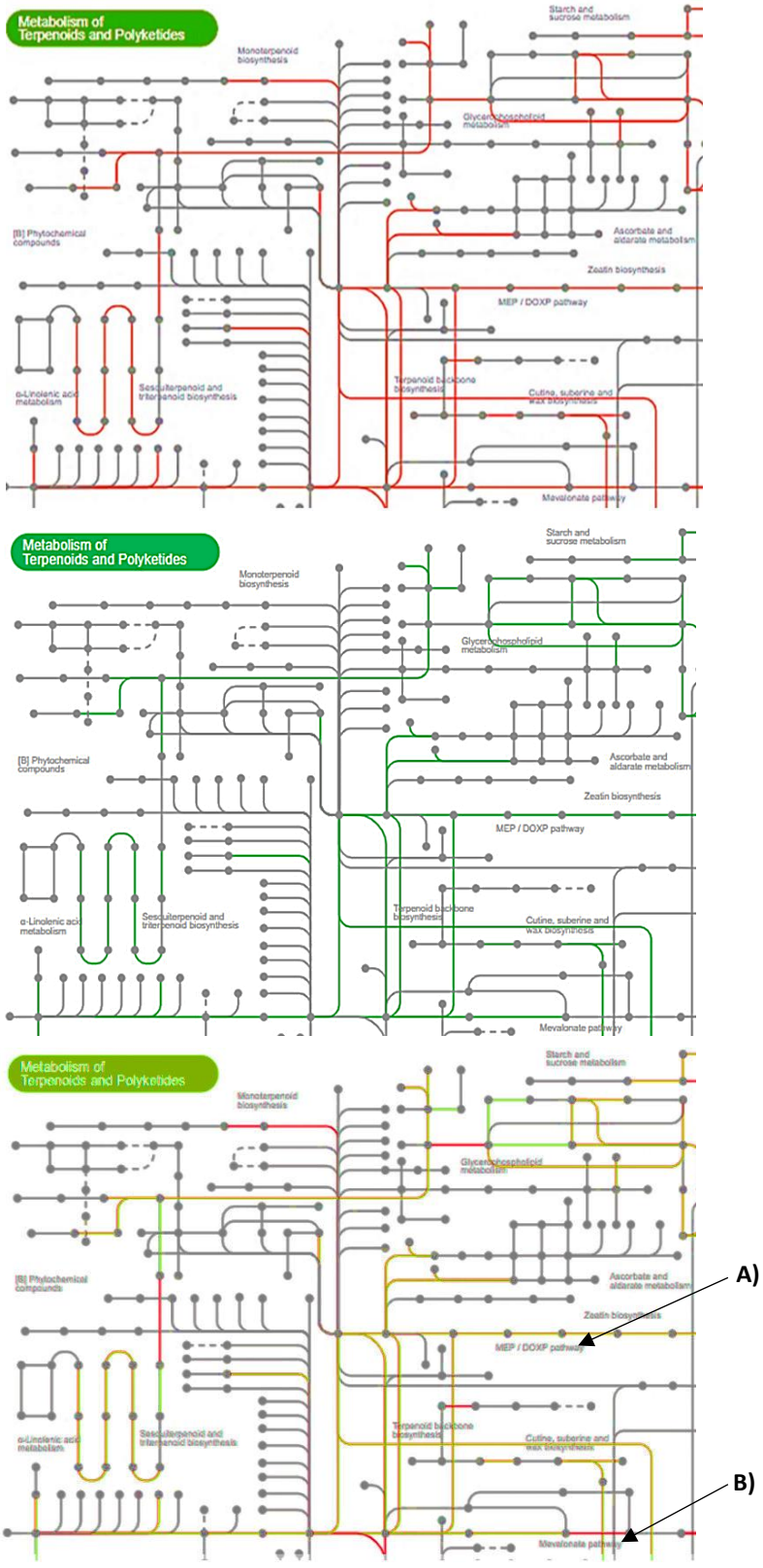


Figure 8.11 Relative responsiveness of terpenoid metabolic pathway under supplementary far-red lighting.

Metabolic pathway showing the relative responsiveness to supplementary far-red lighting of terpenoid pathway genes in two varieties of rosemary under supplementary far-red light conditions, for Perigord (represented by red lines) and for Green Ginger (represented by green lines) and an overlay in which commonly regulated pathways show in yellow. Arrow A) shows the MEP pathway and arrow B) shows the MVA pathway. Gene expression data from RNA sequence analysis, filtered to include top differentially expressed genes >0.5 log2 fold change. Map made on iPath interactive pathway explorer, software created by [*Letunic et al.,* 2008].

Primers were designed from gene sequences obtained from the annotated transcriptome (see chapter 6) to target key terpene synthases genes. The qPCR primers were used to validate RNA seq expression of terpene synthases under different environmental conditions and between different cultivars as an indicator of improved aroma. qPCR was performed on both varieties under while lighting as a control and the Log2 foldchange difference was calculated based on white light expression levels from both cultivars (figure 8.12). The expression of 5 terpene synthases was upregulated under far-red light in Perigord (Figure 8.12 A). This follows a similar trend to the RNA sequencing data showing upregulation of cineole synthase, Linalool synthase, Myrcene synthase and Lupeol synthase. Upregulation of these genes in Perigord would be consistent with a change in aromatic volatiles under supplementary far-red lighting. The upregulation of these terpene synthases would be consistent with the emission of the following volatiles: gamma bisabolene, (E)-nerolidol,α-bisabolol, linalool, b-ocimene, a-farnesene, b-amyrin and lupeol. For Green Ginger, shown on the graph in figure 8.9 B), there was downregulation of six terpene synthases under supplementary far-red lighting with the exception of cineole synthase, which was upregulated under far-red light. The downregulation of linalool under supplementary far-red light was -6.4 log2foldchange with other downregulated genes falling between -3.2 to -3.7 log2foldchange. The downregulation of these genes would mean a change in aroma for Green Ginger as synthesis of products from these terpene synthases would be affected.

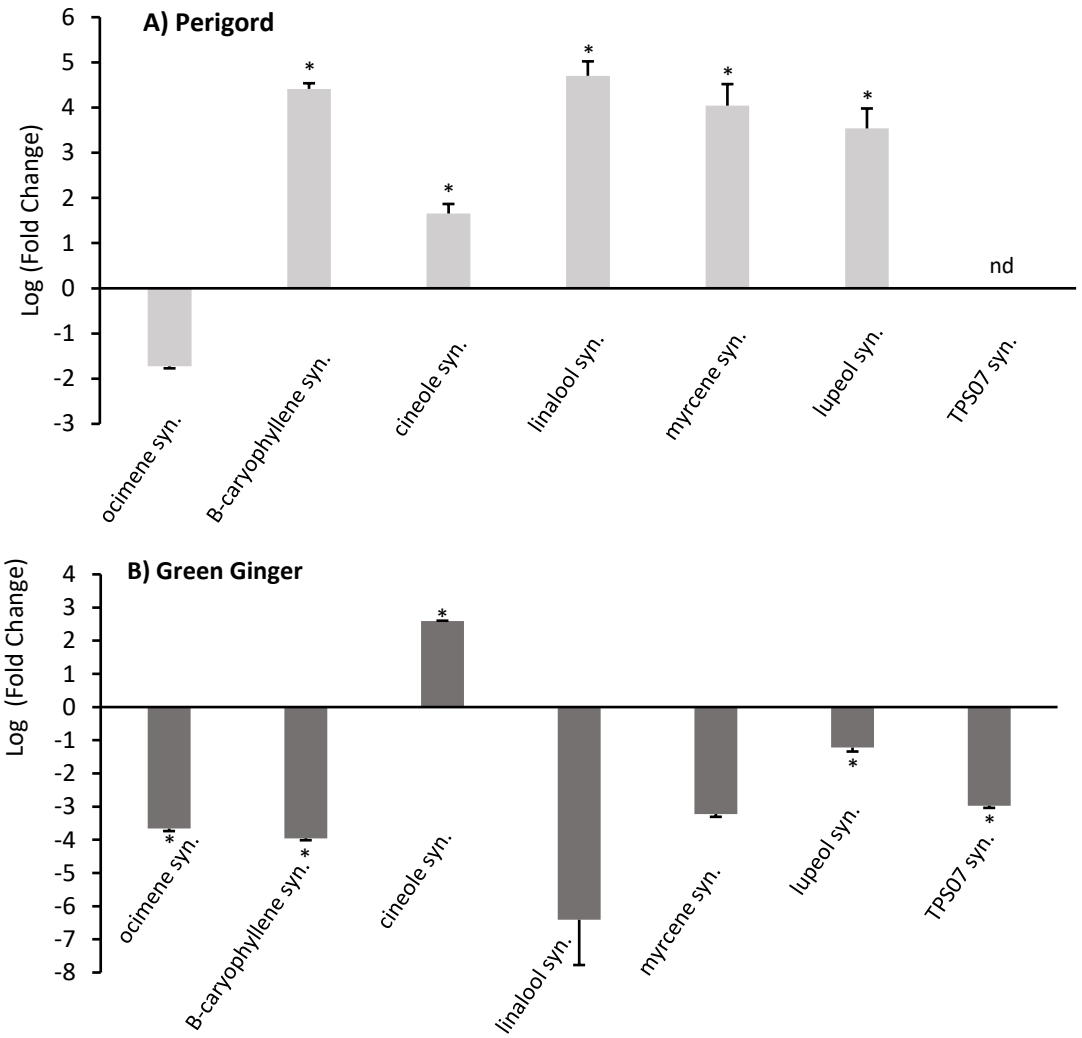


Figure 8.12 Terpene synthase gene expression in two varieties of rosemary treated with supplementary Far-red lighting.

A) Rosemary variety Perigord Log (Fold change) gene expression of 7 terpene synthases compared with control condition of white light. B) Rosemary variety Green Ginger Log (Fold change) gene expression of 7 terpene synthases compared with control condition of white light. Error bars = SEM * indicates statistical significance in gene expression between light treatments.

8.2.5 Correlation of terpenoid pathway genes with volatiles present in two rosemary cultivars in white and supplementary far-red light

Both cultivars and treatments groups were analysed to investigate correlations by including the influences of environmental or genetic factors. The correlation analysis was carried out for both cultivars in both treatment conditions, using Spearman correlation on Past3, version 4.04 (Hammer *et al.,* 2013). . The selected variables were the volatile chemicals emissions and the relative gene expression in the terpenoid pathways. Since most of the volatiles did not correlate with the gene expression a condensed figure was produced (figure 8.13). This figure shows correlation of volatiles with significant correlation to one or more genes in the terpenoid pathways. Significant correlation is indicated by a correlation coefficient of 0.33 or higher (p-value significance is lower than 0.05). The analysis confirms some strong correlations between expression on the terpenoid pathway genes and the emission of volatiles.

The MEP and MVA pathway genes show strong positive correlations with volatile emissions. IPP1 and IPP2 have jointly, positively correlated with the expression of near all volatiles shown. Their function in the terpenoid pathway is to convert IPP to DMAPP for mono-, di-, triterpene synthesis. Sesquiterpenes are synthesised from the other precursor, FPP which is produced by FPS2. In the analysis FPS2 shows positive correlations with the sesquiterpenes cubenene and β-cubenene.

The gene expression of terpene synthases showed both positive and negative correlations with the volatile emissions. TPS-CIN, cineole synthase, had positive correlation with phellandrene, decanal and heptadecane. The major product of cineole synthase is eucalyptol (1,8 cineole) has a positive correlation but has a p-value >0.05 and was not significant. LUP1, lupeol synthase 1, showed a positive correlation with α-pinene and α-pinene isomers. LUP2 correlated negatively with eucalyptol, α-pinene and its isomers. TPS14 is a linalool synthase had negative correlation with m-cymene isomer. TPS07 a putative terpene synthase correlated positively with m-cymene isomer and cubenene (a sesquiterpene). The results of the correlation analysis seem to indicate the complexity in terpene synthase activity in relation to their synthetic products and does seem to inform on the multi-product ability of terpene synthases.

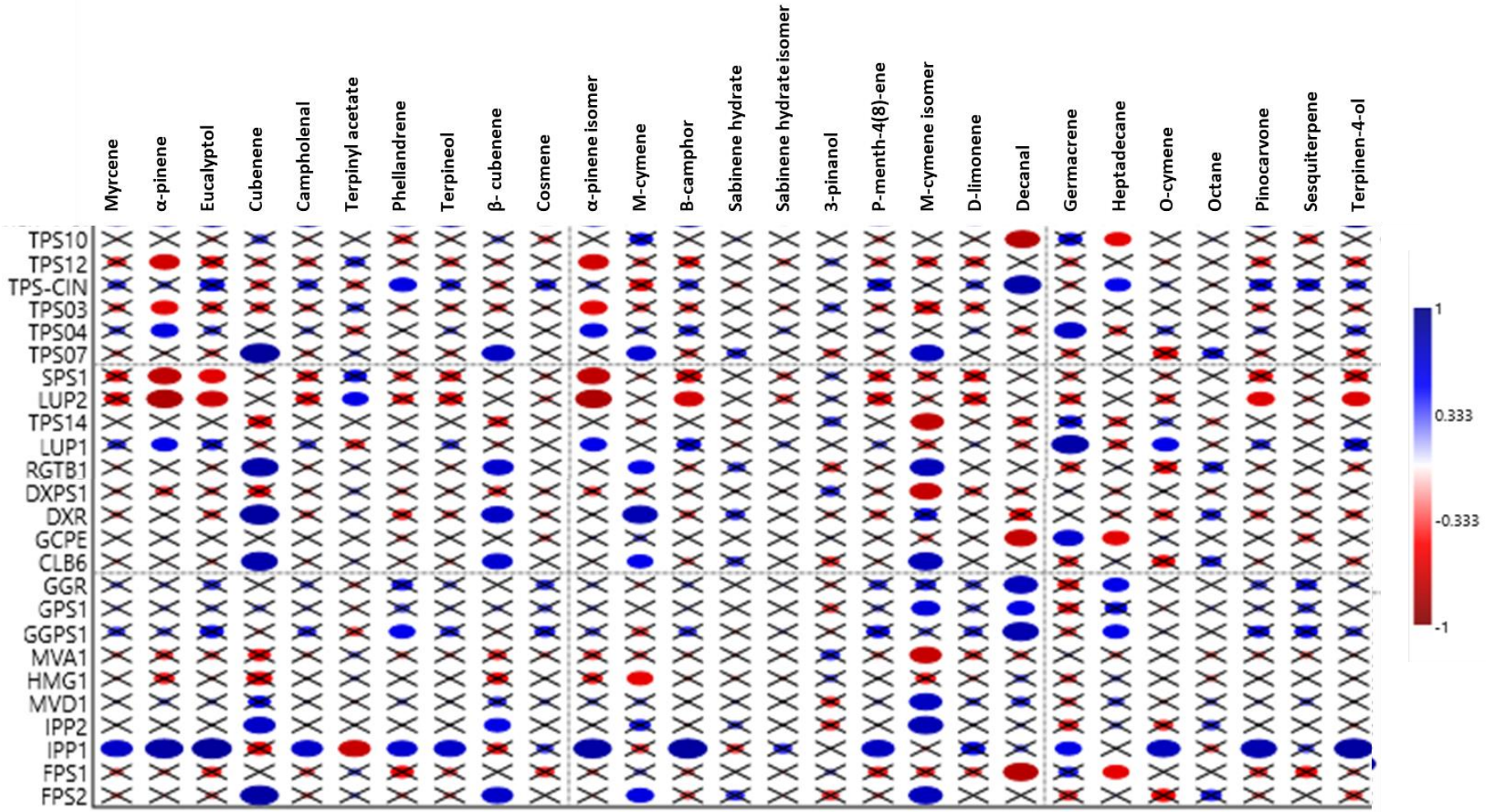


Figure 8. 13 Correlation of terpenoid pathway genes with volatile aromatic emissions from two rosemary cultivars in two light conditions.

Correlation of terpenoid pathway gene expression with the emission of aromatic volatiles from two cultivars of rosemary 'Perigord' and 'Green ginger' plants grown in white light conditions and plants grown in supplementary far-red lighting. Positive correlation is shown in blue and negative correlation is shown in red. Non-significant values are marked with an X.

8.3 Discussion

8.3.1 Differences in volatile content of Perigord and Green Ginger

The volatile emissions were different between the two rosemary cultivars in white light conditions. The SIMPER analysis highlighted the differences between the two cultivars and indicates that rosemary as a species has a large variety of volatile chemicals and these two cultivars with different aromas is due to a largely different volatilome. This diversity in volatiles would be consistent with a large diversity in terpene synthases within the rosemary species. The two contrasting rosemary varieties in morphology and fragrance has demonstrated the diversity in genetics and genetic control of the terpene pathways. The terpene diversity and subsequent terpene synthases may well be useful to horticultural breeding of rosemary, in the selection of cultivars with favourable aromatics. Rosemary essential oil is classified based on the chemical composition, and are consistent with four main types; 1,8 cineole (eucalyptol) types, camphor types, myrcene types and verbenone types. This is depending on the main constituent of the essential oil (Agriculture and Horticulture Development Board, 2018). Both Green ginger and Perigord had high levels of camphor and eucalyptol in their volatilome.

Red and far-red light have been found to influence plant morphology and oil production in rosemary (Mulas *et al.*, 2006). The use of end of day far-red light treatments over the course of five days changed the composition of essential oil and increased oil production in rosemary. In the far-red light experiment above, the treatment had the overall effect of decreasing leaf antioxidant content for Green Ginger. With continued exposure to supplementary far-red lighting, it is possible that Perigord would also show a reduction in antioxidant content as Green Ginger did. This may be consistent with the morphological changes that occurred in response to the far-red lighting, which were more pronounced in Green Ginger than in the supermarket variety suggesting that green ginger may be generally more sensitive to shade. The change to an upright growth form is a beneficial trait for Green Ginger, but as there was a reduction in secondary metabolite production and terpene synthase expression meaning there may be a need to alter the supplementary lighting. While there was an overall reduction in antioxidant content for Green Ginger under supplementary far-red lighting, there was increase in phenolic content and this paired with the increase in gene expression of key terpene synthase genes suggests a possible enhancement of the quality of the rosemary oil. This change to the oil could include more volatile organic compounds contributing to enhanced aroma and taste of rosemary as supplementary far-red lighting switched the production of secondary metabolites in Green Ginger and in Perigord to favour the MEP pathway (shown in figure 8.6). the change in aroma described here may be due to an upregulation of cineole synthase, a MEP pathway monoterpene synthase with upregulation of expression seen in both qPCR analysis of Green Ginger and Perigord cultivars (Figure 8.9). Mulas *et al,* 2006 found that far-red light influenced the constituents of the rosemary essential oil, with far-red light promoting the synthesis of a-pinene, camphene, p-cymene, linalool and geranyl acetate as measured by GCMS (Mulas *et al.*, 2006). This GCMS analysis supports the differential expression data above, as the terpene synthases responsible for the synthesis of some of these products are being expressed such as cineole synthase producing a-pinene as one of its main products and linalool synthase upregulation in Perigord (figure 8.2).

Since there is an interest in using supplementary lighting to increase plant productivity, this treatment may need refinement in order to increase antioxidant capacity. For example, instead of supplementary far-red lighting for a short period it may be more effective to use end of day treatment for shorter time intervals. This will still have the same morphological effects, as end of day treatment with red and far-red lighting was also previously found to significantly increased internode length in rosemary plants (Mulas *et al.*, 2006) Other wavelengths could also have beneficial effects, UV-B and combinations of narrow-bandwidth light treatments. In basil, combination of narrow-bandwidth LED lighting was found to increase the biosynthesis of the volatile constituents monoterpenes, sesquiterpenes and phenylpropanoids under blue/red/green lighting combination (Carvalho *et al.*, 2016a).

8.3.2 Changes in terpene synthase gene expression could be altering the volatile oil composition

Since terpene synthases are responsible for the synthesis of volatile aromatics any changes could influence the volatiles. The gene expression analysis in combination with the GCMS profiling has shown correlation between terpene synthases and their respective volatile products and how influencing the gene expression of the terpene synthases can lead to changes in volatile products. Perigord and Green Ginger had similar terpene synthases detected in the RNA sequencing, but the expression levels of each gene differ in the cultivars. Both the RNA sequencing and qPCR found β-caryophyllene synthase to be expressed at high levels in Perigord, which may contribute to high levels of α-terpinene in the volatile fraction (table 6.2). The opposite effect for β-caryophyllene synthase is seen for Green Ginger which is not expressed as highly and has lower levels of β-caryophyllene present in the volatile fraction. As previously mentioned, the volatilome of these cultivars are very different, and these differences are also present in the expression of the terpene synthases. To better compare the differences between the two cultivars the pronounced effect of supplementary far red lighting on the gene expression of terpene synthases was evaluated.

The secondary aim using supplementary far red light was designed to elucidate changes in the volatile pathways in a controlled environment. As a result, the change in lighting by the addition of supplementary far red light revealed changes in both the genetic expression of terpene synthases and the composition of the volatiles. Although the chemical composition of Perigord had changed when given supplementary far red lighting, there was no noticeable change in the aroma. This may be due to the presence of camphor-like volatiles in the white light and far red light samples and camphor is contributing the most to aroma type. There was a decrease in β-pinene for Perigord under supplementary far red lighting (figure 8.2), the qPCR results also detected a decrease in gene expression of ocimene synthase which is responsible for the synthesis of β-pinene (figure 8.9). This could be a response to the decreased expression of ocimene synthase which lead to less β-pinene production.

In Perigord, ocimene synthase was the only terpene synthase to decrease in expression in response to supplementary far red lighting. All other terpene synthases increased significantly in expression with supplementary far red lighting. B-caryophyllene synthase, that produces α-terpinene, was expressed in both white light and far red light treated samples of Perigord as detected by qPCR (figure 8.9). There was a decrease in volatile quantity in the far red samples, as α-terpinene accounts for the largest degree of differences

between the two treatment groups in the SIMPER analysis (table 8.2). It is possible that β-caryophyllene synthase changes its synthesis activity to one of the other products that it can synthesise, besides β-caryophyllene. It may also be the case for Perigord that the activity in the MEP pathway, which had undergone change in gene expression when treated with supplementary far red light, had influenced the products available for terpene synthases resulting in a switch of production by β-caryophyllene synthase.

Changes in synthetic activity could be due to availability of substrates for the MEP and MVA pathways. In Perigord the RNA sequencing indicates Perigord favours the MEP pathway for volatile production and the expression of GCPE increased with supplementary far red light. GCPE synthesises precursors for terpene production, therefore its increase in activity would have a positive effect on substrate availability for terpene synthases. Mongélard et al (2011) investigated light regulation of the MEP pathway enzyme GCPE. It was found that GCPE activity increases with light intensity, this is a post-transcriptional regulation of enzyme activity and can increase carbon flux through the MEP pathway with high light intensities (Mongélard *et al.*, 2011). GCPE functions to provide substrates for upstream processes in the MEP pathway, and its increased enzymatic activity in high light intensity could increase the synthesis of volatile products upstream in the MEP pathway.

The analysis of terpene synthase expression in Green Ginger showed a decrease in expression of ocimene synthase and caryophyllene synthase under far red light (figure 8.4). The decrease in expression of ocimene synthase could be the reason for lower levels of o-cymene and pinene in the volatile fraction (table 6.3). Caryophyllene synthase has limited activity for geranyl diphosphate and can synthesise limonene as well as other minor products. The RNA sequencing analysis showed geranyl synthase, in the MEP pathway (figure 8.6), decreases in expression under far-red light and may be contributing to lower levels of limonene in the volatile fraction. In Green Ginger, cineole synthase was the only terpene synthase to increase expression with far-red lighting. Cineole synthase produces 1,8 cineole (also known as eucalyptol) and the results from the GCMS detected high quantities of eucalyptol in white light, however eucalyptol levels were lower in far-red light (table 6.3). The RNA sequencing did show a decrease in cineole synthase under far-red light, and it may be that cineole synthase is catalysing other volatile products instead of eucalyptol under far red lighting. The RNA sequencing also allowed for a detailed analysis of the MEP and MVA pathways, and Green Ginger had a downregulation of genes in the MVA pathway indicating that Green Ginger may be utilising the MEP pathway under far red light and may in part be due to the lack of availability of substrates for the MVA pathway to continue production. Two genes have been found to increase availability of FPP for the MVA pathway; the HMGR and the IPK genes (Gutensohn *et al.*, 2021). The HMGR gene expression was detected in the RNA sequencing for Green Ginger, which showed a lower level of expression with supplementary far red light. This could mean less availability of FPP for the MVA pathway and sesquiterpene synthesis. There was a significant visual change to Green Ginger under far red light, but also physiological changes were evident. These changes seen in Green Ginger may be re-directing the specialised metabolism and synthesising other products instead of volatiles and essential oils.

8.3.3 Expression of genes in the terpenoid pathway correlate with expression of aromatic volatiles

Correlation analysis was performed to elucidate possible relationships between volatile emissions and genes in the MEP and MVA pathways including the expression of terpene synthases. Several terpene synthase genes and genes along the MEP and MVA pathways are positively corelating with aromatic volatile emission (figure 8.13). Strong correlations between volatile emissions and terpenoid pathways genes may indicate possible candidate genes which, if upregulated, could improve the synthesis of desirable aromatic volatiles.

TPS04 is a linalool synthase and is positively correlated with α -pinene and germacrene (figure 8.13). This may be due to the catalytic steps towards linalool synthesis, using α -pinene as a substrate. This step includes consecutive α-pinene hydrogenation to form pinane and further hydrogenation of the substrate to synthesise linalool (Semikolenov *et al.*, 2001). It may be that linalool synthase is involved in the later stages of the catalytic steps and the availability of α-pinene in an oxygenated form required gene expression of linalool synthase to catalyse non-oxygenated linalool for plant-cell protection. α-pinene has been shown to have negative effects on root growth and can cause oxidative damage (SINGH *et al.*, 2006). Singh et al. 2006, found elevated activity on antioxidant enzymes in response to α-pinene exposure indicating the plant is undergoing a secondary defence mechanism in response to α-pinene. Linalool synthase may be using the by-products of the defence mechanism to synthesise linalool which would then be expelled from glandular trichomes as part of the essential oils. The rosemary plants used in the correlation analysis included plants exposed to supplementary far-red light and may be undergoing stressors associated with changing light conditions, therefore defence mechanisms and antioxidative pathways may be upregulated in these plants.

TPS-CIN which is a cineole synthase is correlating with emissions of phellandrene, decanal and heptadecane. Cineole synthase had a non-significant correlation with its products eucalyptol (1,8 cineole) and other minor products not shown on the analysis due to non-significant p-values. The correlations with phellandrene, decanal and heptadecane may be due to the synthesis of more complex monoterpenes using TPS-CIN synthetic products in a similar way to the use of α-pinene in further catalytic steps before being a substrate for linalool synthase.

TPS07 is a putative terpene synthase positively correlating with cubenene and myrcene isomers. A similar pattern of correlation with these volatiles is also seen in the MVA pathway gene FPS2 and may be due to the function of these gene in the MVA pathway providing more substrate for this terpene synthase. FPS2 encodes a cytosolic farnesyldiphosphate synthase, FDP (farnesyldiphosphate) is used as a precursor for sesquiterpene synthases (Bhatia *et al.*, 2015). Cubenene is a sesquiterpene, this may represent the increased synthesis of sesquiterpene products seen in Green Ginger plants under supplementary far-red light. Green Ginger also showed an increased expression of FPS2 in the RNA sequencing analysis, suggesting that FPS2 expression could increase the synthesis of sesquiterpenes in rosemary plants.

LUP1, lupeol synthase, has a strong positive correlation with germacrene a terpenoid. Lupeol synthase is a triterpenoid synthase and is known to be multifunctional enzyme (Segura *et al.*, 2000). Lupeol synthase products are mainly lupeol followed by β-amyrin and further investigation by Segura et al. (2000) showed other products including germanicol which may be derived from germacrene. There were weaker correlations

of lupeol synthase gene expression with α-pinene and o-cymene volatile emissions. TPS03 (ocimene synthase) is negatively correlated with α pinene and α-pinene isomers. Ocimene synthase is a multi-product synthase mainly responsible for the synthesises of **β**-pinene as well as other monoterpene products. None of the products from this ocimene synthase were had a significant correlation and were therefore excluded from the analysis (figure 8.10).

The correlation analysis could also be validating the RNA seq annotation of the MEP and MVA pathway genes as described in chapter 6. IPP1 (Isopentenyl-diphosphate Delta-isomerase 1) is a downstream gene in the IPP pathway which shows strong positive correlations with monoterpene emissions, such as myrcene, α pinene and eucalyptol (1,8 cineole). IPP1 is the enzyme responsible for converting IPP to DMAPP which is the precursor of all terpenoid products. Th presence of multiple positive correlations between IPP1 And IPP2 with emission of volatile chemicals is consistent with the important function of these genes at the start of the isoprenoid pathways. Similarly, DXR and DXS are the first enzymes of the MEP pathway involved in the catalytic steps of isoprene, the building-block of terpene products (Tetali, 2019). In the correlation analysis DXR gene expression is seen to correlate with cubenene, **β**-cubenene and m-cymene volatile emissions. Interestingly, the MEP pathway genes RGTB1 (geranyl transferase type-2 beta 1) and DXR seem to be positively correlated with the emission of cubenene and m-cymene, suggesting that GGPP (the precursor of terpenoids in the MEP pathway) is being used for synthesis of these volatiles. Yet, cubenene and m-cymene isomers seem to positively correlate with both genes in the MEP and MVA pathway, further indicating the overlapping functions of these pathways in the production of volatile emissions.

Chapter 9

9.1 Discussion

The use of different environmental conditions has shown differences in the quality of rosemary aroma, and it is suggested that amendments to horticultural growing of rosemary should be taken into consideration. The result of changing environmental conditions through additional light sources, temperature or soil conditions had an impact on the volatile emissions resulting in changes to the aroma of rosemary (as summarised on table 9.1).

The addition of AMF to the soil had the overall effect of increasing the gene expression of several terpene synthases, which combination of terpene synthases upregulated depended on the rosemary cultivar. For some terpene synthases, such as Cineole synthase, there were patterns of gene expression in multiple cultivars with the addition of AMF to the growing media. Cineole synthase responded positively to AMF in every cultivar, except for Vatican Blue. Cineole synthase is known to synthesise 1,8 cineole (eucalyptol) among other minor products; sabene, β-pinene, limonene, β-ocimene, α-terpineol, α-pinene, terpinolene, α thujene. The increased gene expression of Cineole synthase could influence the aroma of rosemary through the increased emissions of 1,8 cineole, giving the rosemary aromas of eucalyptus, minty and herbal notes. In the correlation analysis, Cineole synthase correlated with the production of phellandrene, which would give the rosemary aromatic qualities of citrus, herbal, terpene, wood, and black pepper. These changes in aromatic quality would be of benefit to the consumer, furthermore the stronger aromatic volatiles are favoured by consumers as indicated by the consumer testing in chapter 3.3.5 (figure 3.6). Lupeol synthase was downregulated in some rosemary cultivars in response to AMF colonisation. AMF colonisation increases gene expression of monoterpene synthases, and this may be a result of increased gene expression along the MEP pathway. For the commercial grower it is important to consider the variety of rosemary when interpreting the results from AMF addition to the soil.

Table 9.1 Summary of rosemary responses to the environmental conditions used to alter volatile production and improve aroma

Environmental condition	Benefits for Horticultural Production	Drawbacks for Horticultural Production
Arbuscular mycorrhizal fungi (AMF) addition to the soil	• Stimulates the specialised metabolism including the MEP and MVA pathways • Increase in phenolic chemicals, some associated with aroma and taste • No morphological change under optimized glasshouse conditions, best for grower as it retains desirable visual characteristics of cultivar • Two application methods allow for integration into current growing practices in glasshouse environments • AMF increase gene expression of terpene synthases which may correspond to a boost in aromatic volatiles or changes in the composition of the volatile fraction	• Cultivar dependent, some cultivars showed no difference in production of phenolic compounds with AMF addition to the soil • Morphological changes may be observed under sub-optimal growing conditions • Colonisation rates are different depending on application method • Gene expression increases are also cultivar dependent; some terpene synthases are downregulated in response to AMF
Supplementary UV-C light	• UV-C light induced changes in terpene gene expression, upregulation of some terpene synthases which produce desirable aromatics • Change in volatilome composition. Increases of volatiles with strong aromas; D-limonene, α-pinene and m-cymene • Low- level UV-C light induced gene expression increases in lupeol synthase and linalool synthase	• High-level exposure (11mJ/m^2) caused downregulation of caryophyllene synthase and linalool synthase • Decreases in linalool and phellandrene • The aromatic volatiles Caryophyllene and Ocimene had decreased in emissions • High exposure of UV-C light can trigger plant stress responses, which could slow plant growth
Cold Storage	• Upregulation of β-caryophyllene synthase • Increases in o-cymene and phellandrene volatile emissions • Lupeol synthase increased in expression, produced epoxy-dihydrosqualine required for photo quenching during cold stress. This is evidence that rosemary adapts readily to cold conditions.	• Decreases in expression of some terpene synthases were not balanced with increases in β-caryophyllene synthase, indicating there may not be improvement to aromatic volatile production • Decreased antioxidant and phenolic content • Plant stress indicated by increased expression of lupeol synthase, part of cold stress response included increases in cymene volatile emissions • decreased production of aromatic compounds 1,8 cineole (eucalyptol), α-pinene and D-limonene.
Supplementary Far-red light	• Positively altered the visual appearance of cultivar Green Ginger • Significant increase in gene expression of β-caryophyllene synthase, responsible for the synthesis of α-terpinene and limonene • Increased emissions of α-pinene, camphene, eucalyptol, and tricyclene in cultivar Perigord	• Decreases in volatiles are cultivar dependent; Decreased expression of ocimene synthase and β-caryophyllene in Green Ginger

Rosemary cultivar Perigord treated with supplementary far-red light showed increases in α-pinene and eucalyptol, two major components of rosemary volatile fraction which give the aroma of fresh pine, and eucalyptus respectively. The increase in emissions of α-pinene correlated with expression of linalool synthase, which uses α-pinene as substrate for synthesising linalool. Linalool is a volatile with a strong aroma of fresh, sweet, pine and floral qualities. Upregulation of linalool synthase could enhance the aroma of Perigord with fresh, sweet and pine scents preferable to the public, and this can be achieved through the addition of AMF to the soil with plug application method. Increases in gene expression of linalool synthase can also be achieved through supplementary UV-C light at low dosages. Cultivar Vatican Blue has significantly high expression of linalool synthase with addition of AMF, this cultivar was also second favourite in the aroma testing performed at RHS Garden Wisley, Wisley UK, and has aromatic notes of herbal, sweet, and pine. Targeted genetic engineering of linalool synthase in combination with an α-pinene synthase, such as cineole synthase or ocimene synthase, would be a good solution for a rosemary high in aromatic volatiles as this gene expression combination may increase the accumulation of linalool in the volatile fraction.

Supplementary UV-C lighting at low frequency exposure had a positive effect on the volatile emissions of D-limonene, α-pinene and m-cymene in rosemary cultivar Abraxas. TPS07, a putative terpene synthase, which currently has no published volatile products associated with the gene (according to the Uniprot database), correlated with the emission of cubenene and m-cymene. M-cymene is an aromatic volatile with aroma notes of fresh, citrus, terpene, woody and spice. Increased emissions of m-cymene in combination with α-pinene and D-limonene contribute to a stronger aroma of rosemary for cultivar Abraxas which may be more favourable to the public. However, there is a need for further study into the usage of UV-C lighting for enhancing aroma as it is dosage dependent, and the effects may not be as long lasting as the AMF addition to the soil.

Cold storage is beneficial for the growers to preserve stock and prevent the overgrowth of potted rosemary while awaiting transportation. It was found to have drawbacks in terms of aromatic production due to the cold stress and subsequent activation of cold tolerance pathways to mitigate the stress by the plant. There was also a decrease in the antioxidants the plant produced while in cold storage. Antioxidants are an important part of human dietary health and there is a need for foods high in antioxidants to supplement diets. Rosemary cultivars that contain high quantities of antioxidants include Vatican Blue, Green Ginger, and Logee Blue. The compounds in rosemary found to be good for human health are carnosic acid and carnosol (Tada *et al.*, 2010). A third antioxidant of rosemary essential oil is Lupeol, a triterpene known for its cholesterol-lowering properties, anti-inflammatory and anti-cancer properties (Saleem, 2009). All three cultivars (Vatican Blue, Green Ginger and Logee Blue) have high expression levels of lupeol synthase and high antioxidant contents. Low dosage UV-C lighting increased the gene expression of lupeol synthase in Abraxas, while high dosage showed an increase in antioxidant content compared to white light conditions. Lupeol is an important antioxidant that is in current research for human health. A review by Sohag et al. (2022) summarised the potential of lupeol in treating diseases such as diabetes, cardiovascular disease, kidney and liver disease, skin diseases and neurological disorders such as Alzheimer's disease (Sohag *et al.*, 2022). Further antioxidant analysis of leaf extracts from these rosemary varieties would be required to determine which antioxidants are

present and their biochemical properties important for human health. These cultivars could be the basis of research into rosemary cultivars with high content of dietary antioxidants, through plant breeding and enriching the gene pool with rosemary varieties identified as high antioxidant producers or through genetic engineering with targeted crispr/cas technology of lupeol synthase to increase expression in existing commercial cultivars.

9.2 Conclusion and Future Works

Rosemary is a diverse taxon of morphological, physiological, and genetic traits that can be influenced through environmental conditions to enhance aroma. The two environmental conditions that can successfully be implemented in commercial horticulture are low-dosage supplementary UV-C lighting and addition of arbuscular mycorrhizal fungi (AMF) to the soil. Both of which showed great benefits to volatile production for better aroma and increased the antioxidants in the leaves for dietary health. AMF addition to the soil is a cost-effective method that requires minimal infrastructure to be implemented in commercial glasshouses and is suggested here to growers as a protocol to enhance the aromatics of rosemary. In this research, volatiles were perturbed through environmental conditions to better understand the activity of terpene synthases in volatile production. From this, three candidates for genetic breeding of rosemary cultivars with increased expression of terpene synthases were found Green Ginger, Logee Blue, and Vatican Blue. These candidates showed good visual aspects in selection trials, high antioxidant contents, and high levels of terpene synthase gene expression, which correlated with volatile products with strong aromas favoured by the public during blind aroma trials. The gene expression of four terpene synthases correlated with their respective volatile products, of which TPS07, a putative terpene synthase with no previously published products, was found to correlate with the volatiles cubenene and m-cymene. The genetic work presented puts forward three candidate terpene synthases that can be targeted for genetic engineering; overexpression of Linalool synthase in combination with an α-pinene synthase may increase the aromatic content and increasing expression of lupeol synthase may increase antioxidant levels through increased levels of dietary triterpenoids. In summary, the in-depth RNA sequencing provided new insights into the biosynthetic pathways of volatiles in different rosemary taxa. Further analysis including targeting of specific genes along volatile biosynthetic pathways would allow for further investigation into environmental conditions, and suggest new growing techniques for commercial growers, and to identify candidate terpene synthases for targeted genetic engineering for future research.

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11 Appendix

Appendix 1, Table 1: Current list of selected rosemary varieties based on morphological and physiological characteristics and aroma. 'Perigord' and 'Abraxas' are current commercial varieties and are included as they have desirable morphological characteristics such as leaf size and plant height/width.

Variety	Density index	Leaf size	Height/width	Aroma	TPC mg	Antioxidant mg
'Perigord'	**1**	**30.3 mm long, 4.1 mm wide**	**1.22**	**Weak aroma**	**9.57**	**1.86**
'Abraxas'	**1**	**29.5 mm long, 4.2 mm wide**	**1.10**	**Weak aroma**	**10.03**	**3.30**
24 'Roman Beauty'	3	15.7 mm long, 1.3 mm wide	0.36	Good aroma	8.39	2.59
36 'Arp'	2	19.8 mm long, 1.9 mm wide	0.80	Good aroma	6.99	5.11
41 'Logee Blue'	2	21 mm long, 2mm wide	0.75	Strong menthol scent	13.44	4.81
56 'Wonderful'	2	24.7 mm long, 2 mm wide	0.68	good aroma	7.63	4.07
59 'Blue Boy'	2	18.7 mm long, 3.7 mm wide. Oval in shape	0.83	Good aroma	5.86	3.67
60 'Mendizaballi'	2	13.7 mm long, 1.8 mm wide	0.54	Strong aroma	7.39	3.23
72 'Israel'	1	27 mm long, 3.3 mm wide	Not recorded	Sweet scent	10.1	5.01
81 'Starvordale'	2	28 mm long, 2.2 mm wide	0.57	Strong scent	-	-
44 'Mrs Furneux'	2	24 mm long, 2mm wide	0.79	Good aroma	8.85	3.99
52 'Suffolk Blue'	2	20 mm long, 2mm wide	0.82	Good aroma	7.34	2.42
65 'Balham Blue'	2	18 mm long, 2mm wide	0.46	Strong aroma	9.02	4.85
83 'Vatican Blue'	2	25 mm long, 2 mm wide	0.82	Strong aroma	10.31	3.38
38 'Green Ginger'	2	20 mm long, 2mm wide	0.49	Strong ginger aroma	9.55	4.03
69 'Blue Spire'	2	23 mm long, 2.5 mm wide	0.89	Good aroma	6.17	2.80
17 'Escondido'	1	16 mm long, 2 mm wide	0.42	Good aroma	8.48	3.08

61 'Trusty'	1	17 mm long, 1.6 mm wide	1.04	Good aroma	-	-
66 'Barbecue'	1	20 mm long, 2.5 mm wide	0.92	Sweet aroma	7.45	2.87
87 'Primley Blue'	2	29 mm long, 3 mm wide	Not Recorded	Good aroma	5.65	2.79

Appendix 1, Table 2: Pearson's Correlation Calculation performed on selected rosemary varieties in comparison with two commercial cultivars named Abraxas and Perigord. P values < 0.05 signify that varieties are similar in their morphological characteristics.

Variety number	Mean leaf length (mm)	Mean leaf width (mm)	Mean internode length (mm)	Leaf density	Height/width	R^2	P value
24	15.67	1.333	7.6	27	0.361	0.329	0.253
36	19.83	1.917	21.9	13.25	0.801	0.271	0.316
41	22.17	2.083	14.8	16.25	0.753	0.61	0.056
56	24.33	2	16	14	0.679	0.635	0.046
59	18.67	3.667	17.8	14.5	0.832	0.383	0.203
60	13.67	1.833	23	13.5	0.546	0.069	0.647
72	27	3.25	9.1	17.25	0	0.88	9E-04
81	28	2.167	17.9	15.5	0.569	0.65	0.04
'Perigord'	30.33	4.167	2.1	12	1.22	-	-