Determination of induced changes in foliar emissions of terpene-accumulating plants

By

Dimitrios Zabaras

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College of Science, Technology and the Environment
University of Western Sydney
Richmond, NSW, Australia.

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Please note

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
Model\textsuperscript{1} in which signals produced by insect and pathogen attack activate lipase activity that releases linolenic acid into the cytoplasm. An intracellular pathway then is initiated that results in the production of defensive substances.

\textsuperscript{1} (Farmer E.E, Ryan C.A. Octadecanoic precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. \textit{Plant Cell} 4, 129-134).
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This thesis is presented in loving memory of my late uncles, Vasilios Zabaras and Aristidis Nikas, who tragically left us during its preparation. Their inspiration and support will never be forgotten.
STATEMENT OF AUTHENTICATION

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in whole or in part, for a degree at this or any other institution.

Signed by:

Dimitrios Zabaras
30/05/2003


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ABBREVIATIONS

Car/PDMS: Carboxen/Polydimethylsiloxane
FPLC: Fast-protein liquid chromatography
GC: Gas chromatograph(y)
GC-MS: Gas chromatography-mass spectrometry
GF: Gel filtration
GPP: Geranyl diphosphate
HIC: Hydrophobic interaction chromatography
HS-SPME: Headspace solid phase microextraction
IEC: Ion exchange chromatography
IPP: Isopentenyl diphosphate
IT-GC-MS: Ion-trap gas chromatography-mass spectrometry
JA: Jasmonic acid
K_{fg}: SPME fibre/gas phase distribution constant for an analyte
K_{wa}: Water/gas phase distribution constant for an analyte
KI: Kovats index
LOD: Limit of detection
LOX: Lipoxygenase
LTPRI: Linear Temperature Programmed Retention Index
PCA: Principal component analysis
PDMS: Polydimethylsiloxane
RIC: Reconstructed ion counts
RRF: Relative response factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>SAR</td>
<td>Systemic acquired resistance</td>
</tr>
<tr>
<td>STD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TF</td>
<td>Tailing factor</td>
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GLOSSARY

necrotrophic (fungus): a fungus that invades and grows on dead tissue

semiochemical: a substance that mediates interactions between individuals and their surroundings

Melaleuca alternifolia: paperbark tree that flourishes in swamps

Ocimum minimum: annual subshrub with aromatic leaves

Salvia officinalis: perennial shrub that is well known for its medicinal and cosmetic properties

Note on Nomenclature

The naming of the sabinene hydrates (I, II) can be confusing due to differences between classical terpenoid, preferred and Chemical Abstracts Service (CAS) nomenclature. The nomenclature style followed in this thesis refers to the relationship between the methyl- and isopropyl groups; \(^1\)H-NMR evidence confirmed that these are the "correct" names for the hydrates.

I. Z-sabinene hydrate
(1R, 4R, 5S)-
CAS No.: 15826-82-1
KI on BPX-5: 1118\(^{\text{II}}\)

II. \(E\)-sabinene hydrate
(1R, 4S, 5S)-
CAS No.: 17699-16-0
KI on BPX-5: 1085\(^{\text{b}}\)


\(^{\text{II}}\) These values were determined under the conditions described in Chapter 4.
ABSTRACT

Stress-induced changes in the emissions of volatiles from many economically-important plants have been demonstrated in studies over the past decade\(^1\). Plants such as cotton and corn change both the composition and concentration of their emissions when subjected to wounding, herbivory and pathogen attack\(^1\).

Terpene-accumulating plants have been overlooked as potential objects of such studies although work on conifers has shown that species rich in constitutive defenses can also exhibit induced responses\(^2\).

The aim of this study was to investigate whether terpene-accumulating plants respond to stress by altering their foliar emissions qualitatively and/or quantitatively. Species examined included *Salvia officinalis* (common sage), *Melaleuca alternifolia* (Australian tea tree) and *Ocimum minimum* (bush basil).

An experimental design was developed to eliminate factors such as leaf ontogeny that can affect the obtained results and complicate their interpretation. Small-scale solvent extraction and HS-SPME-based techniques were also developed; they enabled the quantitative determination of treatment-induced changes over periods ranging from 10 minutes to 6 months.

Treatment of plants included mechanical wounding, herbivory, pathogen attack and chemical elicitation. Overall, statistically significant induced-changes were observed for both leaf-oil composition and concentration. The response of the different species used varied. *O. minimum* exhibited the greatest compositional changes whilst *M. alternifolia* was the only species for which oil-concentration changes were observed.
The demonstrated changes were not as great as those reported in similar studies with non-terpene producing plants. The results indicate that the high metabolic costs associated with the production and storage of constitutive defenses may be responsible for the limited induction of further defensive responses.

---


CHAPTER 1

GENERAL INTRODUCTION

1.1 Plant chemical defense

All plants use chemicals to protect themselves against pathogens and herbivores (Baldwin, 1998). The chemical defense of plants has been the object of investigations for more than a century (e.g., Ray, 1901). As a result of these efforts, a large number of secondary metabolites including alkaloids, anthocyanins, flavonoids and terpenoids, have now been identified that possess ecological properties associated with plant defense against microbes, fungi and herbivores (Schröder, 1998).

Chemical defense against herbivores can be direct (via plant toxins, digestion inhibitors, and other deterrents) or indirect by promoting the effectiveness of the herbivores' predators (e.g., Price et al., 1980).

1.2 Induced and constitutive chemical defenses in plants

Chemical defenses in plants can be constitutive or induced. The term constitutive denotes the defenses that are expressed via substances that are preformed and thus, are present on the leaves before herbivory or pathogen invasion occurs. Induced are those defenses that are expressed by chemicals formed as a result of the attack and thus, appear from the leaves at various times after herbivory or pathogen invasion (Derckel et al., 1999).

Inducible defenses are believed to be inferior to constitutive defenses due to the time required for them to be activated; during this period the plant is not
protected and can be exploited by herbivores and pathogens (Baldwin and Preston, 1999). On the other hand chemicals that express constitutive defenses (e.g., terpenoids, phenolics) are costly to produce, store (Gershenzon, 1994) and perhaps, maintain at high levels in the plant. As a consequence, constitutive defenses are thought to disadvantage the plants that rely on them in times and environments lacking herbivores and pathogens, by competing for resources with plant-growth or reproduction mechanisms (Baldwin, 1998; Lerda et al., 1994). Inducible defenses allow plants to produce protective chemicals only when conditions warrant such an action and thus forgo the costs of defense when not needed (Baldwin, 1998).

1.2.1 Stages of induced chemical defense

The production of defensive phytochemicals in response to pathogen/herbivore attack is a process that may be divided into four stages (Stout and Bostock, 1999). The plant-phytophage interaction produces an elicitor (section 1.2.2) which then must be recognised by the plant. Utilisation of a signal transduction pathway follows (section 1.2.3) and finally defensive substances (section 1.2.4) are synthesised and are used to express the response (Stout and Bostock, 1999).

1.2.2 Events/elicitors known to induce plant chemical defenses

Plant chemical defenses may be initiated by abiotic (e.g., ultraviolet light, wounding) or biotic factors referred to as elicitors (Strange, 1992). Biotic elicitors are those that originate from living organisms such as herbivores, pathogens or stressed leaves of neighboring plants. Elicitors can be further sub-divided into endogenous and exogenous depending on whether they are produced by the attacked leaves themselves for their own benefit (endogenous)1 or the

---

1 Endogenous elicitors may also be referred to as (pathway)-translocational signals.
herbivores/pathogens/neighbors leaves (exogenous) (e.g., Karban \textit{et al.}, 2000; Mattiacci \textit{et al.}, 1995).

Research has identified many exogenous elicitors that are utilised by the plants in order to recognise attacking herbivores and pathogens. The enzyme \( \beta \)-glucosidase was one of the first herbivore-derived elicitors to be implicated in induction of chemical defense (Boland \textit{et al.}, 1992). The presence of this elicitor has now been confirmed in secretions from spider mites (Hopke \textit{et al.}, 1994) and caterpillar-regurgitants (Mattiacci \textit{et al.}, 1995). More recently, a putative non-protein elicitor, volicitin (N-(17-linolenoyl)-L-glutamine), was identified from the regurgitants of the beet armyworm (Alborn \textit{et al.}, 1997).

In contrast to the few known herbivore-derived exogenous elicitors, many substances produced by pathogens and especially fungi have been identified that are used by plants to recognise their invaders: enzymes, polypeptides, glycoproteins and low molecular weight organic compounds (Strange, 1992). In particular, the fungal cell wall fragment chitosan, a \( \beta \)-1,4-glucosamine, was reported to elicit production of lodgepole pine monoterpenes even on large, fast-growing trees (Miller \textit{et al.}, 1986).

Exogenous elicitors may also originate from neighboring stressed/damaged leaves. Although early evidence of plant communication (e.g., Baldwin and Schultz, 1983) has been discounted by some scientists (e.g., Karban and Baldwin, 1997) recent rigorous experiments have confirmed that plants may respond to cues, such as methyl jasmonate, produced by stressed neighbors (Karban \textit{et al.}, 2000).
1.2.3 Endogenous elicitors and stress-induced defensive pathways

Activation of a stress-induced response follows the recognition by the plant of the exogenous elicitor (section 1.2.2). It has been known for many years that there are several mechanisms that contribute in the production of an induced-response although they may or may not act in a coordinated fashion (Karban and Kuć, 1999). Despite this variation, research efforts conclude that jasmonic acid (I, Figure 1.1) and its derivatives, salicylic acid (II, Figure 1) and ethylene (ethene) are the endogenous elicitors that mediate the major pathways producing the defensive phytochemicals (e.g., Baldwin, 1999; Baldwin and Preston, 1999; Farmer and Ryan, 1992; Hammerschmidt and Smith-Becker, 1999; Schröder, 1998). These three elicitors are naturally occurring compounds present in a wide variety of plant species (Enyedi et al., 1992).

![Figure 1.1](image)

**Figure 1.1.** Structures of the endogenous plant-defense elicitors jasmonic acid (I) and salicylic acid (II).

Salicylic acid is known to mediate a pathway that provides resistance against a variety of pathogens. This response, termed systemic acquired resistance (SAR), has been known for many years (e.g., Chester, 1933) and has been described in over

---

2 Further details about the defensive pathways can be found in Chapter 8.
3 Other synonyms include induced resistance (IR) and induced systemic resistance (ISR).
30 plant species (Karban and Kuć, 1999). Jasmonic acid is known to activate the octadecanoic pathway which is protective against herbivores (e.g., Farmer and Ryan, 1992). Although this pathway was discovered more recently than SAR (e.g., Green and Ryan, 1972) it has been described for over 100 plant species (Karban and Kuć, 1999). Ethylene is produced at the site of wounding and induces phytochemicals that are active against pathogens and herbivores (Enyedi et al., 1992). It is also involved (with jasmonic acid but not salicylic acid) in a pathogen-induced pathway, distinct from SAR, but the details of this pathway are not fully determined (Pieterse et al., 1998).

It must be noted that the above classification is a guide only; the expression of defense in planta can be quite complicated (Baldwin and Preston, 1999). Research findings suggest that the above (and perhaps any other similar) pathways are not completely independent from each other as induction of one may affect expression of the other (Maleck and Dietrich, 1999).

1.2.4 Plant phytochemicals and their role in chemical defense

Many thousands of phytochemicals have been isolated and described in the plant kingdom (Dey and Harborne, 1997). It is believed that most of them have co-evolved with insects (Ehrlich and Raven, 1964; Harborne, 1993) although it has been shown that phytochemicals may also be implicated in other, equally important, ecological functions besides defense such as nutrient cycling and attraction of pollinators (e.g., Langenheim et al., 1994; Mitchell-Olds et al., 1998).

1.2.4.1 Proteins

Proteins are considered to be important factors of plant defense especially against herbivores (e.g., Constabel 1999 and references therein). Protease inhibitors

---

4 The term phytochemicals is used loosely here and includes defensive proteins/enzymes.
are the best studied defensive plant-proteins; they serve as digestibility reducers or
toxins by targeting the proteolytic digestive enzymes of herbivores (Baldwin and
Preston, 1999; Broadway, 1996).

Polyphenyl oxidases are enzymes which use molecular oxygen to catalyse the
oxidation of phenolic compounds to highly reactive o-quinones (Constand, 1999).
During herbivory the immediately formed quinones polymerise and link with other
biomolecules such as dietary proteins thereby preventing efficient digestion and
assimilation by the herbivores thus impacting herbivory (Duffey and Stout, 1996;
Felton et al., 1992).

Lectins and chitinases are also defensive proteins. Lectins are carbohydrate-
binding proteins toxic to insects (e.g., Powell et al., 1993) while the (lectin-like)
chitin-binding chitinases are known for their high antifungal activity (Collinge et al.,
1993; Derckel et al., 1999).

1.2.4.2 Phenolics

Plant phenolics and their polymers (tannins and lignins) are mostly derived
from the shikimic acid and phenylpropanoid pathway (Constand, 1999). These
chemicals are known to be present in high constitutive concentrations in the bark and
leaves of many plants and they are particularly prevalent in woody perennials
(Swain, 1979). Phenolics have been traditionally associated with plant chemical
defense and they are believed to primarily function as feeding deterrents against
herbivores (Swain, 1979; Baldwin and Schultz, 1983; Schultz and Baldwin, 1982).
Herbivore-induced accumulation of phenolics has been demonstrated in several
species including sugar maple (Baldwin and Schultz, 1983), poplar ramets (Baldwin
and Schultz, 1983), oak (Schultz and Baldwin, 1982), birch (Bergelson and Lawton,
1988) and trembling aspen (Mattson and Palmer, 1988).
1.2.4.3 Alkaloids

Some of the most potent bioactive phytochemicals known are alkaloids (Harborne, 1993). All alkaloids are defined by the presence of a nitrogen-containing structure which is believed to be important for their activity (Constabel, 1999). The bioactivity of alkaloids arises from their ability to interfere and cause malfunctions in the nervous system of herbivores (Hartmann, 1991). Most alkaloids are constitutive in plants although there are a few examples of herbivore- and wound-induction. Mechanical wounding, insect herbivory and jasmonate application was found to increase the nicotine content of tobacco leaves between five and ten-fold (Baldwin, 1999; Baldwin et al., 1994).

1.2.4.4 Phytoalexins

Phytoalexins are low molecular weight antimicrobial compounds induced in plants after pathogen infection (Ebel 1986). Phytoalexins may be derived from a number of different secondary metabolic pathways but despite their different origins most of them are synthesised and accumulate near or at the site of pathogen invasion causing necrosis of the area (Kuć, 1995). The pathogen is then either trapped and killed (Gilchrist, 1998) or starved due to the lack of nutrients and thus its growth is significantly reduced (Govrine and Levin, 2000).

1.2.4.5 Lipoxygenase-derived volatiles

Lipoxygenases, described in more detail in Chapter 6, are considered to be ubiquitous in plant tissues. These enzymes use molecular oxygen to produce fatty acid hydroperoxides from unsaturated fatty acids (Siedow, 1991). These hydroperoxides, perhaps due to their cytotoxicity (Siedow, 1991), are metabolised further to short chain (usually C6-C9) aldehydes and alcohols (Rosahl, 1996) as well as other products such as jasmonates (Weiler, 1997). These volatile aldehydes and
alcohols are known to possess antimicrobial properties against a wide range of plant pathogens (Rosahl, 1996; Vaughn and Gardner, 1993). Lipoxygenase-derived products are emitted immediately or very soon after wounding or herbivory occurs in the leaves (Loughrin et al., 1994; Scutareanu et al., 1997; Turlings et al., 1998).

1.2.4.6 Terpenoids

Terpenoids are the largest group of phytochemicals and are usually found in plants as constitutive and/or induced mixtures of compounds (Langenheim, 1994). Mixtures consisting of mono- (C_{10}) and sesquiterpenoids (C_{15}) are called essential oils whilst the term resin refers to combinations of non-volatile (C_{20} diterpenoids and C_{30} triterpenoids) and volatile (mono- and/or sesquiterpenoids) terpenoids (Langenheim, 1990).

Terpenoids are synthesised\(^5\) by the mevalonate (Goodwin and Mercer, 1990) or the deoxyxylulose pathway (Rohmer et al., 1993). After synthesis the mixtures are stored in specialised secretory structures (section 1.4.1).

The occurrence of terpenoids in plants is likely to be influenced by genetic, biochemical, ecological and other factors (Langenheim, 1994 and references therein). As a result, variations in the compositional profile and/or concentration of the mixture within populations (e.g., Cates et al., 1983; Hall and Langenheim, 1987) and between different-age individuals (ontogenetic variation) (e.g., Crankshaw and Langenheim, 1981; Southwell and Stiff, 1989) are commonly encountered characteristics of terpenoids.

The role of terpenoids in plant defense will be described in detail in section 1.3 because of their direct relevance with this study.

---

\(^5\) Further details about terpenoid biosynthesis are given in Chapter 7.
1.3 Review of chemical defenses expressed by plant terpenoids

1.3.1 Non terpene-accumulating species

1.3.1.1 Released terpenoids and their production

Many non terpene-accumulating plants are known to release terpenoids, synthesised *de novo* within hours of herbivore-attack, as cues to attract herbivore-parasitoids and/or predators (Coleman *et al*., 1997; Paré and Tumlinson, 1996; Paré and Tumlinson, 1997; Takabayashi *et al*., 1995; Takabayashi and Dicke, 1996; Turlings *et al*., 1990; Turlings *et al*., 1995) (Table 1.1).

**Table 1.1.** Summary of herbivore-induced terpenoids released by various non terpene-accumulating plants.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Terpenoids released</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>linalool, dimethyl nonatriene, α-E-bergamotene, E-β-farnesene, trimethyl tridecatetraene</td>
<td>Turlings <em>et al</em>., 1990; Turlings <em>et al</em>., 1993</td>
</tr>
<tr>
<td>Lima beans</td>
<td>linalool, dimethyl nonatriene, E-β-ocimene</td>
<td>Dicke, 1994</td>
</tr>
<tr>
<td>Apple</td>
<td>E-α-farnesene, trimethyl tridecatetraene</td>
<td>Takabayashi <em>et al</em>., 1994a</td>
</tr>
<tr>
<td>Cucumber</td>
<td>E-β-ocimene, dimethyl nonatriene</td>
<td>Takabayashi <em>et al</em>., 1994b</td>
</tr>
<tr>
<td>Cabbage</td>
<td>myrcene, limonene, 1,8-cineole</td>
<td>Mattiacci <em>et al</em>., 1995</td>
</tr>
<tr>
<td>Crabapple</td>
<td>E-β-ocimene, dimethyl nonatriene, E-α-farnesene</td>
<td>Loughrin <em>et al</em>., 1995a</td>
</tr>
<tr>
<td>Pear</td>
<td>E-α-farnesene</td>
<td>Scutareanu <em>et al</em>., 1997</td>
</tr>
<tr>
<td>Potato</td>
<td>dimethyl nonatriene, trimethyl tridecatetraene, several terpenoids in minor amounts</td>
<td>Bolter <em>et al</em>., 1997</td>
</tr>
<tr>
<td>Maize</td>
<td>dimethyl nonatriene, α-E-bergamotene, E-β-farnesene, nerolidol, trimethyl tridecatetraene</td>
<td>Turlings <em>et al</em>., 1998</td>
</tr>
<tr>
<td>Field elm</td>
<td>E-β-ocimene, β-ylangene, β-copaene, α-humulene, germacrene D, E-α-farnesene</td>
<td>Wegener <em>et al</em>., 2001</td>
</tr>
</tbody>
</table>
As can be seen from Table 1.1, the homoterpenes 4,8-dimethyl-1,3(E),7-nonatriene (Figure 1.2, I) and 4,8,12-trimethyl-1,3(E),7(E),11-tridecatetraene (Figure 1.2, II) are the most often reported herbivore-induced volatiles although it would be incorrect to exclusively associate these volatiles with herbivory (Dicke, 1994). These induced components are produced from the terpene alcohols nerolidol and geranyllinalool, respectively, through oxidative bond cleavage (Boland et al., 1992; Gäbler and Boland, 1991). Thus, a proposed mechanism for the regulation of emission of these two homoterpenes involves the storage of these metabolites as non-labile glycoside-coupled components. Herbivore-damage then cleaves the sugars releasing the two homoterpenes (Boland et al., 1992).

\[ \text{Figure 1.2.} \] Structures of some herbivore-induced terpenoids. 4,8-Dimethyl-1,3(E),7-nonatriene (I), 4,8,12-trimethyl-1,3(E),7(E),11-tridecatetraene (II), α-E-bergamotene (III), E-β-farnesene (IV).
A differential timing of the release of the stress-induced terpenoids has been observed for several species including maize (Turlings et al., 1998). This indicates that the released terpenoids are products of distinct biosynthetic (mevalonate and deoxyxylulose) pathways (Baldwin and Preston, 1999; Turlings et al., 1998) or originate from different steps within one pathway (Turlings et al., 1998).

Most of the induced terpenoids described in Table 1.1 are acyclic. The reason behind the preference shown by the plants towards acyclic terpenoids is not known. It has been suggested that lack of cyclase\textsuperscript{6} activity might be responsible for the absence of cyclic terpenoids in stress-induced emissions of plants (Paré et al., 1999).

1.3.1.2 Characteristics of the emitted signal

The stress-induced emitted signal must be readily detectable by the parasitoids/predators of the attacking herbivore and must also be distinct from background plant-emissions (Turlings et al., 1995). In the case of non terpene-accumulating plants the production and release of terpenoids due to herbivory automatically alters the normal plant-odour thus making it easily distinguishable by the parasitoids/predators. However, variations in the induced-signal between individuals within a species (Paré and Tumlinson, 1996) or young and mature leaves of the same plant (Takabayashi et al., 1994b) do exist.

To ensure the signal is detectable, the plants emit a great amount of volatiles; several micrograms of a terpenoid can be released by a corn seedling within a single hour (Turlings et al., 1995). The emitted signal is also amplified by the systemic release of the induced terpenoids. For example, in corn the induced volatiles are

\textsuperscript{6} Cyclases are enzymes that catalyse the cyclisation of isopentenyl pyrophosphate to produce cyclic terpenes (more details in Chapter 7).
released throughout the damaged plants and are not limited to the site(s) of injury (Turlings and Tumlinson, 1992).

If the emitted components are to be effective they need to be produced during, or immediately after, herbivore attack. In addition, the emission is likely to be more efficient if released during the period of day when predators/parasitoids forage (Turlings et al., 1995). Damaged corn plants treated with caterpillar regurgitate were observed to release a significantly higher amount of induced volatiles during daytime compared to night hours (Turlings et al., 1995).

1.3.2 Terpene-accumulating species

Terpenoids found in terpene-accumulating plants are believed to have multiple ecological roles (Langenheim, 1994). When considering the variety of toxic, inhibitory and deterrent effects exhibited by the various terpenoids (Gershenzon and Croteau, 1991; Harborne, 1991) it is sensible to assume that one of those roles is the protection of the plant against (generalist) herbivores and pathogens. This view is supported by indirect evidence obtained over the years. For example, secretory glands found in green tissues of cultivated cotton contain, in addition to mono- and sesquiterpenes (Elzen et al., 1985), gossypol and other related terpenoid aldehydes that are known to possess strong anti-herbivore properties (Bottger and Patana, 1966; Stipanovic et al., 1977). When plant-breeders developed glandless cotton varieties in the 1960's to allow for the seed to be used for human or animal consumption or as an oil source (Cherry and Leffler, 1994), herbivores that were previously minor pests of the glanded variety started causing significant damage to the glandless plants (Jenkins et al., 1966).
Today it is well established that the presence or absence of secretory glands in cotton affects the behaviour of generalist-feeding insect herbivores (McAuslane and Alborn, 1998).

Apart from the constitutive terpenoids, cotton is known to produce increased amounts of heliocides (C_{25} terpenoid aldehydes that are natural insecticides) in response to exposure to volatiles originating from pathogen (*Aspergillus flavus*)-infected cotton leaves (Zeringue, 1987). Herbivore-induced increased production of gossypol and derived terpenoids has also been observed in glanded plants although this behaviour was not detected in glandless individuals (McAuslane and Alborn, 1998). However, both ganded and glandless cotton plants produce and release LOX-derived products and certain acyclic terpenes (including the homoterpenes dimethyl nonatriene and trimethyl tridecatetraene) as a result of herbivore attack (McAuslane and Alborn, 1998; Loughrin *et al.*, 1995b; McCall *et al.*, 1994; Paré and Tumlinson, 1997). The emission of these acyclic terpenes was found to be systemic (Paré and Tumlinson, 1998) and exhibited a diurnal pattern with maximum levels of terpenes released during the afternoon (Loughrin *et al.*, 1994).

Conifers also exhibit *de novo* synthesis of oleoresin-terpenoids (such as α- and β-pinene, α-phellandrene, sabinene, limonene) as a result of bark beetle damage and to a lesser extent mechanical damage (Lewinsohn *et al.*, 1991; Raffa and Berryman, 1982; Raffa and Smalley, 1995). In members of the *Abies* and *Picea* genera, biosynthesis of the induced terpenoids occurs in the cells surrounding tissue damage (Lewinsohn *et al.*, 1992); in members of the *Pinus* genus the induced oleoresin flows into the wound zone on preformed resin-cannals (Raffa and Smalley, 1995). Differences in the chemical expression of the response were also observed between the genera. The damage-induced oleoresin changes seen in *Pinus* species
were related to the resin total concentration rather than the composition (Raffa and Smalley, 1995). In contrast, compositional and concentration changes were detected in Abies conifers as a result of beetle wounding (e.g., Lerdau et al., 1994).

Once at the site of damage, the oleoresin (through the action of its constituent terpenoids) seals off the wound and traps and kills the attacking beetles (Raffa et al., 1985).

1.4 Background of plants examined in this study

1.4.1 Secretory structures in terpene-accumulating plants

The production of essential oil (or resins) by plants is associated with the presence of specialised secretory structures (Fahn, 1979; Venkatachalam et al., 1984). These structures are the primary sites for oil accumulation thus protecting the other cells from the toxic effects of the essential oils/resins.

Several different types of secretory structures exist (Gershenzon, 1994) although glandular trichomes, secretory cavities and resin ducts are those encountered most frequently in plants (Figure 1.3). Glandular trichomes are multicellular epidermal hairs that usually secrete terpenes into an external cavity found at the apex of the trichome (Gershenzon, 1994; Werker et al., 1993) (Figure 1.3). Members of the Lamiaceae, Asteraceae and other families possess glandular trichomes (Gershenzon, 1994). In contrast to glandular trichomes, secretory cavities are internal structures in which large intercellular spaces are filled with terpenoids (Gershenzon, 1994) (Figure 1.3). Secretory cavities are found in the Myrtaceae and Rutaceae among other families (Gershenzon, 1994). Finally, the resin ducts found in the Pinaceae and many other families are also internal secretory structures but they exhibit an extensive elongation of the intercellular space compared to the secretory cavities found in the Myrtaceae for example (Gershenzon, 1994) (Figure 1.3).
**Glandular trichome**
- cuticle
- subcuticular space
- secretory cells
- basal cell

**Secretory cavity**
- cavity lumen
- epithelial cells

**Resin duct**
- cavity lumen
- epithelial cells

**FIGURE 1.3.** The three main secretory structures used for the accumulation of essential oils/resins (Redrawn by Regina Zabaras from Gershenzon, 1994).
1.4.2 *Melaleuca alternifolia* Cheel (Australian tea tree)

*Melaleuca alternifolia* Cheel (family Myrtaceae) is a paperbark tree that flourishes in swamps in the north coast region of New South Wales, Australia (Southwell and Stiff, 1989). *M. alternifolia* is very closely related to *M. linariifolia* and initially it was believed that the two plants were the same species; Cheel (1924) however recognised that the alternate, much narrower leaves of *M. alternifolia* compared to those of *M. linariifolia* together with their geographic isolation were adequate evidence for *M. alternifolia* to be raised to specific rank.

The aerial parts of *M. alternifolia* produce oil that is known to have germicidal properties mainly due to the its high (+)-terpinen-4-ol (~40 %) content (Southwell, 1988). Chemical varieties affording oils high in 1,8-cineole (>15 %) (Southwell et al., 1996) and terpinolene (>20 %) (Southwell et al., 1992) also exist however these oils are medicinally undesirable because of the concomitant decrease in the active constituent, terpinen-4-ol (Southwell et al., 1996). Other constituents of *M. alternifolia* oil include γ-terpinene (10-20 %), α-terpinene (3.5-10 %), α- and β-pinene and sesquiterpenes (Brophy et al., 1989; Southwell, 1988) (Figure 1.4).

1.4.3 *Ocimum minimum* L. (bush basil)

*Ocimum minimum* L. (family Lamiaceae) is an annual subshrub with leaves usually shorter than 20x10 mm (Paton et al., 1999). As with all *Ocimum* L. species, *O. minimum* has square stems with opposite, decussate leaves containing external trichomes (Paton et al., 1999).

The species is native to India and is known worldwide mainly due to the aromatic and medicinal properties of the essential oil obtained from its aerial parts (Martins et al., 1999). The acyclic monoterpene linalool and the allylphenol eugenol together with 1,8-cineole (Figure 1.4) are known to be the major constituents of the
oil from most chemotypes/varieties although methyl cinnamate-rich oils have also been described (e.g., Suh and Park, 1999; Tansi and Nacar, 1999).

1.4.4 *Salvia officinalis* L. (common sage)

*Salvia officinalis* L. (family Lamiaceae) is a perennial shrub that originates from Southern Europe (Karamanos, 2000). The species has long, angular and erect stems that support opposite, ovate leaves (Karamanos, 2000).

*S. officinalis*, as well as several other members of the genus *Salvia*, is known for its medicinal and cosmetic properties since earliest times (Dweck, 2000) and this plant is still present in the Pharmacopoeias of several countries including Britain, Germany and Russia (Todd, 1967).

*S. officinalis* is believed to have the highest essential oil yield among *Salvia* species (Ivanic and Savin, 1976). The major components of the essential oil of *S. officinalis* are α- and β-thujone with 1,8-cineole, camphor and caryophyllene being present to a lesser extent (e.g., Newall *et al.*, 1996) (Figure 1.4). *S. officinalis* oil exhibits tremendous variability in relation to the levels of α- and β-thujone, 1,8-cineole and camphor. As a result, at least 5 distinct chemotypes of *S. officinalis* have been identified (Tucker and Maciarello, 1990).
**Figure 1.4.** Structures of selected terpenoids found in the essential oils of the species examined here.

### 1.5 Statement of the problem

#### 1.5.1 Lack of studies in stress-induction of volatiles from terpene-accumulating plants

Investigations of stress-induced changes in the production/release of volatiles from terpene-accumulating plants are limited. Cotton and conifers (section 1.3.2) have been the only species examined but none of these plants produces terpenoid mixtures that could be called essential oils using the classic meaning of the term; cotton volatiles are dominated by diterpenoid aldehydes (McAuslane and Alborn, 1998) whilst conifers are characterised by resins (section 1.2.4.6). Studies focusing
on stress-induction of volatiles in other economically important plants such as bush basil or Australian tea tree, that produce essential oils used worldwide in a variety of applications, are completely lacking.

The considerable amount of constitutive components present in such species may have "deterred" efforts for stress-induced responses. However, the demonstration of induced responses in cotton and conifers (section 1.3.2) has indicated that some plants possess both constitutive and induced defensive responses. This view is further supported by the behaviour of sawfly larvae; these chewing pests of *Eucalyptus* have been observed to completely remove (by chewing the petiole) partially-eaten leaves before they abandon them (Edwards and Wanjura, 1989). Although the purpose behind this tactic is not known, it is possible that leaf-removal by the insects could sabotage the production and/or release of signals that could alert sawfly-predators thus prevent further damage to the plant.

Although the topic of terpene-turnover is controversial (Gershenzon *et al.*, 1993 and references therein), studies have shown that the concentration and/or composition of essential oil produced by a plant can change as a result of cytokinin application (El-Keltawi and Croteau, 1987a), herbicide application (El-Keltawi and Croteau, 1987b), water stress (Simon *et al.*, 1992), ethylene application (Katoh *et al.*, 1993), and fungal mycorrhization (Kapoor *et al.*, 2002). Thus, it is possible that herbivory and pathogen attack may also induce changes in essential oil concentration and composition.

1.5.2 The need for a different approach

The insect pest problems of *Eucalypt* forests in Australia (reviewed by Stone, 1993) have motivated several investigations into the relationship between herbivory damage and levels of foliar terpenoids found in various *Eucalypt* species (e.g.,
Edwards et al., 1993; Morrow and Fox, 1980; Stone and Bacon, 1994). The levels of defoliation caused by beetle feeding were found to be strongly correlated with the proportion of 1,8-cineole in the essential oil in several eucalypts; this led investigators to suggest that this monoterpenoid was responsible for the observed insect-resistance (Edwards et al., 1993; Stone and Bacon, 1994).

However, correlation of feeding damage with specific plant chemical attributes may lead to unrealistic conclusions (Morrow and Fox, 1980; Southwell et al., 1995). For example, 1,8-cineole is known to be metabolised by the pyrgo beetle, a pest of *M. alternifolia* (Southwell et al., 1995). Although this action could simply serve detoxification purposes, it is also possible that 1,8-cineole is being utilised as part of the beetle's diet; if this is the case then high levels of herbivory on low-cineole trees/species may be the result of increased feeding by the pests in order to obtain the required intake of 1,8-cineole (Southwell et al., 1995).

The use of an orthodox approach, where qualitative and quantitative changes in essential oil would be measured after the challenging of terpene-accumulating plants by herbivores/pathogens, is likely to produce more reliable conclusions (about the role of the various terpenoids) than those originating from studies based on levels of defoliation alone.
1.6 Aims and Objectives

The main aim of this study was to determine qualitative and quantitative changes in the production/release of volatiles by the leaves of three economically-significant terpene-accumulating plants (*M. alternifolia, O. minimum* and *S. officinalis*) as a result of mechanical wounding, herbivore/pathogen attack, and jasmonic acid elicitation.

The above aim was achieved by:

➢ the design of an experimental protocol that minimised/eliminated all interfering factors (Chapter 2);

➢ the optimisation of existent and development of novel sample preparation/analytical techniques (Chapters 3 and 4);

➢ the use and development of appropriate data analysis approaches (Chapter 5);

➢ the use of mechanical wounding, herbivory, pathogen attack, and jasmonic acid to induce changes in volatile production/emission (Chapters 6, 7 and 8).

A schematic diagram outlining the major experiments carried out in this study is given in Figure 1.5.
Melaleuca alternifolia Cheel, Ocimum minimum L., Salvia officinalis L.

Mechanical wounding

Herbivore attack

Pathogen attack

Changes in terpene biosynthesis (i.e. synthase activity)

Immediate changes in headspace oil concentration

Non-immediate changes in leaf-oil concentration and composition

analysed by solvent extraction
analysed by HS-SPME

FIGURE 1.5. An overview of the major experiments carried out in this study.
REFERENCES


CHAPTER 2

EXPERIMENTAL DESIGN

2.1 Introduction

The planning and execution of experiments associated with the monitoring of plant-derived volatiles that mediate a wide range of interactions between organisms from different trophic levels is a delicate task. This arises from the numerous factors (e.g., analytical, chemical, horticultural, biological) that need to be cautiously considered if reliable and realistic results are to be obtained.

The major problem revolves around the choice of a suitable and valid "control" which reflects accurately the status of the untreated plant. This Chapter describes the development of methods to reliably detect the changes in volatiles' composition and concentration when plant materials are subjected to external stress such as wounding or other treatment. It examines a number of different approaches and provides a rationale for the particular methods chosen for application in subsequent experiments.

2.2 Factors that must be considered when investigating the emissions of plants under stress

2.2.1 The use of intact versus detached plant parts

The use of intact plants (e.g., Loughrin et al., 1995a; Bolter et al., 1997; Paré and Tumlinson, 1997a; Paré and Tumlinson, 1997b; McAuslane and Alborne, 1998; Turlings et al., 1998) or detached aerial or other plant-parts (e.g., Zeringue 1987; Dicke et al., 1990; Zeringue, 1992; McCall et al., 1994; Loughrin et al., 1995b; Mattiacci et al.,
1995) have been equally preferred options for scientists working in the field of plant chemoecology. Experiments based on detached plant-parts are usually easier to design and carry out but several studies have shown that detached or macerated plant material emits a different “blend” of volatiles when compared to intact plants (e.g., Mookherjee et al., 1989; Tollsten and Bergström, 1998; Agelopoulos et al., 1999; Chapter 6). After investigating intact plants, Gershenzon et al. (1993) found no evidence of rapid terpene turnover in several terpenoid-accumulating species and questioned the validity of numerous previous studies that reported rapid turnover in many species but were performed using detached foliage, shoots or flowers (e.g., Banthorpe and Ekundayo, 1976; Burbott and Loomis 1969; Croteau and Loomis 1973; Hefendehl et al., 1967).

All experiments in this study (with the exception of the jasmonic acid experiment, Chapter 8) were designed in such way as to utilize intact plants under normal physiological conditions.

2.2.2 Inter- and intra-plant variation

2.2.2.1 Background

Inter- and intra-plant variation in terpenoid composition and biogenesis has been routinely observed during experiments with terpene accumulating plants even when individuals of the same age and chemotype grown under identical conditions are used (e.g., Brooker et al., 1988; Doran and Brophy, 1990; Perry et al., 1999; Southwell, 1973; Southwell and Stiff, 1990; Zabaras et al., 2002). The use of clonal material has not reduced the amount of variation (Kawakami et al., 1990; List et al., 1995).

For most essential-oil producing species, the observed variation increases even more when (the same or different) leaves at various stages of development are
compared. Chemical and/or enzymatic transformations of terpenoids occurring during leaf development have been demonstrated for many plants including Japanese mint (Duriyaparan and Britten, 1982), peppermint (Maffei et al., 1989) and sweet basil (Werker et al., 1993). One of the most notable ontogenetical transformations occur in *M. alternifolia* leaves where the thujane precursors (sabinene, *E*-sabinene hydrate, *Z*-sabinene hydrate) are converted to the more stable *p*-menthanes (terpinen-4-ol, *α*- and *γ*-terpinene, terpinolene) as the leaves mature (Southwell and Stiff, 1989; Cornwell et al., 1995).

Variation in composition and concentration of essential oil obtained from different parts of the same leaf is also common especially in herbaceous species (e.g., *Lamiaceae*) (Maffei et al., 1989; Werker et al., 1993). Work has shown that the number of secretory structures in such species increases during leaf development (Turner et al., 1980; Croteau et al., 1981; Maffei et al., 1986; Maffei et al., 1989; Werker et al., 1993) and it is not fixed at leaf emergence as was initially claimed (Carliquist, 1958; 1959). Therefore, the within-leaf variation in essential oil simply reflects the fact that the number and age of trichomes, situated at different parts within the same leaf, varies (Maffei et al., 1989; Werker et al., 1993).

Based on the information described above, it was necessary to determine the inter- and intra-plant variation present in the experimental materials used in this investigation. This would ensure the accurate interpretation of subsequent results obtained in this study.
2.2.2.2 Materials and Methods

2.2.2.2.1 Determination of intra- and inter-plant variation in experimental plants

Five month old Ocimum minimum and Salvia officinalis plants were used to demonstrate the oil profile variation between leaves of the same pair (4th from apex). The plants were purchased as seedlings from a local nursery and were grown under field conditions until used. Each leaf of the pair was taken and subjected (separately) to solvent extraction (section 2.2.2.2.2) and GC/GC-MS analysis (section 2.2.2.2.3). In each case eight pairs from the same plant were used. A similar process was used for M. alternifolia but in this case leaves from three pairs (2nd-4th for flush growth, 7th-9th for mature leaves) were used due to the small size of individual leaves. Two-year regrowth M. alternifolia trees were used (part of a small plantation located at the University of Western Sydney, Hawkesbury Campus, New South Wales, Australia).

Inter-plant variation in leaf-oil composition was determined similarly but in this case samples from eight different plants (of the same species, age and chemotype, grown under identical field conditions) were collected and separately analysed.

2.2.2.2.2 Solvent extraction

After sampling, the leaf material (10-25 mg) was immersed in dichloromethane (CH₂Cl₂) (100 μL) spiked with 400 ppm octane (as an internal standard) for 24 hours at room temperature in the dark (a discussion on the selection of these parameters can be found in Chapter 3). GC/GC-MS analysis followed (section 2.2.2.2.3).

Leaf-oil concentration was calculated based on the internal standard and was expressed per sample dry weight (more details and background information to these issues is provided in Chapters 3 and 5).
2.2.2.3 GC/GC-MS analysis

GC analysis was carried out using a HP 6890 gas chromatograph equipped with a flame ionisation detector and a HP 7673 GC/SFE auto-injector. The column used was BPX-5 (50 m length x 0.22 μm ID x 0.25 μm film thickness) (SGE Scientific, Melbourne, Australia). The injection volume was 2 μL, inlet temperature and pressure were 280 °C and 20 psi respectively, carrier gas was H₂ (40 cm sec⁻¹ velocity), split ratio was 1:10, detector temperature at 280 °C, and the oven program was: initial temperature 60 °C for 5 min., increased to 180 °C at 4°C/min., final temperature maintained for 5 min.

GC-MS analysis conditions were as above with He as the carrier gas. The instrument used was a Varian 3800 gas chromatograph connected to a Varian 2000 ion trap detector (0.9 scans/sec, 20 μAmp). Compounds were identified by comparison of their mass spectra and retention indices (based on n-alkanes) with those of authentic standards (Fluka Chemicals, NSW, Australia). The relevant literature was also consulted (Brophy et al., 1989; Adams, 1995; Chalcat et al., 1998; Martins et al., 1999).

2.2.2.4 Determination of leaf-moisture

Leaf-material (collected according to section 2.2.2.1) were placed in a glass petri-dish and dried for 10 days at 52 °C (Appendix 2.1). Moisture was determined by the weighing of the leaves to a constant mass before and after drying (Appendix 2.2).
2.2.2.3 Results-Discussion

From Tables 2.1 and 2.2 it can be seen that the between-leaf differences were smaller than the between-plant differences for all three species. This does not mean that the former were negligible; notable differences were observed for several terpenoids (e.g., ~8.2 % for α-thujone, ~2.4 % for camphor) despite the fact that these values originated from leaves of the same pair. If the induction experiments were designed to utilise same-pair leaves (e.g, one leaf as control, the other as treated) then treatment-induced differences could only be established if they were greater than the differences presented in Table 2.1. Smaller changes would have been “masked” and even an 8 % increase in α-thujone, for example, would have passed undetected.

These experiments demonstrate that the approach of using leaf-pairs (or similarly located leaves on different plants) as samples does not provide the necessary precision to enable even quite large variations in plant volatiles, that may result from stress, to be reliably detected. Therefore, approaches based on these protocols were not further developed.
TABLE 2.1. Difference in essential oil composition and concentration between leaves of the same pair for the species of interest. The averaged relative differences (%) for each terpenoid between leaves from eight pairs are given. NP stands for not present (includes the components present at undetectable levels).

<table>
<thead>
<tr>
<th>Components</th>
<th>Difference in percentage composition between leaves from the same pair (mean, n=8)</th>
<th>O. minimum</th>
<th>S. officinalis</th>
<th>M. alternifolia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-salvene</td>
<td>NP</td>
<td>0.21</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>E-salvone</td>
<td>NP</td>
<td>0.38</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>α-thujene</td>
<td>NP</td>
<td>0.18</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>α-pinene</td>
<td>0.11</td>
<td>0.29</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>camphene</td>
<td>NP</td>
<td>0.50</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>cluster 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.33</td>
<td>0.74</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>α-terpinene</td>
<td>NP</td>
<td>0.11</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>cluster 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.50</td>
<td>0.83</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>0.05</td>
<td>0.20</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>E-sabinene hydrate</td>
<td>0.13</td>
<td>0.03</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>terpinolene</td>
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<td>0.04</td>
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<tr>
<td>β-thujone</td>
<td>NP</td>
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<td>NP</td>
<td></td>
</tr>
<tr>
<td>E-p-menth-2-en-1-ol</td>
<td>NP</td>
<td>NP</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Z-p-menth-2-en-1-ol</td>
<td>NP</td>
<td>NP</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>camphor</td>
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<td>endo-borneol</td>
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</tr>
<tr>
<td>E-piperitol&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NP</td>
<td>NP</td>
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<td>18.5</td>
<td>1.75</td>
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<sup>a</sup> cluster 1 includes sabinene, β-pinene and myrcene

<sup>b</sup> cluster 2 includes p-cymene, (M. alternifolia and S. officinalis only), limonene, β-phellandrene (M. alternifolia only) and 1,8-cineole

<sup>c</sup> tentative identification
<table>
<thead>
<tr>
<th>Components</th>
<th>Percentage (%) composition (min.-max., n=16)</th>
<th>O. minimum</th>
<th>S. officinalis</th>
<th>M. alternifolia</th>
</tr>
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<tr>
<td></td>
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<td>Mature</td>
<td>Fresh</td>
<td>Mature</td>
</tr>
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<td>NP</td>
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<td>1.43-3.08</td>
<td>4.55-9.09</td>
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<td>cluster 2(^b)</td>
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<td>β-thujone</td>
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</tr>
<tr>
<td>E-p-menth-2-en-1-ol</td>
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<td>NP</td>
<td>0.10-0.75</td>
<td>0.21-0.36</td>
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<td>NP</td>
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<td>0.23-0.39</td>
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<td>NP</td>
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</tr>
<tr>
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<td>2.51-5.24</td>
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<td>methyl eugenol</td>
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</tr>
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<td>sesquiterpenes</td>
<td>12.5-27.6</td>
<td>18.9-43.1</td>
<td>9.17-20.71</td>
<td>21.62-34.43</td>
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<tr>
<td><strong>Oil concentration</strong></td>
<td><strong>range (µg g(^{-1}) dry weight)</strong></td>
<td>32.7-118</td>
<td>1.07-3.61</td>
<td>363-666</td>
</tr>
</tbody>
</table>

\(^a\) cluster 1 includes sabinene, β-pinene and myrcene

\(^b\) cluster 2 includes p-cymene (M. alternifolia and S. officinalis only), limonene, β-phellandrene (M. alternifolia only) and 1,8-cineole

\(^c\) tentative identification
2.3 Quantitative determination of volatiles emitted by intact plants using headspace sampling

2.3.1 Introduction

Almost all studies reported in the literature have employed headspace-related techniques for measuring the results of treatment of plants with an inducing "agent" (e.g., Zeringue 1987; Dicke et al., 1990; Zeringue, 1992; McCall et al., 1994; Loughrin et al., 1995a; Bolter et al., 1997; Paré and Tumlinson, 1997a; Paré and Tumlinson, 1997b; McAuslane and Alborne, 1998; Turlings et al., 1998).

The use of a headspace-related technique simplifies the experimental design and minimises the number of additional experiments that may be required to validate the results due to the availability of "true" controls. For example, headspace techniques allow for the sampling of the same plant-material before and after treatment. In such a situation, the before-treatment plant-material results represents a “true” control; thus the requirement of supporting experiments to determine other factors (e.g., inter- and/or intra-plant variation, section 2.2.2) that could affect the results if “identical” plant-material was used instead, is eliminated.

2.3.2 Determination of volatiles emitted by intact leaves using on-plant HS-SPME

2.3.2.1 Introduction

An HS-SPME method was employed for the monitoring of volatile emissions from individual, non-detached leaves over short periods of post-treatment time (≤ 70 minutes).

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1 The term “identical” here denotes plant-material derived from morphologically similar plants of the same species and chemotype or same-age leaves from the same plant.
The protocol involved the placing of an individual leaf (or small twig in the case of *M. alternifolia*) in the apparatus described in section 2.3.2.2. HS-SPME was then carried out before and after treatment of the leaf (or twig) and changes in volatile emissions due to treatment were determined (as per Chapter 6).

Results from experiments investigating headspace depletion effects, and, the importance of a “stabilizing” period between plant sampling and handling, are presented and discussed below.

**2.3.2.2 System design and Methods**

*O. minimum, S. officinalis* and *M. alternifolia* plants similar to those described above (section 2.2.2.2.1) were used. This technique was based on placing an individual leaf (or small twig in the case of *M. alternifolia*) in a small glass cylinder (~20 mL volume) closed at one end with a Teflon cap (Figure 2.1). The cap was pre-drilled to exactly fit a manual solid-phase microextraction (SPME) holder (Supelco, USA). Glass wool was used to restrict the other end after the leaf (or twig) was in place, however, the system was not air-tight.

After 30 minutes the headspace above the branch was sampled using a polydimethylsiloxane (PDMS)-coated fibre (10 minutes, 25 °C). Thermal desorption (10 minutes) of the fibre in the injector of the GC and GC-MS system followed (inlet temperature 220 °C, other conditions as per section 2.2.2.2.3). The process was repeated for a similar plant but this time the branch was sampled immediately (was not allowed to "stabilise" in the cylinder for 30 minutes).
2.3.2.3 Results-Discussion

The handling of a plant just before sampling can affect its emissions even when the plant has sustained no visible damage (Vercammen et al., unpublished). Results from experiments with the species of interest to this study confirmed this effect (Figure 2.2) and showed that the use of a short “stabilising” period between the plant handling and sampling processes is important for the integrity of the obtained results.
Figure 2.2. RIC traces of *M. alternifolia* twigs as obtained by HS-SPME. a) Sampled after 30 minutes “stabilisation” and b) sampled immediately upon placement within the sampling apparatus. Peaks include mono- and sesquiterpenoids and lipoxygenase-derived products (e.g., Z-3-hexenyl acetate).

A stabilising period of at least 30 minutes was employed in all further experiments involving plant-headspace monitoring by HS-SPME; this ensured that the obtained results were “free” from handling-induced volatiles.

Depletion of the headspace due to repetitive sampling or other losses through the system can also be a serious problem when volatiles in the vapour phase are reduced at a faster rate than they are emitted by the plant. In such cases correction of the obtained HS-SPME values is required particularly during quantitative measurements.
Before correction can be applied, the rate of the depletion of each component from the headspace above the plants of interest must be established. This was achieved by repetitive sampling (section 2.3.1.2) of “untreated” leaves by HS-SPME (at 10-minute intervals, up to 70 minutes after the initial sampling). These parameters were used because they were identical to those employed during the monitoring of “treated” plants. The rate of the depletion of selected components from the headspace above a S. officinalis leaf is given below (Figure 2.3).

**Figure 2.3.** Depletion rate of selected components from the headspace above S. officinalis leaves enclosed within the sampling unit described in section 2.3.1.2 and sampled repetitively by HS-SPME. Similar depletion rates were obtained from all components (with the exception of β-thujone).
As can be seen from Figure 2.3, most oil constituents (the only exception being β-thujone) exhibit very similar depletion rates that range between 70-80 % loss of analyte after the 4th sampling. This indicates the importance of taking account of this phenomenon which can seriously affect the integrity of quantitative results if corrective measures are not employed.

The HS-SPME approach discussed in this section allowed the qualitative and quantitative determination of individual-leaf volatiles (with the aid of SPME fibre-gas phase distribution constants calculated in Chapter 4). In particular, this technique was found to be most useful for the measurement of leaf-volatiles known to be released immediately upon wounding such as lipoxygenase-derived products (such as E-3-hexen-1-ol) (e.g., Agelopoulos et al., 1999; Ruther, 2000; Chapter 6 here) and wound-released terpenoids (Zabaras and Wyllie, 2001; Zabaras et al., 2002).

2.3.3 Determination of volatiles emitted by whole, intact plants using purge-and-trap headspace sampling

2.3.3.1 Introduction

Many parameters have to be considered during the development of a system that will allow the non-destructive sampling of volatiles produced by whole, intact plants (Charron et al., 1996; Pham-Tuan et al., 2000). A design that may be efficient from an analytical/chemical point of view may be totally inappropriate and inadequate for the biological requirements (e.g., nutrients, light, moisture, temperature, air) of the plant sampled.

Recently, a few fully automated systems that satisfy the requirements for efficient, non-destructive monitoring of plant-emitted volatiles have been described (Charron et al., 1996; Pham-Tuan et al., 2000; Vercammen et al., 2000) but due to the high cost such equipment was not available during this study.
2.3.3.2 System design and Methods

A potted *Melaleuca alternifolia* plant (approximately 6 months old, grown under field conditions with daily irrigation) was placed inside the system shown in Figure 2.4. The system consisted of a Perspex cylinder (10 mm thickness x 400 mm height x 300 mm diameter) sealed on one end. The sealed (top) end was fitted with an inlet and an outlet line (Teflon® tubing). A water-filled (150 mm in height) container was used to "seal" the open (bottom) end after placement of the potted plant inside the cylinder. A Gilian® (GilAir 5 model) multi-flow air pump was then used to purge air (70 mL min⁻¹) through the system. Plants were left to "stabilise" in the chamber for at least 3 days before sampling. Volatiles were collected on standard GC liners packed with Tenax-TA® (70 mg) (Appendix 2.3). Sampling time of 2 hours was used. Subsequently, the traps were thermally desorbed by insertion into the split/splitless port of the GC-MS system (section 2.2.2.2.3). The inlet was (manually) programmed to remain "cold" (40 °C) until the trap was inserted and then allowed to heat-up (220 °C). The GC column was kept at 0 °C with CO₂ for 6 min. (time it required for the inlet to heat from 40 °C to 220 °C).

2.3.3.3 Results-Discussion

The system shown below (Figure 2.4) satisfied the horticultural requirements for plant growth (natural light, temperature range 23-28 °C, humidity ~85%) and thus plants could be within the system and sampled without any signs of stress for long periods of time (4-5 weeks). However, the desorption and subsequent analysis of the trapped volatiles revealed that only a few (mostly non-oxygenated monoterpenes) components of the expected (Table 2.2, section 2.2.2.3) "suite" of compounds for each plant species could be detected (Figure 2.5).
Figure 2.4. Schematic representation of the developed system for the non-destructive sampling of plant-emitted volatiles (part of the drawing was taken from Verammen et al., 2000). The plant was not placed in the water body but it was irrigated as required.

Figure 2.5. RIC trace of a M. alternifolia (terpinen-4-ol chemotype) plant sampled for 2 hours with the purge-and-trap system. Volatiles detected include α-thujene (1), α-pinene (2), sabinene (3), p-cymene (4), α-terpinene/limonene (5), γ-terpinene (6) and two unidentified peaks (7), (8).
As can be seen from Figure 2.5 higher boiling volatiles such as the oxygenated monoterpenoids and sesquiterpenes could not be detected despite the fact that *M. alternifolia* (terpinene-4-ol chemotype) leaves are known to be “rich” in monoterpene alcohols and to a lesser extent sesquiterpenes (Table 2.2, section 2.2.2.3).

There are likely to be several factors contributing to the inadequacy of the system to collect the above-mentioned volatiles. In the case of non-oxygenated sesquiterpenes (e.g., *E*-β-caryophyllene, aromadendrene) lack of sensitivity may have been the major factor by virtue of their low vapour pressure and low concentration in the sample (Wyllie *et al.*, 1978). Losses due to adsorption onto the walls of the cylinder and other parts of the system (e.g., soil, pot surface) may have also contributed as sesquiterpenes are known to have the ability to sorb at all surfaces with which they come into contact (Matich *et al.*, 1996; Górecki *et al.*, 1998). The rapid removal of organic compounds in the vapour phase by atmospheric species such as O₃, OH, and NO₃ may also be part of the problem (Zini *et al.*, 2001) considering that the half-life of *E*-β-caryophyllene, in air containing 50 ppbv of ozone, is 36 seconds (Grosjean, 1995).

Solubility of sample components in the water body used to “seal” the system (Figure 2.4) and in the water droplets formed on the walls of the cylinder due to humidity/condensation is considered to be the major cause for the loss of the oxygenated monoterpenoids and sesquiterpenoids (e.g., terpinen-4-ol, α-terpineol). Such components are more polar than other non-oxygenated constituents and therefore are more soluble in water (Griffin *et al.*, 1999). Considering the amount of water present in the system and the solubility value of terpinen-4-ol in water (1491 ± 28 mg/L; Griffin *et al.*, 1999) for example, it is not surprising that terpinen-4-ol was
not detected although it is a major component (~40 %; Zabaras et al., 2002) of the volatile "blend" emitted by the leaves of the M. alternifolia plants used herein.

The two unidentified peaks in Figure 2.5 (7, 8) and the unexpectedly high level of p-cymene (4) observed in the trace are likely to be artifacts of the porous, solid sorbent Tenax-TA®. Coeur et al. (1997) found that Tenax-TA® decomposes α-pinene (2) and sabinene (3) to a mixture of other monoterpenes with p-cymene being the major degradation product (25.6%) of sabinene (Coeur et al., 1997).

The problems discussed above meant that the developed volatile collection system and associated headspace sampling could not be used as the method for monitoring of volatiles produced by whole, intact plants. As a consequence, an alternative sampling methodology had to be found (section 2.4).

2.4 Use of leaf division to provide "control" material

2.4.1 Detection of stress-induced changes in essential oil composition and concentration

Used during the determination of the long term (≥ 24 hours) treatment-induced changes in leaf-oil concentration and composition (further details in Chapters 6 and 7), this approach involved treatment of the selected plants with the “agent” of interest (herbivore, elicitor, pathogen) followed by the cutting-off of a strip of treated leaf tissue (this was considered to be time-zero (T_0)). After 24 hours (although this period varied for some experiments) an identical strip from the same leaf (that was still attached to the plant) would be taken (T_{24}). This process was also carried out for T_0 and T_{48} strips with a different set of plants (3 leaves per sample, 1 sample per plant, 8 plants per experiment). After cutting, all strips were subjected to sample preparation and analysis (solvent extraction followed by GC/GC-MS) and

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2 The protocols are described here in general terms only. Please consult Chapters 3-7 for details.
differences between the T₀ and T₂₄, T₄₈ strips in terms of essential oil composition and concentration were sought with the aid of inferential and descriptive statistical techniques (as per Chapter 5). Finally, any established differences were overlaid upon changes occurring in untreated plants (e.g., developmental changes) before the final conclusions were drawn.

Collection of strips from the same leaf (or leaves) before and after treatment rather than collection of "identical" leaves was necessary due to the great intra- and inter-plant variability, in terms of oil composition and concentration, observed in the plants used (section 2.2.2).

2.4.2 Detection of stress-induced changes in terpene synthase activity

The protocol described in section 2.4.1 was also used to investigate treatment-induced changes in terpenoid biosynthesis. In this case the untreated (control) and treated leaves were subjected to procedures suitable for the extraction, isolation and purification of terpene synthases. The detection of any synthases present in the cell-free enzyme preparations (obtained from the untreated and treated leaves) was achieved by the development of a novel HS-SPME-based technique (details in Chapter 4). This approach allowed any treatment-induced changes in the biogenetic activity of the leaves to be “mapped” and complemented similar changes in essential oil composition and concentration observed by the solvent extraction approach.
2.5 The effects of ontogeny and leaf-division on leaf-essential oil

2.5.1 Introduction

The cutting of the leaf tissue strip was the only treatment in the case of mechanical wounding experiments. Any changes in leaf volatile emissions / terpene biosynthesis due to mechanical wounding itself had to be established first; this process formed part of all subsequent plant-induction experiments and thus was required for the accurate interpretation of the results obtained in those later experiments. The nature of the protocol (section 2.4.1) meant that no untreated plants (i.e. "true" controls) were available during the mechanical wounding experiments. In these cases several supporting experiments (described below) were carried out as required in order to ensure the validity and accuracy of the presented results.

2.5.2 Materials and Methods

2.5.2.1 Determination of intra-leaf variation

*M. alternifolia* and *O. minimum* plants, of a similar physiological state to that described in section 2.2.2.2.1, were used. Three *M. alternifolia* mature leaves were cut longitudinally in half; the halves were then combined into a sample containing the leaves’ distal parts and another one containing the leaves’ proximal parts. For *O. minimum*, two adjacent, laterally-cut strips from the same leaf were taken and formed separate samples. All samples were then subjected to solvent extraction (section 2.2.2.2.2) and GC/GC-MS analysis (section 2.2.2.2.3). Eight replicates were used for both species.
2.5.2.2 Determination of ontogenetical changes

*S. officinalis* and *O. minimum* plants, of a similar physiological state to that described in section 2.2.2.2.1, were used. Leaves from five pairs were taken starting from the apex and moving down the branch. All leaf-pairs were subjected (separately) to solvent extraction (section 2.2.2.2.2) and GC/GC-MS analysis (section 2.2.2.2.3).

2.5.3 Results-Discussion

2.5.3.1 Intra-leaf variation

The determination of the intra-leaf differences for all oil-constituents in the species used was essential for the correct interpretation of any results obtained due to the nature of the sampling protocol. Results from appropriate experiments showed that there were no significant differences (for any of the oil-constituents) between the two leaf-halves (for *M. alternifolia*) or between the two leaf-strips (for *O. minimum* and *S. officinalis*) (Wilkoxon signed-ranks test at $p \leq 0.05^3$) (Table 2.3).

The lateral cutting of adjacent leaf-strips is likely to be the reason that no significant oil differences were observed especially for *O. minimum* and *S. officinalis* (Table 2.3). The studies that reported large intra-leaf differences in essential oil composition and concentration in herbaceous species compared distal and proximal leaf-parts where the developmental difference between the two parts can be quite notable especially in species with large leaves such as sweet basil (Werker *et al.*, 1993). The technique of laterally cutting adjacent strips reduces any compositional differences caused by leaf development.

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3 This was the lowest confidence level (i.e 95%) used for statistical inference throughout this study.
TABLE 2.3. Differences in essential oil composition and concentration between different parts from the same leaf (mean ± standard deviation, n=8). No statistically significant differences were detected (Wilkoxon signed-ranks test at p≤0.05). NP stands for not present (includes the components present at undetectable levels).

<table>
<thead>
<tr>
<th>Components</th>
<th>Difference in percentage (%) composition</th>
<th>(mean ± std, n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>O. minimum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Outer-inner strip)</td>
</tr>
<tr>
<td>α-thujene</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>α-pinene</td>
<td>-0.02±0.15</td>
<td>0.03±0.14</td>
</tr>
<tr>
<td>cluster 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.06±0.41</td>
<td>-0.08±0.36</td>
</tr>
<tr>
<td>α-terpinene</td>
<td>NP</td>
<td>0.07±0.12</td>
</tr>
<tr>
<td>cluster 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.43±1.67</td>
<td>0.32±1.21</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>0.08±0.23</td>
<td>0.10±0.36</td>
</tr>
<tr>
<td>E-sabinene hydrate</td>
<td>0.03±0.06</td>
<td>-0.02±0.09</td>
</tr>
<tr>
<td>terpinolene</td>
<td>-0.05±0.09</td>
<td>-0.02±0.04</td>
</tr>
<tr>
<td>linalool</td>
<td>0.33±0.55</td>
<td>NP</td>
</tr>
<tr>
<td>Z-sabinene hydrate</td>
<td>NP</td>
<td>0.02±0.05</td>
</tr>
<tr>
<td>E-p-menth-2-en-1-ol</td>
<td>NP</td>
<td>NP</td>
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<td>NP</td>
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<td>camphor</td>
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<tr>
<td>α-terpineol</td>
<td>0.05±0.14</td>
<td>NP</td>
</tr>
<tr>
<td>E-piperitol&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>bornyl acetate</td>
<td>0.03±0.29</td>
<td>0.02±0.25</td>
</tr>
<tr>
<td>eugenol</td>
<td>0.05±1.30</td>
<td>NP</td>
</tr>
<tr>
<td>methyl eugenol</td>
<td>-0.06±0.05</td>
<td>NP</td>
</tr>
<tr>
<td>sesquiterpenes</td>
<td>-0.54±1.55</td>
<td>-0.38±1.32</td>
</tr>
<tr>
<td><strong>Difference in oil concentration</strong></td>
<td></td>
<td>1.44±13.0</td>
</tr>
<tr>
<td><strong>(mean ± std, n=8)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>(µg g&lt;sup&gt;-1&lt;/sup&gt; dry weight)</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> cluster 1 includes sabinene, β-pinene and myrcene
<sup>b</sup> cluster 2 includes p-cymene (M. alternifolia and S. officinalis only), limonene, β-phellandrene (M. alternifolia only) and 1,8-cineole
<sup>c</sup> tentative identification
As it can be seen from Table 2.3, a large variation in the values for the
difference in oil concentration between the two leaf-parts was obtained especially for
*O. minimum* and *S. officinalis* (shown by the large std values when compared to the
mean values). This variation indicates a large inconsistency in oil production by the
leaf-parts involved and may be associated with the variation in numbers of trichomes
even for neighbouring parts of the leaf (Maffei et al., 1989). Much smaller variation
was obtained by *M. alternifolia* leaves.

The validity of the assumption that $T_0$ values represent pre-wounding values
was also a very critical point of the sampling protocol. Studies on corn (Turlings et.
al., 1995) and cotton (Paré and Tumlinson, 1997b) have shown that there is a delay of
several hours between the start of wounding and the release of induced terpenoids.
This suggests that a series of inducible biochemical reactions are required for those
compounds to be produced and subsequently released. Similarly, if compositional
changes in *M. alternifolia* oil were to be induced by mechanical wounding, the
process would not be instantaneous. Therefore, the assumption that $T_0$ **compositional**
values represent pre-wounding values is valid; this is supported by the results given
on Table 2.4.

The $T_0$ oil **concentration** values do not accurately represent pre-wounding oil
levels (Table 2.4) as wounding ruptures the leaves' secretory structures and this
results in loss of oil into the atmosphere (Zabaras et al., 2002; Zabaras and Wyllie,
2001; Chapter 5). However, these initial oil losses are equivalent (Table 2.3) for both,
$T_0$ and $T_{24}$ or $T_{48}$, leaf strips and thus it can be assumed that concentration changes
over time are not affected.
**Table 2.4.** Comparison of essential oil composition and concentration between intact and T<sub>0</sub> (cut) *M. alternifolia* leaves (from the same plant). As can be seen the cutting of a leaf has no notable instantaneous effects on the composition of the oil in the leaf (considering the between leaf variation shown in Table 2.1).

<table>
<thead>
<tr>
<th>Components</th>
<th>Percentage (%) composition (mean ± std, n=8)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M. alternifolia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intact leaves</td>
<td>T&lt;sub&gt;0&lt;/sub&gt; (cut) leaves</td>
</tr>
<tr>
<td>α-thujene</td>
<td>0.78±0.14</td>
<td>0.75±0.12</td>
</tr>
<tr>
<td>α-pinene</td>
<td>2.08±0.08</td>
<td>2.10±0.10</td>
</tr>
<tr>
<td>cluster 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.43±0.20</td>
<td>1.52±0.42</td>
</tr>
<tr>
<td>α-terpinene</td>
<td>5.81±1.94</td>
<td>6.43±2.31</td>
</tr>
<tr>
<td>cluster 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.52±2.46</td>
<td>5.21±1.45</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>17.97±0.79</td>
<td>17.62±1.10</td>
</tr>
<tr>
<td>E-sabinene hydrate</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>terpinolene</td>
<td>2.47±0.49</td>
<td>2.61±0.36</td>
</tr>
<tr>
<td>Z-sabinene hydrate</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>E-p-menth-2-en-1-ol</td>
<td>0.16±0.11</td>
<td>0.14±0.09</td>
</tr>
<tr>
<td>Z-p-menth-2-en-1-ol</td>
<td>0.17±0.11</td>
<td>0.15±0.10</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>40.5±2.21</td>
<td>40.9±3.21</td>
</tr>
<tr>
<td>α-terpineol</td>
<td>3.05±0.17</td>
<td>3.91±0.32</td>
</tr>
<tr>
<td>E-piperitol&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.13±0.39</td>
<td>0.95±0.21</td>
</tr>
<tr>
<td>sesquiterpenes</td>
<td>18.8±3.47</td>
<td>17.4±2.8</td>
</tr>
<tr>
<td>Oil concentration (mean ±std, n=8) (µg g&lt;sup&gt;-1&lt;/sup&gt; dry weight)</td>
<td>445±77.1</td>
<td>375±85.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> cluster 1 includes sabinene, β-pinene and myrcene

<sup>b</sup> cluster 2 includes p-cymene, limonene, β-phellandrene and 1,8-cineole

<sup>c</sup> tentative identification

### 2.5.3.2 Ontogenetical changes

Developmental processes such as maturation are known to have an impact on the oil profile of terpene accumulating plants (Maffei *et al.*, 1989; Southwell and Stiff, 1989; Werker *et al.*, 1993). Therefore it was essential to determine such effects especially for *O. minimum* and *S. officinalis* because ontogenetical changes in these species had not been previously reported<sup>1</sup>. The results obtained are summarised in Figures 2.6 and 2.7.

<sup>1</sup> The effects of leaf-ontogeny on *M. alternifolia* terpenoids are well documented (section 2.2.2).
**FIGURE 2.6.** Ontogenetical changes occurring in terpenoids produced by *S. officinalis* leaves (from tip to base of plant). a) Major oil-constituents and b) constituents implicated at a later stage in this study (section 6.4.2.3).
**Figure 2.7.** Ontogenetical changes occurring in terpenoids produced by *O. minimum* leaves (from tip to base of plant). Major oil-constituents (sesquiterpenes, 1,8-cineole) and constituents implicated at a latter stage in this study (linalool, eugenol, methyl eugenol, camphor, myrcene) (section 6.4.2.2) are shown.
As can be seen from Figures 2.6 and 2.7 there are some significant changes in the oil-profile from *O. minimum* and *S. officinalis* leaves accompanying their maturation. In *O. minimum* leaves, eugenol increases with age while the level of sesquiterpenes decreases; the trend is reversed in *S. officinalis* where sesquiterpenes increase their contribution to the oil while α- and β-thujone and camphor levels are reduced. Changes in the levels of some of the lesser oil-constituents (e.g., linalool, myrcene) may also be important despite their relatively small presence in the oil; studies have shown that components present in small amounts can also have important ecological roles (e.g., Katoh and Croteau, 1998; Shu et al., 1998).

2.6 Conclusion

A well-planned experimental design that addresses all potentially interfering factors is essential if meaningful and reliable data is to be obtained from experiments focusing on the characterisation of interactions between organisms from different trophic levels.

The non-destructive methodologies described here allowed for the sampling of whole, intact plants and individual, non-detached leaves without requirement of an automated headspace-sampling unit.

The coupling of techniques such as leaf division with solvent extraction and HS-SME enabled the qualitative and quantitative determination of both immediate and non-immediate treatment-induced changes in plant emissions using valid controls.
APPENDICES

Appendix 2.1. Principles behind the selection of the parameters used for the determination of leaf-moisture.

The leaves were dried at 50 °C because it is known to be an appropriate temperature for the removal of the leaf-moisture (over several days) without affecting significantly the oil concentration of the leaves (I.A Southwell, pers.comm.). Experiments in this study showed that 10 days were found to be adequate for “complete” drying (Figure A2.2.1).

![Graph showing moisture removal over drying time](image)

Figure A2.2.1. Effect of drying-time on removal of moisture from *M. alternifolia* leaves.
Appendix 2.2. Values of moisture content for the leaf types used in this Chapter.

Table A2.3.1. Moisture content (% mean ± std, n=3) for the leaves used. Results from three experiments are shown.

<table>
<thead>
<tr>
<th>Species</th>
<th>Leaf part</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distal or Outer</td>
<td>Proximal or Inner</td>
<td>Intact leaves</td>
</tr>
<tr>
<td>M. alternifolia</td>
<td>38.9±2.51</td>
<td>39.0±3.52</td>
<td>39.4±2.6</td>
</tr>
<tr>
<td>(mature)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. alternifolia</td>
<td>58.5±2.55</td>
<td>55.7±4.46</td>
<td>60.5±4.25</td>
</tr>
<tr>
<td>(flush)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O. minimum</td>
<td>88.2±1.1</td>
<td>88.8±1.3</td>
<td>87.9±1.8</td>
</tr>
<tr>
<td>S. officinalis</td>
<td>79.1±0.5</td>
<td>78.6±0.7</td>
<td>79.4±1.2</td>
</tr>
</tbody>
</table>

Appendix 2.3. Trap packing and conditioning protocol.

The volatiles were trapped in standard Pyrex glass GC liners (78.5 mm length, 4 mm ID) (Supelco) packed with Tenax-TA®. Before packing all GC glass liners were rinsed thoroughly with water followed by ethanol and then dried in the oven (200 °C for 2 hours).

Subsequently, a small silanised glass wool plug was inserted into one end of the liner that was then packed with Tenax®-TA (70 ± 2 mg). Adsorbent particles were closely packed using an engraver as a vibrating tool.

After packing Tenax-TA® traps were conditioned at 300 °C for 4 hours under a continuous flow of nitrogen (10 mL/min). After conditioning, the traps were removed, capped immediately with teflon caps, cooled and stored at room temperature until required.
REFERENCES


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CHAPTER 3

ANALYSIS OF TERPENOIDS IN LEAVES USING SMALL-SCALE SOLVENT EXTRACTION

3.1 Introduction

3.1.1 Reasons for the selection of solvent extraction

The selection of an appropriate sample preparation/isolation technique for the target analytes is critical for the success of metabolic and physiological studies of plant material containing terpenoids. Sample preparation/isolation techniques can have a great influence on the composition of the resulting oils especially when hydrolysis- and/or other reaction-sensitive components are involved (Fischer et al., 1987; Kawakami et al., 1990; Cornwell, 1999). “Mild” approaches that have been used in the past due to their ability to minimise degradation or other artefacts in the resulting oils include vacuum-distillation (Bignell et al., 1996; Guillén et al., 1996) and solvent extraction (Weston, 1984; Cornwell et al., 1995). The information obtained from these latter two methods is the same (Cornwell, 1999). Solvent extraction however, was preferred over vacuum-distillation because it is less complex and most importantly can be carried out on the micro-scale required by this project. Micro-scale solvent extraction techniques that require small amounts of sample have been used frequently over the last fifteen years in physiological and metabolic studies (Ammon et al., 1985; Southwell and Stiff, 1989; Brophy et al., 1989; Southwell and Stiff, 1990; List et al., 1995; Cornwell et al., 1995; Cornwell, 1999). Although a small-scale technique that resembles a traditional large-scale steam-distillation has been recently developed (Cornwell et al., 1995; Corwell, 1999;
Corwell et al., 1999), a micro-scale equivalent to vacuum-distillation is not yet described.

3.1.2 Choice of solvent

During the investigation of ontogenetical changes occurring in monoterpenoids of *M. alternifolia* leaves, Southwell and Stiff (1989) used an ethanol-based small-scale extraction method identical to that developed by Ammon et al. (1985) for the determination of terpenoids in *Eucalyptus spp.* Since then methanol (Cornwell et al., 1995) and dichloromethane (List et al., 1995) have also been employed in similar methods.

Following the investigation of the extraction efficiency of various solvents (hexane, acetone, dichloromethane, ethanol), List et al. (1995) concluded that dichloromethane was the most appropriate to use for the small-scale extraction of *M. alternifolia* leaves as it produced oil-yield equivalent to that of the other solvents but exhibited better reproducibility. Another benefit that is derived from the use of dichloromethane instead of a more polar compound (e.g., methanol) as a solvent is the reduced introduction of water soluble or high boiling point compounds onto the capillary GC column (Cornwell, 1999). Such an introduction can seriously affect the chromatography particularly in the sesquiterpene region of a chromatogram as large, very broad background peaks appear (Cornwell, 1999). These polar compounds have been reported to cause deterioration of GC columns (Dunlop et al., 1997). The use of dichloromethane not only reduces this effect but allows for the addition of a water layer just before the GC analysis; many "unwanted" polar compounds are dissolved in the water layer permitting the analysis of the "clean" dichloromethane layer (Cornwell, 1999).
3.1.3 Length of extraction

A minimum time-period of 30 hours is recommended for use during the extraction of leaf-tissue if the absolute oil-concentration of the sample is determined (Brophy et al., 1989; Southwell and Stiff, 1989; List et al., 1995). Studies have also shown that longer extraction does not increase the amount of oil extracted irrespective of solvent used (Brophy et al., 1989; Southwell and Stiff, 1989; List et al., 1995).

3.1.4 Choice of internal standard

The selection of the appropriate internal standard is critical for a successful quantitative analysis. The substance selected must be sufficiently different from the analyte so that, while it is detected, it does not interfere with the analysis; it must also be sufficiently similar so that its recovery reflects that of the analyte (Rubinson and Rubinson, 1998).

Tridecane (C_{13}) has been often used as the internal standard during solvent extraction of *M. alternifolia* leaves (Murtagh and Etherington, 1990; List et al., 1995). On capillary columns coated with a non- or slightly-polar stationery phase (e.g., 100% (poly)dimethylsiloxane (PDMS) or 95% PDMS – 5% phenylpolysiloxane), this hydrocarbon elutes in-between the mono- (C_{16}) and sesquiterpenoids (C_{15}) found in *M. alternifolia* and thus does not interfere with the analysis.

Questions have been raised about the suitability of tridecane as an internal standard as poor reproducibility in recovery values has been obtained during solvent extraction of *Melaleuca* and *Eucalyptus* leaves (Griffin S, unpublished; Cornwell C.P, pers. comm.).
A description of the parameters used during the small-scale solvent extraction of *M. alternifolia*, *O. minimum* and *S. officinalis* leaves is given below. The reasons for their selection are then presented and discussed.

### 3.2 Materials and Methods

#### 3.2.1 Chemicals

Octane and tridecane (>98 % purity) were obtained from Fluka (NSW, Australia).

#### 3.2.2 Plants

Two-year regrowth *M. alternifolia* trees were used (part of a small plantation located at the University of Western Sydney, Hawkesbury Campus, New South Wales, Australia). *Cryptocarya microneura* leaves were collected from local trees whilst *O. minimum* and *S. officinalis* plants were purchased from a nursery.

#### 3.2.3 Solvent extraction

Leaves (10-25 mg) were immersed in CH$_2$Cl$_2$ (100 µL) spiked with 400 ppm octane or 200 ppm tridecane (as an internal standard) for 24 hours at room temperature in the dark. Extracts were then subjected to GC analysis.

#### 3.2.4 Determination of internal standard recovery

Internal standard recovery after the 24-hour leaf-extraction was determined by comparison against values from "blanks" spiked with same amount of octane or tridecane. Eight replicates were used in each case.

#### 3.2.5 Determination of instrumental precision

The reproducibility of the GC system used (section 3.2.7) was determined by repetitive injections of two different leaf-extracts (prepared as per section 3.2.3). The errors introduced to the analysis by the GC system could then be estimated.
3.2.6 Determination of thujane – p-menthane relationship by solvent extraction

*M. alternifolia* leaves of various ages were collected and extracted as described in section 3.2.3. GC analysis followed (section 3.2.7) and the relative amount (%) of thujanes and p-menthanes in the extracts were determined by integration of the areas under the peaks.

3.2.7 GC analysis

GC analysis was carried out using a HP 6890 gas chromatograph equipped with a flame ionisation detector and a HP 7673 GC/SFE auto-injector. The column used was BPX-5 (50 m length x 0.22 µm ID x 0.25 µm film thickness) (SGE Scientific, Melbourne, Australia). The injection volume was 2 µL, inlet temperature and pressure were 280 °C and 20 psi respectively, carrier gas was H₂ (40 mL/min.), split ratio was 1:10, detector temperature at 280 °C, and the oven program was: initial temperature 60 °C for 5 min., increased to 180 °C at 4°C/min., final temperature maintained for 5 min.

3.2.8 Software used for data processing

Data was processed using HP Chemstation® software (version A 3.0.1, Hewlett Packard, Melbourne Australia).

3.3 Results and Discussion

3.3.1 Choice of solvent

As the GC traces in Figure 3.1(a-c) demonstrate, addition of water was not required during the analysis of the dichloromethane extracts of material from the plant species investigated in this study (i.e., *S. officinalis, O. minimum, M. alternifolia*). The obtained baseline for all these species did not exhibit the distortion
shown by the baseline of the *Cryptocarya microneura*\(^1\) extract (Figure 3.1d) possibly due to absence or trace presence of high-boiling, polar compounds. The distortion of the baseline that results from these broad peaks (indicated by the arrow in Figure 3.1d) affects adversely the quantitation of the peaks eluting within the distorted part of the baseline. Addition of water just before analysis is required in such cases (Cornwell, 1999).

### 3.3.2 Length of extraction

A slightly shorter time-period (24 hours) instead of that recommended (i.e. 30 hours, section 3.1.3) was employed during the small-scale dichloromethane extraction of leaf-material in this study. Statistical analysis showed no significant differences (Kruskal–Wallis test at \(p \leq 0.05\), \(n=8\)) between the two (24 and 30 hours) extraction periods in relation to **oil-composition** after extraction of “identical” *M. alternifolia* (terpinen-4-ol chemotype) leaves.

The **oil-concentration** values obtained in this project are likely to be slightly smaller than the “true” values due to the shorter extraction. However, this does not affect the determination of treatment-induced trends over time considering that controls and samples were (strictly) subjected to the same length of extraction (24 hours). The detection of differences in leaf-oil concentration and composition before and after treatment rather than exhaustive extraction was the purpose of this study.

---

\(^1\) Family **Laureceae**, used for comparison.
Figure 3.1. Sesquiterpene-region traces from the dichloromethane extracts of leaf-material from a) S. officinalis, b) O. minimum, c) M. alternifolia and d) Cryptocarya microneura (family Lauraceae; given for comparison). As can be seen, the traces from the species of interest (a-c) do not exhibit the effect shown by the arrow in trace d).
3.3.3 Internal standard used

Tridecane was found to be unsuitable for use as an internal standard during this study. As shown in Figure 3.2, under the analytical conditions used, tridecane eluted very close to bornyl acetate (a component found in the extracts from S. officinalis and O. minimum). Peak overlapping of the two would have had an adverse affect on quantitation as peak-integration would then be less accurate.

**Figure 3.2.** Traces of solvent extracts from a) *M. alternifolia* flush growth with tridecane as internal standard and b) *S. officinalis* leaves with octane as internal standard. The very close elution of tridecane and bornyl acetate is evident.
Octane (C₈) was employed as an alternative internal standard. Octane eluted before all peaks of interest (Figure 3.2) and, in contrast to tridecane, exhibited good reproducibility (seen from the low RSD values in Figure 3.3) and recovery for both, flush and mature, *M. alternifolia* leaves (Figure 3.3).

![Bar chart](image)

**Figure 3.3.** Recovery (mean ± std, n=8) of octane and tridecane after the 24-hour extraction of flush and mature *M. alternifolia* leaves. Tridecane exhibits not only more than 100 % recovery but also poor reproducibility (seen from the high RSD values).

The reason behind the 110 % recovery obtained for tridecane is not known. Possible interpretations may include co-elution with a component present in the *M. alternifolia* extract or interference by “endogenous” tridecane found on the leaves.
3.3.4 GC reproducibility

The repetitive injections of the two different *M. alternifolia* extracts showed that the instrument used introduced negligible errors to the analysis (Table 3.1).

**Table 3.1.** Results obtained from the repetitive injection of two different *M. alternifolia* leaf-extracts. As can be seen the errors introduced to the results by the system are negligible for all components.

<table>
<thead>
<tr>
<th>Components</th>
<th>Percentage (%) composition (mean ± std, n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample 1</td>
</tr>
<tr>
<td>α-thujene</td>
<td>0.23±0.02</td>
</tr>
<tr>
<td>α-pinene</td>
<td>2.01±0.04</td>
</tr>
<tr>
<td>cluster 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.88±0.13</td>
</tr>
<tr>
<td>α-terpinene</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>cluster 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.46±0.04</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>3.96±0.09</td>
</tr>
<tr>
<td><em>E</em>-sabinene hydrate</td>
<td>8.05±0.04</td>
</tr>
<tr>
<td>terpinolene</td>
<td>0.09±0.00</td>
</tr>
<tr>
<td>Z-sabinene hydrate</td>
<td>50.5±0.30</td>
</tr>
<tr>
<td><em>E</em>-p-menth-2-en-1-ol</td>
<td>0.74±0.03</td>
</tr>
<tr>
<td><em>Z</em>-p-menth-2-en-1-ol</td>
<td>0.28±0.01</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>6.46±0.11</td>
</tr>
<tr>
<td>α-terpineol</td>
<td>1.36±0.07</td>
</tr>
<tr>
<td><em>E</em>-piperitol&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.03±0.04</td>
</tr>
<tr>
<td>sesquiterpenes</td>
<td>12.6±0.20</td>
</tr>
</tbody>
</table>
| Oil concentration (mean±std, n=8) (µg g<sup>-1</sup> dry weight) | 1451±35.4 | 931±20.4

<sup>a</sup> cluster 1 includes sabinene, β-pinene and myrcene
<sup>b</sup> cluster 2 includes p-cymene, limonene, β-phellandrene and 1,8-cineole
<sup>c</sup> tentative identification

3.3.5 Reliability of the extraction process

The reproducibility of the extraction process itself could not be determined due to the existing inter-leaf variation (discussed in Chapter 2). However, the results obtained when the extraction process was applied on *M. alternifolia* leaves (of variable age) indicate its reliability as it was able to detect the conversion of thujanes
to \( p \)-menthanes (Figure 3.4), a well-documented process operating during the aging of \textit{M. alternifolia} immature leaves (Southwell and Stiff, 1989; Cornwell \textit{et al.}, 1995; Corwell 1999).

![Graph](image)

**Figure 3.4.** Relationship between thujanes (sabinene, \( E \)- and \( Z \)-sabinene hydrate) and \( p \)-menthanes (\( \alpha \)- and \( \gamma \)-terpinene, terpinolene, terpinen-4-ol) in \textit{M. alternifolia} leaves as obtained by solvent extraction (n=11).

As can be seen in Figure 3.4, the extraction process was able to demonstrate with confidence (\( R^2 = 0.9907 \)) the reciprocal relationship between these two structural groups and confirm the results produced by previous studies (Southwell and Stiff, 1989; Cornwell \textit{et al.}, 1995; Corwell 1999).
3.4 Conclusion

Small-scale solvent extraction is a simple and inexpensive approach for the analysis of volatiles from leaves of terpene-producing plants. The process, as employed in this study, used dichloromethane (as solvent) spiked with octane (as the internal standard) and extraction was performed over 24 hours.

The above parameters were selected because they were found to be sufficient for the purposes of the study (i.e. demonstration of changes over time) and were able to produce reliable results.
REFERENCES


CHAPTER 4

ANALYSIS OF TERPENOIDS IN THE GAS PHASE BY SOLID PHASE MICROEXTRACTION (SPME)

Solid phase microextraction (SPME) is a rapid, solvent-free sample preparation technique developed by Belardi and Pawliszyn for the purpose of extraction of organic pollutants from water bodies (Louch et al., 1992; Schäfer et al., 1995). SPME utilises a small fused-silica fibre usually coated with a polymeric phase to absorb/adsorb the analytes of interest until the system reaches equilibrium (Pawliszyn, 1997). After exposure into the sample the fibre is then thermally desorbed in a split/splitless injection port of a gas chromatograph.

Since its commercial release SPME has become very popular technique employed by scientists across many fields (e.g. Louch et al., 1992; Pawliszyn, 1997; Yang and Peppard, 1994; Zabaras et al., 1999).

PART 1: SPME FIBRE SELECTION

4.1 Introduction

Selection of the type of sorptive fibre used during solid phase microextraction (SPME) is critical, especially for a quantitative analysis, as the selectivity of the fibre coating can affect the detection limit (Louch et al., 1992; Pawliszyn, 1997). In this case the relatively apolar nature of most terpenes indicated that a non-polar (poly)dimethylsiloxane (PDMS)-coated fiber would be more suitable than one employing a polar polymer such as Polyacrylate or Carbowax and would minimize selectivity effects and ensure maximum sensitivity (Schäfer et al., 1995). Initially the

\[1\text{The 75 \( \mu m \) PDMS-divinylbenzene fibre is now also known to be suitable for terpenoid analysis (Zini et al., 2001).}\]
newly marketed (at that time) 75 μm Carboxen-PDMS coated fibre was preferred over the plain PDMS coating due to its bipolar nature (Alpendurada, 2000). However, evidence presented below shows that the Carboxen-PDMS coating was unsuitable for the quantitative determination of some terpenoids and exhibits major disadvantages, such as \( p \)-menthane rearrangement (section 4.2) and \( p \)-cymene peak tailing (section 4.3), when compared to the plain PDMS coating.

4.2 Rearrangement of \( p \)-menthane terpenes by Carboxen during headspace (HS)-SPME

4.2.1 Theoretical background

A large number of SPME fibre-coatings are commercially available and they can be classified into a) pure polymer coatings (e.g., PDMS) and b) mixed coatings (e.g., Carboxen-PDMS) (Górecki et al., 1999; Mani, 1999). PDMS is a non-polar high viscosity rubbery liquid that extracts analytes via absorption (Górecki et al., 1998). In contrast, Carboxen is a solid porous polymer that has an even distribution of micro- (2-20 Å diameter), meso- (20-500 Å) and macropores (>500 Å) and extracts analytes via adsorption (Górecki et al., 1999; Mani, 1999).

The Carboxen-PDMS coating consists of Carboxen particles blended in the PDMS phase and analytes are primarily extracted via adsorption (Górecki et al., 1999; Mani, 1999) although some absorptive processes must still apply to the coating since PDMS is the supporting phase. This bipolar coating exhibits higher distribution constants and thus better sensitivity than PDMS in relation to polar analytes (Alpendurada, 2000) but can also extract non-polar analytes at levels comparable to those obtained by the PDMS coating. The above characteristics and the relatively recent commercial release of the Carboxen-PDMS coating (Mani, 1999) are
responsible for the recent steady increase in reported usage (e.g., Popp and Paschke, 1997; Achten and Puttmann, 2000; Marsili, 2000; Peres et al., 2001).

Preliminary experiments in our laboratory showed that an abnormally high concentration of \( p \)-cymene (Figure 4.1(1)) was obtained at the expense of other \( p \)-menthane monoterpenoid hydrocarbons such as terpinolene (Figure 4.1(2)), \( \alpha \)- (Figure 4.1(3)) and \( \gamma \)-terpinene (Figure 4.1(4)), during attempts to quantitatively determine the headspace composition above *Melaleuca alternifolia* (Australian Tea Tree) leaves using the Carboxen-PDMS coating.

**Figure 4.1.** Structures of monoterpenoids involved in this investigation. 1. \( p \)-cymene (\( p \)-menthane group); 2. terpinolene (\( p \)-menthane group); 3. \( \alpha \)-terpinene (\( p \)-menthane group); 4. \( \gamma \)-terpinene (\( p \)-menthane group); 5. \( \alpha \)-phellandrene (\( p \)-menthane group); 6. \( E \)-sabinene hydrate (thujane group); 7. 1,8-cineole (\( p \)-menthane group); 8. terpinen-4-ol (\( p \)-menthane group); 9. linalool (acyclic geraniolane group); 10. \( \alpha \)-pinene (pinane group); 11. \( \alpha \)-terpineol (\( p \)-menthane group).
This section investigates the reasons for this abnormality using a group of 10 common monoterpenoids spanning several structural groups (Figure 4.1).

4.2.2 Materials and Methods

4.2.2.1 Authentic standards

High purity standards for monoterpenoids α- and γ-terpinene, terpinolene, p-cymene, terpinen-4-ol, α-terpineol, linalool, α-pinene, 1,8-cineole, α-phellandrene were obtained commercially (Sigma-Aldrich or Fluka Chemicals, Castle-Hill NSW, Australia). *E*-Sabinene hydrate was synthesised (Cornwell, 1999) and kindly provided by Dr. C.P. Cornwell.

4.2.2.2 Sample preparation

A small volume (5 μL) of each monoterpenoid was mixed with water (8 mL) and the mixture was vortexed for 5 min. Aliquots (10 μL) of the mixture were then injected into pre-conditioned (300 °C for 2 hrs) glass vials (10 mL). The vials were left to equilibrate for 30 min. at room temperature and then HS-SPME and static HS was carried out as described below (section 4.2.2.3).

4.2.2.3 HS-SPME

A manual SPME holder (Supelco, USA) equipped with a 100 μm PDMS or a 75 μm Carboxen-PDMS fiber (Supelco, USA) was used. Fibre exposure time was 5 min for both fibres although much shorter times (2-15 sec) were employed during the establishment of absorption/adsorption profiles for the analytes of interest. Thermal desorption (220 °C for 10 minutes) of the fiber in the split/splitless injection port of the GC system (as per section 4.2.2.5) followed. Before use the fibres were conditioned according to the manufacturer's instructions (Appendix 4.1).

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2 now at Australian Botanical Products, Hallam, Victoria, Australia.
4.2.2.4 Static HS

A gas-tight Hamilton TLL (10 mL) syringe (Supelco, USA) was used to inject 3 mL of the HS withdrawn from the sample vials (prepared as per section 4.2.2.2) into the split/splitless injection port of the GC system.

4.2.2.5 GC analysis

GC analysis was carried out using a HP 6890 gas chromatograph equipped with a FID. The column used was BPX-5 (50 m length x 0.22 µm ID x 0.25 µm film thickness) (SGE Scientific, Melbourne, Australia) and the oven program was initial temperature 60 °C for 5 min., increased to 150 °C at 6 °C/min., final temperature maintained for 5 min. The inlet temperature and pressure were 280 °C and 20 psi respectively, carrier gas was H₂ (40 mL/min.), split ratio was 1:50, detector temperature at 280 °C.

4.2.2.6 Moisture determination

The absorption/adsorption of moisture by the PDMS and Carboxen-PDMS coatings was determined by exposing the pre-conditioned fibres to vials (10 mL) filled with air for 5 min. Desorption of the fibres into the split/splitless injection port of the IT-GC-MS system followed. Moisture levels were determined by the monitoring of ion m/z 18.

4.2.2.7 Supporting experiment

A small volume (5 µL) of α-phellandrene was mixed with water (8 mL) and the mixture was vortexed for 5 min. Aliquots (10 µL) of the mixture were then injected into warm (~60 °C) glass vials (10 mL) containing anhydrous Na₂SO₄ (2 g). HS-SPME was then carried out as described in section 4.2.2.3 but in this case extra care was taken to keep the fibre free of moisture (desorbed in the hot GC inlet and
immediately exposed to the sample. GC analysis followed (section 2.5). The process was repeated without anhydrous Na₂SO₄ and the results were compared (n=3).

4.2.2.8 Ion trap gas chromatography-mass spectrometry (IT-GC-MS)

The instrument used was a Varian 3800 gas chromatograph connected to a Varian 2000 ion trap detector (1 scan/sec, 20 μA current) operating in the electron ionization mode. The column used was CP-Sil 8CB (30 m length x 0.25 μm ID x 0.25 μm film thickness) (Chrompack, USA). The inlet temperature and pressure were 220 °C and 10 psi respectively, carrier gas was He, split ratio was 1:20, detector temperature at 260 °C, and the oven program was: initial temperature 50 °C, increased to 180 °C at 6 °C/min.

4.2.3 Results-Discussion

An example of the abnormally high concentrations of p-cymene obtained when the Carboxen-PDMS coating was used to quantitatively determine the headspace composition above _M. alternifolia_ leaves is shown below (Figure 4.2).

Ten common monoterpenoids representing various structural and functional groups were used to investigate the rearrangement effect of the Carboxen-PDMS coating (Figure 4.1). By employing such analyte diversity the specificity of the effect could be established.
**Figure 4.2.** GC traces (monoterpene region) obtained by HS-SPME above Australian Tea Tree leaves, a) PDMS and b) Carboxen-PDMS coating. The higher level of p-cymene (2) at the expense of terpinolene (4), α- (1) and γ-terpinene (3) is evident in b) when compared to a).

Table 4.1 presents the ratio of p-cymene/analyte obtained by HS-SPME and static HS for each monoterpene investigated. p-Cymene was detected in the headspace of p-menthane hydrocarbons (α- and γ-terpinene, α-phellandrene, terpinolene) but it was not found in the headspace of any of the other monoterpenoids tested including terpinen-4-ol (p-menthane alcohol, Figure 4.1 (8)). This indicates the high specificity of the rearrangement process for p-menthane hydrocarbons. For the p-menthane hydrocarbons the Carboxen-PDMS coating gave a
very high p-cymene/analyte ratio compared to that obtained from the PDMS coating (between 76% - 843% more) (Table 4.1).

The original purpose of the static HS measurements was to provide a measure of the "endogenous" levels of p-cymene present in the samples. It was expected that the levels of p-cymene in the samples obtained by static HS should be less or at most, equal to those obtained by HS-SPME. As Table 4.1 illustrates this was not the case; the levels of p-cymene as measured by static HS were much higher than those from HS-SPME with PDMS coating. Analyte rearrangement occurring during the static HS process (e.g., interaction with the walls of the syringe) or during injection in the hot GC inlet is likely to be the cause of these unexpected results.

**Table 4.1.** p-Cymene/analyte ratios (%) of 10 common monoterpenoids as obtained by HS-SPME (PDMS and Carboxen-PDMS coatings) and static HS. n/d indicates no detection of p-cymene.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>p-cymene/analyte (%) (mean ± std, n=5)</th>
<th>Rearrangement ratio(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Static HS</td>
<td>Carboxen-PDMS</td>
</tr>
<tr>
<td>(\alpha)-terpinene</td>
<td>7.3 ±0.3</td>
<td>9.0±1.0</td>
</tr>
<tr>
<td>(\gamma)-terpinene</td>
<td>2.5±0.2</td>
<td>15.1±0.8</td>
</tr>
<tr>
<td>(\alpha)-phellandrene</td>
<td>19.8±2.5</td>
<td>10.9±1.8</td>
</tr>
<tr>
<td>terpinolene</td>
<td>0.6±0.1</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>(E)-sabinene hydrate</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>(\alpha)-terpineol</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>(\alpha)-pinene</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>linalool</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>n/d</td>
<td>n/d</td>
</tr>
</tbody>
</table>

\(^a\) Values in this column obtained from Carboxen-PDMS (%) divided by PDMS (%) values.

\(^b\) A value of 1 indicates no rearrangement.
To ensure that the large difference between the $p$-cymene/analyte ratios obtained by the PDMS and those obtained by the Carboxen-PDMS coating (Table 4.1) did not originate from differences in compound affinities, the absorption (PDMS) and adsorption (Carboxen-PDMS) rates of the 4 $p$-menthane hydrocarbons and $p$-cymene were determined for each coating (Table 4.2). This was achieved by the establishment of the absorption and/or adsorption profiles of each compound on the two coatings followed by the calculation of the slopes of the linear section of the absorption/adsorption lines (as per Appendix 4.2).

**Table 4.2.** Absorption and/or adsorption rates of the monoterpene of interest on the PDMS and Carboxen-PDMS coatings. To simplify comparison with the rearrangement ratio presented in Table 3.1 the ratio obtained from the $p$-cymene rate/analyte rate values for each coating is also given.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Rate (pA's/sec)</th>
<th>$p$-cymene rate/analyte rate</th>
<th>Ratio$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carboxen-PDMS</td>
<td>PDMS</td>
<td>Carboxen-PDMS</td>
</tr>
<tr>
<td>$\alpha$-terpinene</td>
<td>32</td>
<td>25</td>
<td>53.4</td>
</tr>
<tr>
<td>$\gamma$-terpinene</td>
<td>52</td>
<td>48</td>
<td>17.3</td>
</tr>
<tr>
<td>$\alpha$-phellandrene</td>
<td>18</td>
<td>17</td>
<td>50.3</td>
</tr>
<tr>
<td>terpinolene</td>
<td>283</td>
<td>300</td>
<td>3.2</td>
</tr>
<tr>
<td>$p$-cymene</td>
<td>909</td>
<td>933</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$^a$ Values in this column obtained from Carboxen-PDMS / PDMS values within the $p$-cymene rate / analyte rate column.

Table 4.2 shows that the absorption and adsorption rates calculated from these slopes for each compound of interest are quite similar between the two coatings resulting in ratio values close to one. This indicates that the much higher $p$-cymene levels obtained by the Carboxen-PDMS versus those from the PDMS coating (Table 4.1) are not due to differences in affinities towards the analytes of interest.
The similarity in the rate of sorption of the various analytes on the two coatings (Table 4.2) is surprising given that adsorption and absorption mechanisms operate in the Carboxen-PDMS when compared to the sole absorption mechanism of the PDMS coating (Górecki et al., 1998; Mani, 1999). Theoretically, this difference in the sorption mechanism should result in differential behaviour of the two coatings toward the various analytes depending on their physical and chemical characteristics. This would then be expressed by dissimilar sorption rates. However, as Table 4.2 shows this was not the case. A possible interpretation for this is that the diffusion of the analyte through the PDMS layer (i.e. absorption) of the Carboxen-PDMS coating is the slower and therefore rate-determining step of the sorption process as compared to the adsorption of the analyte by the Carboxen particles. As a result, the sorption rates for both, PDMS and Carboxen-PDMS coatings, were found to be similar (Table 4.2).

The dehydrogenation of $p$-menthane hydrocarbons to $p$-cymene is a process occurring extensively in poorly stored plant essential oils especially those rich in $p$-menthanes such as Australian Tea Tree oil (Southwell, 1988; 1999) (Figure 4.3). Moisture is known to be one of the factors that "catalyse" the process (Southwell, 1999). Thus, it was of interest to test whether the $p$-menthane rearrangement caused by the Carboxen-PDMS coating was related to moisture absorption/adsorption during HS-SPME. Figure 4.4 shows results obtained from exposing both coatings in laboratory air for 5 min followed by monitoring of their water content by IT-GC-MS. As can be seen, Carboxen-PDMS adsorbs 10.5 times more moisture than the PDMS coating.
**Figure 4.3.** Rearrangement of the menthadienes terpinolene (2), α- (3) and γ-terpinene (4), and α-phellandrene (5) to p-cymene (1).

**Figure 4.4.** Absorption and adsorption of moisture by the two SPME coatings (mean ± std, n=3). Each coating was exposed to 10 mL vials filled with laboratory air for 5 min. An old SPME needle with the attachment-tubing intact but without a fibre was used to mimic fibre insertion into the hot GC-MS inlet (control).
These results support the hypothesis that the rearrangement of the \( p \)-menthane hydrocarbons by the Carboxen-PDMS coating is associated with moisture adsorbed by the polar Carboxen during HS-SPME.

Figure 4.5 below shows that the level of analyte (\( \alpha \)-phellandrene used as an example) rearranged by the Carboxen-PDMS coating to \( p \)-cymene does not increase when longer sampling time is used. This indicates that the rearrangement is likely to occur during the desorption of the coating in the hot GC inlet and not during the sorption of the analytes on the coating. If the later was true, then the extent of rearrangement would be greater with longer sampling time.

**Figure 4.5.** Effect of sampling time on the level of rearrangement of \( \alpha \)-phellandrene by the Carboxen-PDMS coating. As it can be seen the extent of rearrangement is independent from the sampling time used.
The presented evidence suggests the Carboxen-PDMS coating is not suitable for HS-SPME of samples associated with moisture such as fresh plant material especially when the analytes of interest are \(p\)-menthane hydrocarbons. Results obtained from an experiment designed to test this hypothesis (Figure 4.6, below) confirmed that Carboxen-PDMS will readily rearrange \(p\)-menthane hydrocarbons when moisture is present in the sample; the extent of rearrangement was notably reduced when anhydrous Na\(_2\)SO\(_4\) was used in an attempt to minimise the level of moisture available in the sample.

**Figure 4.6.** Extent of rearrangement (mean ± std, \(n=3\)) of \(\alpha\)-phellandrene by the Carboxen-PDMS coating in the presence and absence of anhydrous Na\(_2\)SO\(_4\) from identical samples. The difference is evident.
4.3 *p*-Cymene peak tailing

The chromatographic efficiency of an analytical separation resulting from SPME sample introduction depends on rapid sample desorption from the fibre. If this is not achieved, peak tailing is observed in the chromatogram. The extent of peak tailing can be determined; the U.S. Pharmacopoeia recommends that the *tailing factor* (TF) value of a peak is used to express the magnitude of the effect (Felinger, 1998).

*p*-Cymene peak tailing is another disadvantage of the Carboxen-PDMS coating compared to the PDMS coating. To demonstrate the effect the two coatings were exposed for 10 min to identical samples of *p*-cymene (prepared as outlined in section 4.2.2.2) and desorbed for 10 min. by insertion into the hot injection port of the GC-FID system (section 4.2.2.5). The process was repeated for desorption temperatures recommended by the manufacturer 200-280 °C for PDMS, 220-300 °C for Carboxen-PDMS. The tailing factor (TF) value of each peak was then calculated (as per Appendix 4.3) and the values obtained were plotted in relation to the desorption temperature (Figure 4.7). As can be seen from Figure 4.7, the Carboxen-PDMS coating exhibits much higher TF values than the PDMS coating especially within the critical, for quantitative desorption of terpenoids, temperature range (240-280 °C) (Schäfer *et al*., 1995).
The shape of the pores in a carbon matrix can affect the rate of analyte desorption (Mani, 1999). In relation to Carboxen matrix, slow desorption of molecules (and thus peak tailing) is known to occur especially for analytes trapped in its meso- and macropores (Mani, 1999).

Although peak tailing was not observed for any of the other terpenoids encountered in this study, $p$-cymene peak tailing can be a problem particularly in quantitative analyses involving terpenoids with close retention times to that of $p$-cymene (e.g., limonene and $\beta$-phellandrene for a non-polar phase column (Adams, 1995) due to peak overlapping. Integration (manual or automated) of overlapping peaks is a difficult and time-consuming process that requires high levels of expertise if reliable results are to be obtained.
4.4 Film-thickness of the PDMS fibre used

In addition to the type of sorptive fibre used, the volume of coating (i.e. thickness of the fiber) is another factor that has an effect on the detection limit and thus need to be considered during quantitative HS-SPME.

In this study the 100 μm-thick fibre was chosen because all analytes of interest were lower molecular weight compounds, such as mono- and sesquiterpenes, where the 100 μm coating ensured higher adsorption capacity and therefore lower detection limits than the 30 μm or 7 μm PDMS coating, without a significant increase of the enrichment time required (Louch et al., 1992).

4.5 Conclusion

The specific, terpene-related problems associated with the Carboxen-PDMS coating (i.e. p-menthane monoterpen rearrangement and p-cymene peak tailing) and some other more generalised criticisms about its poor reproducibility (Popp and Paschke, 1997) meant that the Carboxen-PDMS fibre was unsuitable for use during this study, and more generally, for analysis of p-menthane-containing terpenoid mixtures. As a result the PDMS-coated fibre was the one selected and used for all further work.
PART 2: SPME METHOD DEVELOPMENT

4.6 Introduction

This section presents the development of solid phase microextraction methods for a) the quantitative determination of terpenoids in the gas phase and b) the detection of terpene-synthase products in cell-free enzyme preparations. These methodologies are simpler and less expensive alternatives to existing approaches and although they were developed for the analytes of interest to this study, can be readily adjusted to suit any volatile organic compound.

4.7 Quantitative analysis of terpenoids in the gas phase using HS-SPME

4.7.1 Theoretical background

Dynamic or purge-and-trap headspace analysis was the method of choice for workers in the field of "scent-chemistry" for many years (review by Raguso and Pellmyr, 1998) but lately the use of SPME in plant headspace studies has been a popular alternative (e.g., Schäfer et al., 1995; Field et al., 1996; Vercammen et al., 2000; Zini et al., 2001).

Quantitative SPME of compounds in the gas phase is possible using external calibration, standard addition or the partition coefficient $K_{fg}$ of an analyte between the fibre coating and the gas phase (Pawliszyn, 1997). The latter option was considered to be the most practical given that authentic standards of many terpenoids are very costly or they are not commercially available.

Recently it has been shown that there is a linear relationship between log $K_{fg}$ and the Linear Temperature Programmed Retention Index (LTPRI) at least for alkanes and aromatic hydrocarbons (Equation 1) (Martos et al., 1997):
\[
\log K_{fg} = \alpha (\text{LTPRI}) + \beta
\]  
Equation 1

where \(\alpha\) is the slope and \(\beta\) is the intercept. This relationship is valid for all compounds in the gas phase as long as the fibre coating is the same as the coating of the column used to obtain the LTPRI values (Pawliszyn, 1997). Once the \(K_{fg}\) value for an analyte is known then its concentration in the headspace can be determined by Equation 2 (Pawliszyn, 1997):

\[
C_{\text{headspace}} = \frac{C_{\text{fiber}}}{K_{fg}}
\]  
Equation 2

where \(C_{\text{headspace}}\) and \(C_{\text{fiber}}\) are the analyte concentrations in the headspace and the fibre coating, respectively. \(C_{\text{fiber}}\) can be determined readily by measuring the analyte quantity extracted onto the fibre coating provided that the volume of the coating used is known.

To establish a relationship between \(\log K_{fg}\) and LTPRI for a particular category of organic compounds, \(K_{fg}\) must be experimentally measured for a selected set of model compounds. This is usually carried out by performing HS-SPME and static headspace analyses on identical samples under experimental conditions similar to those used during analysis of the sample of interest. \(K_{fg}\) is then determined according to Equation 3 (Matich, 1999):

\[
K_{fg} = \frac{(A_f V_g)}{(A_g V_f)}
\]  
Equation 3

where \(A_f\) and \(A_g\) are the peak areas obtained by HS-SPME and static HS and \(V_f\) and \(V_g\) are the volumes of the fibre coating and the static HS injected into the GC, respectively.

However, it should be noted that Equations 2 and 3 can be used for the determination of \(K_{fg}\) only for systems at equilibrium (Pawliszyn, 1997) and produce reliable results only when the volume of the vial used during the measurement of the \(K_{fg}\) is relatively large (Górecki and Pawliszyn, 1997; Górecki et al., 1998).
The following section establishes the relationship between log $K_{fg}$ and LTPRI for terpenoids, using a group of common monoterpenoids. The calculated and experimentally measured $K_{fg}$ values are then compared for the same group of monoterpenoids and the $K_{fg}$ values for another 37 plant-derived essential-oil constituents are calculated. The calculated $K_{fg}$'s will be used to quantify mechanical wounding-induced differences in the headspace profile above intact-plant leaves in a subsequent part of this thesis (Chapter 6).

4.7.2 Materials and Methods

4.7.2.1 $K_{fg}$ determination

Leaves (~30 g in total) from two oil-bearing plants (*Melaleuca alternifolia* Cheel or Australian tea tree and *Salvia officinalis* L. or common sage) were placed in a 500 mL glass flask capped with a pre-drilled Teflon cap equipped with a septum. The flask was left to equilibrate for at least 1 hour at 25 °C before HS-SPME and static HS sampling. Three samples were used for the determination of each $K_{fg}$ value.

4.7.2.2 Fibre absorption profiles

The fibre absorption profile for each terpenoid was determined by varying the exposure of the fibre to the sample (5, 10, 30 and 60 min.). The same sample was used for all exposure periods but the sample was left to equilibrate for 1 hour before each re-sampling.

4.7.2.3 HS-SPME

A manual SPME holder (Supelco, USA) equipped with a 1 cm-long polydimethylsiloxane (100 μm PDMS)-coated fibre (Supelco, USA) was used with a sampling time of 1 hour. Thermal desorption (260 °C for 10 minutes) of the fibre in the split (1:10) injection port of the GC/GC-MS system followed. Before and
between sampling the fibre was conditioned according to the manufacturer's instructions (Appendix 4.1).

4.7.2.4 Static HS

A 10 mL Hamilton TLL gastight syringe (Supelco) was used to inject part of the sample headspace (10 mL) into the split/splitless injection port of the GC.

4.7.2.5 External calibration

Solutions of varying concentrations (20-1000 ng) of the monoterpene \( \alpha \)-pinene and the sesquiterpene E-\( \beta \)-caryophyllene in \( \text{CH}_2\text{Cl}_2 \) were used to construct calibration curves used for the determination of the analytes (each solution injected in triplicate). The curve from \( \alpha \)-pinene was used for all monoterpennoids and that of E-\( \beta \)-caryophyllene for the sesquiterpenoids.

4.7.2.6 GC analysis

GC analysis was carried out using a HP 6890 gas chromatograph equipped with a flame ionization detector and a HP 7673 GC/SFE auto-injector. The stationary phase of the column used contained 5% phenyl-PDMS and 95 % PDMS (BPX-5, 50 m length x 0.22 µm ID x 0.25 µm film thickness) (SGE Scientific, Melbourne, Australia) and the oven program was initial temperature 60 °C for 5 min., increased to 180 °C at 4 °C/min., final temperature maintained for 5 min. The inlet temperature and pressure were 240 °C and 20 psi respectively. Carrier gas was \( \text{H}_2 \) (40 mL/min.), split ratio was 1:10, detector temperature 280 °C. A BPX-5 column was employed instead of a BP-1 (100 % PDMS phase) because it provided a better separation of the terpenoids involved in this study.

4.7.2.7 GC-MS analysis

The instrument used was a Varian 3800 gas chromatograph connected to a Varian 2000 ion trap detector (0.9 scans/sec, 20 µA current) operating in the electron
ionization mode. The column used was CP-Sil 8CB (30 m length x 0.25 μm ID x 0.25 μm film thickness) (Chrompack, USA). The inlet temperature and pressure were 220 °C and 10 psi respectively, carrier gas was He, split ratio was 1:10, detector temperature at 260 °C, and the oven program was: initial temperature 60 °C, increased to 180 °C at 6 °C/min.

4.7.3 Results-Discussion

The determination of the experimental $K_{fb}$ values was carried out using plant material instead of standards to ensure that conditions (e.g., matrix effects) are identical to those encountered during actual sampling. HS-SPME followed by static HS was applied to each (and the same) sample. The large volume of the flasks (500 mL), and the relatively large amount of leaves used, ensured that the depletion of the headspace by HS-SPME sampling was negligible and therefore the static determination of the headspace was not affected. Table 4.3 shows that the difference between two successive SPME determinations from the same sample for all monoterpenoids was within the typical error expected in any SPME analysis (5 %) (Pawliszyn, 1997).

Since the relationship between $\log K_{fb}$ and LTPRI values (Equation 3) is valid only under equilibrium conditions (Pawliszyn, 1997; Matich, 1999), the absorption profiles of selected terpenoids were determined to establish appropriate sampling protocols. Figure 4.8 (below) shows that the monoterpenoids but not the sesquiterpenoids reach equilibrium between matrix, gas phase and the fibre-coating within the 60 minute HS-SPME sampling time employed during the determination of experimental $K_{fb}$'s.
TABLE 4.3. Effect of an initial HS-SPME exposure on the headspace concentration of samples similar to those used for the determination of the $K_{fg}$ values. R.S.E. is the relative standard error between the two means.

<table>
<thead>
<tr>
<th>Monoterpeneid</th>
<th>FID Response (pA's)$^a$ (mean, n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1$^{st}$ exposure</td>
</tr>
<tr>
<td>$\alpha$-thujene</td>
<td>40.6</td>
</tr>
<tr>
<td>$\alpha$-pinene</td>
<td>69.1</td>
</tr>
<tr>
<td>$\beta$-pinene</td>
<td>20.6</td>
</tr>
<tr>
<td>myrcene</td>
<td>41.9</td>
</tr>
<tr>
<td>$\alpha$-phellandrene</td>
<td>14.1</td>
</tr>
<tr>
<td>$\alpha$-terpinene</td>
<td>333.7</td>
</tr>
<tr>
<td>$p$-cymene</td>
<td>111.3</td>
</tr>
<tr>
<td>limonene</td>
<td>47.4</td>
</tr>
<tr>
<td>$\beta$-phellandrene</td>
<td>15.9</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>56.9</td>
</tr>
<tr>
<td>$\gamma$-terpinene</td>
<td>605.3</td>
</tr>
<tr>
<td>terpinolene</td>
<td>104.6</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>290.7</td>
</tr>
</tbody>
</table>

$^a$ pA stands for pico ($10^{-12}$) Amps.

Other studies have shown that sesquiterpenoids require a very long time to reach equilibrium (Field et al., 1996; Matich et al., 1996). This is expected for compounds that exhibit low vapour pressure in combination with high $K_{fg}$ values (Pawliszyn, 1997). During HS-SPME such compounds present in the gas phase are absorbed by the fibre coating at a much faster rate than their release from the matrix thus a long time is required to reach equilibrium (Pawliszyn, 1997). In addition, sesquiterpenoids are expected to have very large $K_{fg}$ values based on their long retention time on PDMS-coated gas chromatography capillary columns. The experimental determination of $K_{fg}$ for such compounds is likely to be erratic due to their semi-volatile nature and their ability to sorb at all surfaces with which they come into contact (Górecki et al., 1997).
**Figure 4.8.** Absorption profile (mean ± standard error, n=3) of selected mono- (α-pinene, α-terpinene) and sesquiterpenoids (α-gurjunene, viridiflorene) obtained with the 100 μm PDMS-coated SPME fibre (25 °C).

The experimentally determined $K_{fg}$ values of ten monoterpane hydrocarbons and one monoterpane ketone were used to construct a regression plot against their LTPRI values (Figure 4.9) calculated using homologous $n$-alkanes (C₈-C₁₇) as advised by Martos et al. (1997). These monoterpenoids were chosen because the precision in the experimental determination of their $K_{fg}$'s was deemed to be satisfactory (RSD below 10 %, n=3).
**Figure 4.9.** Regression plot of $\log K_{fg}$ experimentally determined against LTPRI for selected monoterpenoids (for PDMS-coated SPME fibre, 25 °C). Compounds used included $\alpha$-thujene, $\alpha$-pinene, camphene, sabinene, $\beta$-pinene, myrcene, $\alpha$-terpinene, limonene, $\gamma$-terpinene, terpinolene and $\alpha$-thujone.

The regression plot between $\log K_{fg}$ and LTPRI (Figure 4.9) yields a line with a slope $\alpha$ of 0.0044 and an intercept $\beta$ of 0.2741 ($r^2 = 0.9917$). When these values are substituted in Equation 1, an equation that describes a relationship between $\log K_{fg}$ and LTPRI for terpenoids can be derived:

$$\log K_{fg} = 0.0044 \times \text{(LTPRI)} + 0.2741 \quad \text{Equation 4}$$

The above equation was then used to calculate the $K_{fg}$ values of the same monoterpenoids (including those not used in the regression plot) based on their LTPRI values. These calculated values were then compared to values experimentally determined by HS-SPME and static-HS techniques (Table 4.4).
As can be seen from Table 4.4 the agreement between the two values is acceptable for most compounds shown even though the GC column used for the determination of the LTPRI values contains 95 % PDMS and 5 % phenyl-PDMS.

**TABLE 4.4.** Comparison between calculated and experimentally measured (25 °C) \( K_{fg} \) values for 13 monoterpenoids. R.S.E. represents the relative standard error between the two \( K_{fg} \) values.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LTPRI</th>
<th>( K_{fg} ) measured (n=3)</th>
<th>( K_{fg} ) calculated</th>
<th>RSE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )-thujene</td>
<td>929</td>
<td>2.58E+04</td>
<td>2.31E+04</td>
<td>10</td>
</tr>
<tr>
<td>( \alpha )-pinene</td>
<td>938</td>
<td>2.84E+04</td>
<td>2.53E+04</td>
<td>11</td>
</tr>
<tr>
<td>camphene</td>
<td>957</td>
<td>3.22E+04</td>
<td>3.05E+04</td>
<td>5</td>
</tr>
<tr>
<td>sabinene</td>
<td>980</td>
<td>3.84E+04</td>
<td>3.86E+04</td>
<td>1</td>
</tr>
<tr>
<td>( \beta )-pinene</td>
<td>987</td>
<td>3.96E+04</td>
<td>4.13E+04</td>
<td>4</td>
</tr>
<tr>
<td>myrcene</td>
<td>994</td>
<td>5.29E+04</td>
<td>4.47E+04</td>
<td>16</td>
</tr>
<tr>
<td>( \alpha )-terpinene</td>
<td>1026</td>
<td>6.42E+04</td>
<td>6.13E+04</td>
<td>5</td>
</tr>
<tr>
<td>limonene</td>
<td>1039</td>
<td>7.15E+04</td>
<td>6.99E+04</td>
<td>2</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>1044</td>
<td>8.45E+04</td>
<td>7.37E+04</td>
<td>13</td>
</tr>
<tr>
<td>( \gamma )-terpinene</td>
<td>1068</td>
<td>1.03E+05</td>
<td>9.43E+04</td>
<td>9</td>
</tr>
<tr>
<td>terpinolene</td>
<td>1095</td>
<td>1.34E+05</td>
<td>1.24E+05</td>
<td>7</td>
</tr>
<tr>
<td>( \alpha )-thujone</td>
<td>1124</td>
<td>1.85E+05</td>
<td>1.66E+05</td>
<td>11</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>1200</td>
<td>7.93E+05</td>
<td>3.58E+05</td>
<td>55</td>
</tr>
</tbody>
</table>

* Determined on a BPX-5 column

From Table 4.4 it can be seen that the only analyte showing a large difference (55 %) between the calculated and experimentally determined \( K_{fg} \)'s is terpinen-4-ol. Reports in the literature involving compounds with large \( K_{fg} \)'s (Matich et al., 1996; Górecki et al., 1998; Matich, 1999) suggested that most of this error may originate from adsorptive losses of the analyte onto the wall of the syringe during static HS sampling. This would result in a much higher than actual value for \( K_{fg} \). However, further evidence presented and discussed below indicates that the hydroxyl group of terpinen-4-ol may have a major effect on its LTPRI value and this effect was likely to be the cause of the observed discrepancy between the two \( K_{fg} \)'s.
Using Equation 4 and LTPRI values determined by HS-SPME sampling of terpene accumulating plants, $K_{fg}$ values for 33 commonly-found essential-oil constituents were calculated (Table 4.5). The identity of all oil components was established based on mass spectral information, retention times and use of authentic standards when available. The appropriate literature was also consulted (Brophy et al., 1989; Adams, 1995; Martins et al., 1999).

**Table 4.5.** Calculated $K_{fg}$'s for a wide range of essential-oil constituents. These values are valid only when sampling is performed at 25 °C.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LTPRT $^a$</th>
<th>$K_{fg}$ calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-phellandrene</td>
<td>1015</td>
<td>5.47E+04</td>
</tr>
<tr>
<td>$p$-cymene</td>
<td>1036</td>
<td>6.82E+04</td>
</tr>
<tr>
<td>$\beta$-phellandrene</td>
<td>1042</td>
<td>7.21E+04</td>
</tr>
<tr>
<td>$E$-sabinene hydrate</td>
<td>1085</td>
<td>1.12E+05</td>
</tr>
<tr>
<td>$l$-fenchone</td>
<td>1107</td>
<td>1.40E+05</td>
</tr>
<tr>
<td>fonalool</td>
<td>1112</td>
<td>1.47E+05</td>
</tr>
<tr>
<td>Z-sabinene hydrate</td>
<td>1118</td>
<td>1.57E+05</td>
</tr>
<tr>
<td>$\beta$-thujone</td>
<td>1135</td>
<td>1.86E+05</td>
</tr>
<tr>
<td>endo-fenchol</td>
<td>1141</td>
<td>1.97E+05</td>
</tr>
<tr>
<td>Z-$p$-menth-2-en-1-ol</td>
<td>1142</td>
<td>1.99E+05</td>
</tr>
<tr>
<td>$E$-$p$-menth-2-en-1-ol</td>
<td>1162</td>
<td>2.43E+05</td>
</tr>
<tr>
<td>camphor</td>
<td>1170</td>
<td>2.64E+05</td>
</tr>
<tr>
<td>menthone</td>
<td>1176</td>
<td>2.81E+05</td>
</tr>
<tr>
<td>$\alpha$-terpineol</td>
<td>1217</td>
<td>4.27E+05</td>
</tr>
<tr>
<td>Z-dihydrocarvone</td>
<td>1222</td>
<td>4.48E+05</td>
</tr>
<tr>
<td>$E$-dihydrocarvone</td>
<td>1229</td>
<td>4.81E+05</td>
</tr>
<tr>
<td>$E$-piperitol</td>
<td>1238</td>
<td>5.28E+05</td>
</tr>
<tr>
<td>pulegone</td>
<td>1263</td>
<td>6.78E+05</td>
</tr>
<tr>
<td>carvone</td>
<td>1271</td>
<td>7.35E+05</td>
</tr>
<tr>
<td>menthyl acetate</td>
<td>1304</td>
<td>1.03E+06</td>
</tr>
<tr>
<td>terpinen-4-ol acetate</td>
<td>1357</td>
<td>1.75E+06</td>
</tr>
<tr>
<td>eugenol</td>
<td>1380</td>
<td>2.21E+06</td>
</tr>
<tr>
<td>$\beta$-elemene</td>
<td>1402</td>
<td>2.76E+06</td>
</tr>
<tr>
<td>$\alpha$-gurjunene</td>
<td>1422</td>
<td>3.41E+06</td>
</tr>
<tr>
<td>$\beta$-gurjunene</td>
<td>1449</td>
<td>4.46E+06</td>
</tr>
<tr>
<td>$E$-iso-eugenol</td>
<td>1465</td>
<td>5.23E+06</td>
</tr>
<tr>
<td>$\alpha$-humulene</td>
<td>1476</td>
<td>5.85E+06</td>
</tr>
<tr>
<td>$\gamma$-muurolene</td>
<td>1501</td>
<td>7.59E+06</td>
</tr>
<tr>
<td>germacrene D</td>
<td>1504</td>
<td>7.77E+06</td>
</tr>
<tr>
<td>viridiflorene</td>
<td>1511</td>
<td>8.36E+06</td>
</tr>
</tbody>
</table>

(continued on next page)
Equation 4 was established by using compounds with an LTPRI less or equal to 1124 (Figure 4.8, Table 4.4). Therefore, it was of interest to investigate the accuracy of the $K_{fg}$'s predicted by extrapolation of Equation 4 for compounds with LTPRI's higher than 1124 (shown in Table 4.5). This could only be achieved by the experimental determination of $K_{fg}$'s for such compounds and their subsequent comparison with calculated values. A comparison between experimentally determined (after five hours HS-SPME sampling to ensure equilibrium) and calculated $K_{fg}$'s for a group of four terpenoids with LTPRI's higher than 1124 (Table 4.6) showed that Equation 4 can be extrapolated for compounds with LTPRI's at least as high as 1481 with acceptable results.

**Table 4.6.** Comparison between calculated and experimentally measured $K_{fg}$'s (25 °C) for selected terpenoids. R.S.E. represents the relative standard error between the two $K_{fg}$ values.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LTPRI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$K_{fg}$ measured&lt;sub&gt;n=3&lt;/sub&gt;</th>
<th>$K_{fg}$ calculated</th>
<th>R.S.E (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>menthol</td>
<td>1199</td>
<td>9.95E+05</td>
<td>3.55E+05</td>
<td>64</td>
</tr>
<tr>
<td>E-β-caryophyllene</td>
<td>1439</td>
<td>3.61E+06</td>
<td>4.01E+06</td>
<td>11</td>
</tr>
<tr>
<td>aromadendrene</td>
<td>1458</td>
<td>4.03E+06</td>
<td>4.90E+06</td>
<td>21</td>
</tr>
<tr>
<td>alloaromadendrene</td>
<td>1481</td>
<td>5.59E+06</td>
<td>6.16E+06</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup> determined on a BPX-5 column

However, as it was observed with terpinen-4-ol (Table 4.4), menthol also exhibited a large difference (< 64 %) between its calculated and experimentally determined $K_{fg}$'s although it has a much smaller LTPRI compared to the three other terpenes used (Table 4.6). This indicated that the increased polarity of these two
compounds and its effect on their LTPRI's is the major cause of the large error obtained rather than their loss during static HS-SPME due to their semi-volatile nature. The data presented in Table 4.4 also shows that differences between the calculated and experimentally determined K_{fg}'s appear to be slightly higher for more polar terpenes such as 1,8-cineole and α-thujone when compared with the non-polar terpene hydrocarbons.

K_{fg}'s calculated based on Equation 4 can be used for the quantitative determination of gas-phase terpenoids in any sample provided that (PDMS) HS-SPME is performed at 25 °C because K_{fg} values are temperature dependent (Martos et al., 1997; Pawliszyn, 1997). Therefore, this work eliminates the need to re-determine K_{fg}'s for every system. Results from the application of this method are presented in Chapter 6.

4.8 Detection of terpene-synthase products in enzyme preparations by HS-SPME

4.8.1 Theoretical background

The in vitro activity of terpene synthases is routinely determined by assays in which the appropriate substrate is incubated with the preparation containing the enzyme(s) of interest (e.g., Gambriel and Croteau, 1984; Lewinsohn et al., 1992; Croteau et al., 1994; Pichersky et al., 1995). The identity and overall profile of the products formed during the assay are then used to determine and characterize the synthase(s) present in the initial enzyme preparation.

The use of radiolabelled substrate during the assay followed by radio-gas chromatographic (radio-GC) analysis of the extracts is usually the procedure employed for the separation and identification of the enzyme products (e.g.,
Gambriel and Croteau, 1984; Lewinsohn et al., 1992; Croteau et al., 1994; Pichersky et al., 1995). This process is often supported by the solvent extraction of the substrate-enzyme mixture and subsequent analysis of the extracts by gas chromatography-mass spectrometry (GC-MS) (e.g., Gambriel and Croteau, 1984; Lewinsohn et al., 1992; Croteau et al., 1994; Pichersky et al., 1995).

However radio-GC detectors are expensive and thus inaccessible to many researchers in the field. In addition, the cost of radiolabeled substrate can also be a problem if numerous assays need to be performed.

This section describes the use of headspace solid phase microextraction (HS-SPME) combined with GC-MS as an alternative method to radiolabeling and solvent extraction for the detection and characterization of synthase activity in cell-free enzyme preparations.

Fuchs et al. (1999; 2000) reported specific cases of detection of terpene biosynthesis by SPME GC-MS but those studies were carried out in vivo and were not associated with an enzyme preparation. The simple and inexpensive technique described below can be used reliably on its own where a radio-GC detector is not available and should be preferred over solvent extraction as it offers higher sensitivity and solvent-free analysis.

4.8.2 Material and Methods

4.8.2.1 Terpene source

The products evolved from the acid hydrolysis of the ubiquitous monoterpane-precursor geranyl diphosphate (GPP) were used as target analytes. GPP was obtained from Echelon Research Laboratories Inc. (USA).
4.8.2.2 HS-SPME

A manual SPME holder (Supelco, USA) equipped with a 1 cm-long (PDMS)-coated fiber (Supelco, USA) was used. GPP (2.74 mM)(20 μL) was mixed with assay buffer (50 mM Tris, 1 mM MnCl₂, 10 mM MgCl₂, 10 % glycerol, adjusted to pH 2 with HCl) (980 μL) in a 2 mL screw-top glass vial (Agilent Technologies, USA). The total volume of the GPP-buffer mixture was kept to 1 mL to allow the full length of the fiber to be exposed to the headspace of the mixture within the vial. The mixture was left to equilibrate for 15 minutes at 32 °C and then the fiber was exposed to the headspace of the mixture for a further 15 minutes (32 °C). Thermal desorption (220 °C for 10 minutes) of the fiber in the split/splitless injection port of the GC-MS system followed. Before use the fiber was conditioned according to the manufacturer's instructions.

4.8.2.3 Solvent extraction

The GPP-buffer mixture described above was gently topped with a layer (400 μL) of pentane (Fluka, USA). After 15 minutes the vial was vortexed and the pentane (top) layer was concentrated with under a stream of N₂ to 30 μL and then subjected to GC-MS analysis (1μL injection volume).

4.8.2.4 Determination of linearity

The appropriate amounts of linalool (Fluka, USA) were mixed with assay buffer (pH 7.2) in order to obtain solutions of four different concentrations (4.16, 8.32, 20.80 and 33.28 μg/mL⁻¹). HS-SPME was then carried out as described above. The process was repeated three times.

4.8.2.5 Limit of detection (LOD) determination

HS-SPME was carried out as described above (section 4.8.2.2) on a low concentration (8.16 μg/mL) solution of linalool in assay buffer (pH 7.2).
standard deviation (multiplied by 3) from 5 injections in the IT-GC-MS system was then used to determine the LOD as advised by Pawliszyn (1997).

4.8.2.6 Determination of HS-SPME equilibrium

An amount of terpenoid (5 μL) was mixed with water (10 mL) and the mixture was vortexed for 5 min. Part of this mixture (10 μL) was then placed in 2 mL screw-top vials together with Tris assay buffer (pH 7.2) (990 μL). The vials were left to equilibrate at 32 °C for at least 15 minutes. Then HS-SPME was carried out (also at 32 °C) with exposure times ranging from 2 to 10 minutes (one exposure time per vial). The process was carried out for linalool, γ-terpinene and aromadendrene (Figure 4.11).

4.8.2.7 Enzyme extraction

Bush basil (Ocimum minimum L.) leaf material (~1 g) was ground with liquid N₂ to a fine powder. Extraction buffer (50 mM Tris, 1 mM MnCl₂, 10 mM MgCl₂, 20 % (v/v) glycerol, adjusted to pH 6.9 with HCl) (25 mL), polyvinylpolypyrrolidone (0.1 g) and amberlite XAD-4 (1 g) were added and the slurry was filtered through cheesecloth. The filtrate was centrifuged (JA 20 rotor) at 10,000g for 10 min. at 4 °C, the supernatant was collected and further centrifuged at 20,000g for 60 min. at 4 °C. The supernatant was then passed through a 0.45 μm syringe filter (Nalgene), placed in a centrifugal concentrator and centrifuged (6,000g at 4 °C) to a volume of about 10 mL. An equal volume of assay buffer (pH 7.2) was then added and the extract was again concentrated (6,000g at 4°C) to about 10 mL. This process was repeated five times (5x10 mL) to ensure the extract was free of contaminant terpenoids. The extract was then subjected to HS-SPME (as described above, section 4.8.2.2).

4.8.2.8 GC analysis

GC analysis was carried out as outlined in section 4.7.2.6.

- 111 -
4.8.2.9 GC-MS analysis

GC-MS analysis was carried out according to section 4.7.2.7.

4.8.3 Results-Discussion

The acid-catalysed hydrolysis\(^3\) of GPP to various acyclic and cyclic monoterpenes is a process investigated by Cramer and Rittersdorf (1967) who reported linalool (76-78 %) and geraniol (17-19 %) to be the major products of the conversion under mild acidic conditions. Under more vigorous conditions the initially formed linalool generates monocyclic monoterpenes (Cramer and Rittersdorf, 1967; Erman, 1985) (Figure 4.10). This chemical transformation (rather than an enzymatic one) was chosen as the source of monoterpenes during this investigation so a more accurate estimation of the HS-SPME method's reproducibility could be obtained. The level of enzyme activity can vary even in aliquots from the same preparation especially when very sensitive and unstable enzymes are involved. In addition, the GC profile obtained from the hydrolysis of GPP (as per Table 4.7 and Figure 4.11a) is very similar to those from previously isolated terpene synthases in terms of product distribution; the yield of a major product together with a few other smaller in amount byproducts is a behaviour commonly exhibited by terpene synthases (Gambriel and Croteau, 1984; Lewinsohn, 1992; Wise et al., 1998).

\(^3\) Bivalent metal ions can also affect the hydrolysis of GPP (Vial et al., 1981) but this topic is described in detail in Chapter 7.
**Figure 4.10.** IT-GC-MS traces of the GPP degradation products at various pH’s as obtained by HS-SPME. a) pH 2, b) pH 4, c) pH 6 and d) pH 7.1. The effect of pH on the process is evident. Please refer to Table 4.7 (below) for the identity of the numbered peaks.
TABLE 4.7. Monoterpenes obtained from the acid hydrolysis of GPP as detected by HS-SPME coupled IT-GC-MS. Peak numbers refer to Figures 4.10 and 4.11. STD represents the standard deviation obtained from three experiments.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Monoterpene</th>
<th>Quantitation ion</th>
<th>Percentage RIC&lt;sup&gt;a&lt;/sup&gt; (mean ± STD) (n=3)</th>
<th>Relative STD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Myrcene</td>
<td>93</td>
<td>3.2 ± 0.3</td>
<td>9.4</td>
</tr>
<tr>
<td>2</td>
<td>Limonene</td>
<td>67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.9 ± 0.1</td>
<td>11.1</td>
</tr>
<tr>
<td>3</td>
<td>Z-β-Ocimene</td>
<td>93</td>
<td>4.1 ± 0.1</td>
<td>2.1</td>
</tr>
<tr>
<td>4</td>
<td>E-β-Ocimene</td>
<td>93</td>
<td>4.7 ± 0.3</td>
<td>7.2</td>
</tr>
<tr>
<td>5</td>
<td>Terpinolene</td>
<td>93</td>
<td>1.3 ± 0.1</td>
<td>9.0</td>
</tr>
<tr>
<td>6</td>
<td>Linalool</td>
<td>93</td>
<td>76.4 ± 1.8</td>
<td>2.3</td>
</tr>
<tr>
<td>7</td>
<td>α-Terpineol</td>
<td>41</td>
<td>trace</td>
<td>n/a</td>
</tr>
<tr>
<td>8</td>
<td>Geraniol</td>
<td>69</td>
<td>9.3 ± 0.6</td>
<td>6.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage RIC is given because no account has been taken for the different response factors.

<sup>b</sup> m/z 67 (instead of m/z 68) was used for quantitation because it was observed to be the base peak in the limonene mass spectrum (as per Appendix 4.4). This is in accordance with the limonene spectrum published by Adams (1995) (also by IT-GC-MS).

HS-SPME is fast, simple to perform and exhibits good reproducibility as shown by the RSD values presented in Table 4.7. Slightly higher RSD values were observed with analytes present at low concentrations but these values are still satisfactory considering that the typical error for any SPME determination is around 5 % (Pawliszyn, 1997). However, the biggest asset of HS-SPME when compared to solvent extraction is its much higher sensitivity. This is evident from the comparison of the chromatograms obtained with the two methods on identical samples under the same conditions (Figures 4.11b and 4.11c). Most of the minor products shown by HS-SPME (Figure 4.11b) were not detected by the solvent extraction approach even after concentration of the solvent layer (Figure 4.11c).
FIGURE 4.11. Typical chromatograms obtained from acid hydrolysis of GPP. a) Reconstructed ion count (RIC) trace from HS-SPME, b) extracted (m/z 69, 93) trace from HS-SPME and c) extracted (m/z 69, 93) trace from solvent extraction after concentration of the pentane layer. Traces b and c are presented under the same scale. The identity of the numbered peaks can be found in Table 4.7. Peaks labelled with an X represent extraneous impurities.
The HS-SPME's LOD for linalool in buffer (pH 7.2) was determined to be 2.30 μg/mL (using IT-MS detection under the routine-operation conditions described above).

It is possible to reduce this LOD figure to sub-ppm values if the ion trap detector is set to higher sensitivity parameters. The age of the capillary column also has an effect on the LOD with old, deteriorating columns exhibiting "abnormally" high LOD's due to loss of chromatographic performance over time (e.g., Jackman et al., 2001). Higher sensitivity is also expected for less polar terpenes showing better affinity (i.e., higher partition coefficient \( K_{dL} \)) for the PDMS coating (Pawliszyn, 1997).

The developed method also exhibits good linearity \( (R^2=0.9829) \) over the range of linalool concentrations tested \( (4-33 \mu g/mL) \) (Figure 4.12).

**Figure 4.12.** Graph showing the linearity of HS-SPME over a range of linalool concentrations (mean ± std, n=3).
The determination of the effect of the matrix pH on the absorption of volatiles was not one of the objectives of this study. The LOD and linearity experiments were conducted at almost neutral pH (7.1) because all plant-derived terpene synthases characterised so far are known to exhibit maximum activity at similar pH values (e.g., Croteau and Karp, 1977; Croteau and Cane, 1985; Croteau, 1987; Hallahan and Croteau, 1988; Hallahan and Croteau, 1989), and therefore identical experiments at other pH values were not required. The method's reproducibility (RSD's in Table 4.7) will certainly be the same at any pH value. Changes in pH are known to have an effect on the sensitivity of SPME methods for certain analytes (Pawliszyn, 1997; Shirey, 2000). However, if analyte RSD's for a particular method are obtained at constant pH (irrespective of the value) then changes in sensitivity between replicates are eliminated and the true reproducibility of the method is obtained.

The "aging" of the fibre coating can become a problem in cases where accurate quantitation is required for a large number of samples over a long period of time. This can be solved by the addition to the mixture of an internal standard just before GPP is added. The compound to be used must be chosen cautiously; not only does it have to elute well clear of all product-peaks but it must also be completely "inert" in relation to the synthase of interest (i.e. not being able to be used as an alternative substrate).

The solvent-free nature of HS-SPME means that there is no need for any type of solvent to be used during the enzyme assay. This eliminates any possible harmful effects to enzyme properties caused by the contact between the solvent and the buffer layer during the all-important enzyme-substrate incubation period. Furthermore, it can be difficult to completely separate the two layers at room temperature and buffer constituents such as glycerol can be very detrimental to GC-MS analysis if
"accidentally" introduced in the capillary column. Caution is also required during HS-SPME as contact between the fiber (even of the non-polar PDMS-coated) and the buffer layer will have the same effect. Even if contamination was not a problem, direct SPME sampling of the buffer layer would, under equilibrium conditions, produce comparable results with the sampling of the headspace in terms of the detection limit and the amount of analyte extracted (Pawliszyn, 1997).

Knowledge of an ion or ions characteristic of the analytes expected to be evolved during an enzyme-substrate assay is very beneficial especially when accurate characterisation/quantitation of the assay products is required. "Masking" of the peaks of interest by "unwanted" peaks originating from the buffer or other reagents can become a problem unless single ion monitoring or post-run extraction of the characteristic ion(s) is employed during analysis. The difference between a reconstructed ion count (RIC) trace and a specific ion trace from the same ion-trap scan can be seen in Figures 4.11a and 4.11b. Several extraneous peaks can be seen on the RIC trace (Figure 4.9a) but not on the specific ion trace (Figure 4.9b) where only the peaks of interest appear. For the same reasons coupling of HS-SPME with GC-MS is evidently more rewarding than HS-SPME-GC unless very high sensitivity (ppb) is required (Louch et al., 1992).

A problem, which arises during the isolation of terpene-synthases from plant material, is the contamination of the enzyme preparation by water-soluble terpenoids. These compounds can complicate the interpretation of the assay results and in cases where the carried-over contaminants coincide with the products expected from the targeted enzyme(s) the assay becomes useless. There are procedures however, which can be used to eliminate this problem and ensure that the enzyme preparation is terpenoid-free before the assay. "Washing" of the preparation with several aliquots
of buffer (as per section 4.7.2.7) can be time-consuming but it is an easy, inexpensive and effective process as is shown in Figure 4.13a and 4.13b. Another alternative is the use of ion exchange and/or hydrophobic interaction chromatography but more skill and equipment is required in this approach (e.g., Lewinsohn et al., 1992; Croteau et al., 1994).

**Figure 4.13.** Trace (m/z 93) from a cell-free extract from bush basil (*Ocimum minimum* L.) leaves a) before and b) after the elimination of the contaminating background. The effectiveness of the clean-up procedure is clear.
The equilibration time between the analyte and the fiber coating is also important if accurate quantitation results are to be obtained from headspace sampling above a static aqueous solution. In such systems extraction equilibrium is reached only when the analyte concentration becomes homogeneous within each of the three phases (matrix, headspace, fibre) (Scheppers-Wereinski and Pawliszyn, 1999). Experiments have shown that the rate-determining step in the process is the diffusion of the analytes from the matrix into the headspace rather than from the headspace onto the fiber-coating (Louch et al., 1992; Matich et al., 1996; Field et al., 1996). HS-SPME of conifer needles (with a PDMS-coated fibre) has shown that an enrichment period of 5 minutes is sufficient for quantitative purposes when the analyte of interest is a monoterpane such as α-pinene or camphene (Schäfer et al., 1995). Much longer enrichment (> 60 minutes) is known to be required for less volatile terpenes (Matich et al., 1996; Field et al., 1996).

Figure 4.14 illustrates that the above findings are also valid during HS-SPME above a static buffer matrix. The monoterpenes γ-terpinene (MW 136) and linalool (MW 154) reach extraction equilibrium much faster than the sesquiterpene aromadendrene (MW 204) (Figure 4.14). The absorption profile for aromadendrene (Figure 4.12) is typical for compounds with low headspace/water (K_{hw}) distribution constants, but large coating/headspace (K_{hk}) distribution constants (Pawliszyn, 1997; Scheppers-Wereinski and Pawliszyn, 1999; section 4.7.3). Agitation of the sample during extraction may be used to facilitate faster mass transport of the matrix into the headspace for such compounds, therefore decreasing the required extraction time (Pawliszyn, 1997).
**Figure 4.14.** Absorption profiles for selected terpenoids with the 100 μm PDMS fibre (static HS-SPME above buffer matrix). a) γ-Terpinene, b) aromadendrene and c) linalool.

Results from the application of this method to terpene-synthase assays will be presented in Chapter 6. Although developed in relation to terpenoids, the conditions described above can be modified to suit the requirements for the determination of a wide range of synthase-products.
4.9 Conclusion

The quantitative monitoring of volatiles emitted by terpene accumulating plants using HS-SPME is a simple and reliable process when the $K_{fb}$ value for the analytes of interest is known. Equation 4 allows the simple determination of $K_{fb}$ just by insertion of the LTPRI value for a particular terpenoid.

HS-SPME was also found to be a reliable and cheaper alternative to radiolabelling for the detection of terpene synthase products formed during enzyme-substrate assays. This technique is characterised by high sensitivity, good reproducibility and linearity and was found to be superior to solvent extraction mainly due to its higher sensitivity, simplicity and solvent-free nature.

However, as with all SPME applications, caution is required when selecting thickness and type of fibre to be used as these two factors affect the sensitivity of the technique.
APPENDICES

Appendix 4.1. SPME conditioning parameters for the types of fibres used in this study.

<table>
<thead>
<tr>
<th>Coating (thickness)</th>
<th>Recommended operating temperature (°C)</th>
<th>Conditioning temperature (°C)</th>
<th>Conditioning time (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDMS (100 µm)</td>
<td>200-270</td>
<td>250</td>
<td>1</td>
</tr>
<tr>
<td>Carboxen-PDMS (75 µm)</td>
<td>240-300</td>
<td>280</td>
<td>0.5</td>
</tr>
</tbody>
</table>

(Supelco, PA, USA)

Appendix 4.2. Determination of the absorption (PDMS coating) and adsorption (Carboxen-PDMS) rates of the analytes of interest on each coating.

The determination of the absorption rate of p-cymene on the PDMS coating is given as an example. First of all the absorption profile of the analyte on the particular coating was established by exposing the fibre-coating (as per section 4.2.2.3) to identical samples of p-cymene (prepared as per section 4.2.2.2). Exposure times in this case varied between 2-16 seconds (Figure A4.2.1).

Figure A4.2.1. Absorption profile of p-cymene on the 100 µm PDMS coating (25 °C).
Then the linear section of the curve was taken (points between 0-5 sec. for Figure A4.2.1) and the slope of the resulting line was calculated (Figure A4.2.2).

![Graph showing FID response vs. time with linear regression line and R^2 = 0.9947]

**Figure A4.2.2.** Linear part of the absorption profile shown in Figure A4.2.1.

For the line in Figure A4.2.2 the slope was determined as follows:

\[
slope = \frac{\text{rise}}{\text{run}} = \frac{(4665-0) \text{ pA's}}{(5-0) \text{ sec}} = 933 \text{ pA's/sec.}
\]

(Borowski and Borwein, 1989).

**Appendix 4.3.** Details regarding the calculation of the tailing factor (TF) for a particular peak.

The TF as recommended by the United States Pharmacopeia is based on the measurement of the half-width parameters \(a\) and \(b\) at 5% of the peak height. For the peak shown the TF is calculated as: \(T = \frac{(a+b)}{2a}\) (Felinger, 1998).
Appendix 4.4. Experimentally obtained a) and published b) (Adams, 1995) ion trap mass spectra of limonene.
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CHAPTER 5

DATA TREATMENT AND STATISTICAL ANALYSIS

5.1 Introduction

Appropriate data treatment and correct statistical analysis are vital for the success of any study as they are, directly or indirectly, related to all other major processes activated during the addressing of complicated research questions (Figure 5.1).

![Diagram showing relationships between experimental design, analytical technique, facts, measurements, dataset, data analysis, information, and decision-making.]

**Figure 5.1.** Schematic representation of the relationships between the major processes activated during the addressing of a research question (modified from Mellinger, 1987).

Data analysis (term includes both, data treatment and statistical analysis) investigates the data and aids the extraction of information from the dataset; then a decision can be made based on the information extracted (Mellinger, 1987).
There are numerous examples in the research literature that describe and/or apply specific, data treatment and statistical analysis techniques. As is often the case with analytical techniques, an adjustment or modification of a published approach may be required in order to suit the needs of a specific study.

Coupling of techniques that belong to different categories (e.g., multivariate and univariate statistics) and/or serve distinct purposes (e.g., inferential and descriptive statistics) may also be required in order to interpret complex datasets.

A description (and the reasoning behind the selection) of the data treatment processes and the subsequent statistical analyses employed during the "before and after treatment" experiments (experimental design described in section 2.2.3) is given below\(^1\).

### 5.2 Pre-statistical data treatment

#### 5.2.1 Data preparation for investigating treatment-induced changes in essential oil concentration

The concentration of each oil component \((C_i)\) was determined by the equation:

\[
C_i = \left(\frac{C_{istd}}{A_{istd}} \times A_i\right) \times RRF
\]

where \(C_{istd}\) is the concentration of the internal standard, \(A_{istd}\) is the peak area of the internal standard\(^2\) and \(A_i\) is the peak area of each component after normalisation (in this case per sample fresh weight) (Rouessac and Rouessac, 2000). The relative response factor (RRF) of the internal standard in relation to all oil components was assumed to be 1. Any differences in the detector response between the internal

---

\(^1\) Details in relation to the processing of the data obtained from all the other (especially HS-SPME-based) experiments are given within the sections associated with a particular experiment in Chapter 6. For these experiments complex data processing and statistical analysis approaches were not required.

\(^2\) Octane (400 ppm in CH\(_2\)Cl\(_2\)) was used as the internal standard (as per Chapter 3).
standard and the oil components and also between the various oil components could introduce small bias in the determination but these are known to be consistent and do not affect trends over time (Murtagh and Smith, 1996).

The oil concentration (expressed in μg g⁻¹ dry weight) of each sample represented the total of the sum of the concentrations of each oil component. Although the extraction was carried out on fresh leaves the values had to be expressed on a dry weight basis due to the substantial variations that can occur with moisture content (Murtagh and Smith, 1996). This was achieved by incorporation of data obtained from separate, moisture-determining experiments involving "identical" leaves (as per Chapters 3 and 6).

5.2.2 Data preparation for investigating treatment-induced changes in essential oil composition

The normalised (per sample fresh weight) peak areas of the integrated gas-chromatograms were used to express the percentage contribution of each monoterpenoid (and of the sesquiterpenoids measured as a group) in the oil. This was necessary in order to compare leaf-oil profiles before and after treatment. If absolute (e.g., area counts g⁻¹ or μg g⁻¹) rather than relative (e.g., percentage) values were to be used for the comparison, then changes in any factor that can affect the weight of the sample (e.g., leaf structural development, oil concentration) would have been superimposed on the data and the results obtained would be inaccurate and misleading. The difference between the two approaches is demonstrated by the example given below (Table 5.1).
Table 5.1. Absolute (µg g\(^{-1}\), based on internal std) and relative (%) values indicating the contribution of selected components in the essential oil of *M. alternifolia* unwounded (\(T_0\)) and wounded (\(T_{24}\)) leaves. Results from the cold solvent extraction of a **single sample** are presented in order to show data treatment differences only; evaluation of the data for other purposes (e.g., to determine wound-induced changes in terpenoid levels) is not recommended. Please refer to Chapter 6 for methodological details.

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount in essential oil (µg g(^{-1}) dry weight)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(T_0)</td>
<td>(T_{24})</td>
<td></td>
</tr>
<tr>
<td>(\alpha)-thujene</td>
<td>4.70</td>
<td>(0.9)</td>
<td>3.33</td>
</tr>
<tr>
<td>(\alpha)-pinene</td>
<td>12.65</td>
<td>(2.4)</td>
<td>9.97</td>
</tr>
<tr>
<td>cluster 1(^a)</td>
<td>9.40</td>
<td>(1.8)</td>
<td>7.60</td>
</tr>
<tr>
<td>(\alpha)-terpinene</td>
<td>36.26</td>
<td>(6.8)</td>
<td>29.42</td>
</tr>
<tr>
<td>cluster 2(^b)</td>
<td>51.46</td>
<td>(9.7)</td>
<td>44.63</td>
</tr>
<tr>
<td>(\gamma)-terpinene</td>
<td>101.18</td>
<td>(19.0)</td>
<td>80.92</td>
</tr>
<tr>
<td>terpinolene</td>
<td>14.73</td>
<td>(2.8)</td>
<td>11.78</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>214.95</td>
<td>(40.4)</td>
<td>180.20</td>
</tr>
<tr>
<td>(\alpha)-terpinol</td>
<td>17.50</td>
<td>(3.3)</td>
<td>14.65</td>
</tr>
<tr>
<td>sesquiterpenes</td>
<td>58.30</td>
<td>(11.0)</td>
<td>58.43</td>
</tr>
<tr>
<td>Oil concentration (µg g(^{-1}) dry weight)</td>
<td>521.13</td>
<td></td>
<td>440.93</td>
</tr>
</tbody>
</table>

\(^a\) cluster 1 includes \(\beta\)-pinene and myrcene  
\(^b\) cluster 2 includes \(\pi\)-cymene, limonene, \(\beta\)-phellandrene and 1,8-cineole

As can be seen from Table 5.1, all \(T_{24}\) absolute values are smaller than those for \(T_0\). Apart from the fact that no conclusion about the essential oil composition could be established based on these (absolute) values alone, if they were to be used in analysis the result obtained would indicate a wound-induced decrease in the levels of all oil components. This conclusion would certainly be inaccurate as this change simply reflects the changes in the essential oil concentration occurring during the 24-hour post-wounding period (Zabaras et al., 2002; Chapter 6 herein). By placing the absolute values under a "common base" (in this case total peak area) and therefore obtaining relative (e.g., %) values, it is possible to eliminate such effects and the "true" wound-induced changes in the essential oil composition can be established (\(T_0\) and \(T_{24}\) percentage values, Table 5.1).
5.3 Statistical analysis

5.3.1 Inferential methods used

A combination of inferential and descriptive techniques was employed during
the statistical analysis of the data after the treatment processes (described in sections
5.2.1 and 5.2.2). Inferential techniques are used to test hypotheses about differences
in populations on the basis of measurements made on samples of subjects
(Tabachnick and Fidell, 2001).

All inferential techniques used in this study were non-parametric due to the
relatively small sample size. Data from independent samples (e.g., comparison
between $T_{24}$ and $T_{48}$ treatments, comparison of leaf-oil concentration differences
between control and treated plants) was tested by the Kruskal–Wallis test (Sall and
Lehman, 1996). Similar data but from dependent (paired) samples (e.g., comparison
of oil composition of the same leaf before and after treatment) was tested for
statistical significance using the Wilkoxon signed ranks test (Sall and Lehman,
1996).

In addition to the above univariate techniques, a multivariate (Spearman's
test, Sall and Lehman, 1996) inferential method that tests correlations between
variables, was also employed to investigate the effect of wounding on the non-
parametric measures of association between the major thujanes and $p$-menthanes
based on their percentage contribution to the flush-growth solvent extracts (further
details in Chapter 6).

5.3.2 Descriptive methods used

Descriptive statistics describe samples of subjects in terms of variables or
combinations of variables (Tabachnick and Fidell, 2001). Their purpose is the
detection and interpretation of data patterns in large and/or complex datasets
(Mellinger, 1987). If a descriptive technique is used subsequently to inferential analysis to provide estimations of central tendency or other similar information about the population, then it is termed parameter estimate (Tabachnick and Fidell, 2001).

The descriptive technique used in this study was principal component analysis (PCA). PCA is a feature-reduction technique first formulated by Pearson (1901) who interpreted the analysis as finding "lines and planes of closest fit to systems of points in space". Steps in PCA include selection of a set of variables, preparation of a correlation matrix, extraction and selection of a set of components and finally interpretation of the results (Tabachnick and Fidell, 2001).

PCA extracts independent factors from a number of variables by decomposing a data matrix, $X$, into two smaller matrices, $T$ (scores matrix) and $P$ (loadings matrix), that capture the essential data patterns of $X$ (Dunlop et al., 2000; Wold et al., 1987). Plotting the columns of $T$ results in a scoreplot that provides information about object patterns (in this case leaf-oils) within $X$ while the plotting of rows of $P$ shows similarities or differences between the variables (in this case terpenoids) used for the analysis (Wold et al., 1987). Therefore, in this case similarity in scores indicates similarity between essential oils; inspection of the scoreplot of the sources of two or three principal components can yield natural groupings in the initial data matrix, $X$ (Dunlop et al., 1999; 2000). The loadings indicate which variable(s) (i.e terpenoid) contribute(s) most to each extracted principal component (Dunlop et al., 1999; 2000).

Since its inception, PCA has been used in many diverse scientific fields including analytical chemistry (e.g., Ramos et al., 1986) and essential oil chemistry (e.g., Gaydou et al., 1988; Nogueira et al., 1999; Sacco et al., 1992; Whiffin and Hyland, 1989). Australian workers have extensively used PCA during taxonomic
studies of *Eucalyptus* and *Angophora* (Myrtaceae) species based on their leaf-
essential oils (Dunlop et al., 1997; 1998; 1999; 2000).

In the above studies PCA was performed using all the detected oil-
components as variables. Then the *loading* matrices obtained were used to determine
which oil-component contributed most to the observed oil similarities or differences
between species (Dunlop et al., 1997; 1998; 1999; 2000).

In this study PCA was used as a parameter estimate to aid in the visualisation
of trends in the data. There are not many examples in the terpenoid/essential oil
literature reporting the use of PCA as a parameter estimate. The approach employed
by Mateus et al. (1995) for the demonstration of “bark-odour” differences between
borer-attacked and unattacked *Eucalyptus globulus* tree-logs, is the only PCA
application found to be similar to the novel statistical treatment described below.

PCA was carried out using as variables only those oil-components whose
presence was found to be significantly different (by the inferential tests described in
section 5.3.1) in treated and untreated leaves. This approach was required due to the
small differences (much smaller than those observed between different species)
found between oil-profiles of treated and untreated leaves. If PCA alone was to be
used, then these small differences could not be demonstrated as clearly as when they
are when a combination of an inferential method and PCA is used. This can be
illustrated by the hypothetical example below.

Assume the pre- (*T₀*) and post-wounding (*T₂₄*) oil-profiles from *E.
camaldulensis* leaves were as given in Table 5.2.
**Table 5.2.** Hypothetical $T_0$ and $T_{24}$ oil-profiles of *E. camaldulensis* leaves.

<table>
<thead>
<tr>
<th>Components</th>
<th>Oil composition (%) (mean ± std, n=16)¹</th>
<th>$T_0$</th>
<th>$T_{24}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-pinene</td>
<td>4.5 ± 0.1</td>
<td>4.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Limonene</td>
<td>4.8 ± 0.1</td>
<td>4.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>67.6 ± 0.2</td>
<td>66.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>$\gamma$-terpinene</td>
<td>1.5 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>$p$-cymene</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>aromadendrene</td>
<td>5.5 ± 0.1</td>
<td>6.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>allo-aromadendrene</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>globulol</td>
<td>1.5 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

¹ The true oil-profile of *E. camaldulensis* (clone no. 6) grown at Bolivar, Southern Australia (Dunlop *et al.*, 2000) was used as a guide to create these hypothetical values.

As can be seen from Table 5.2 the data set was created to show a decrease in the level of 1,8-cineole while at the same time an increase in aromadendrene and $\alpha$-pinene is observed. The differences were kept small (0.8 % change was the maximum) to simulate those encountered in this study.

Figure 5.2a shows the scatterplot obtained from the scores of the *E. camaldulensis* leaves on the first two principal components. Scores in this Figure (5.2a) were obtained from PCA carried out using all components in Table 5.1 as variables. Figure 5.2b shows the same scatterplot but here the scores are derived from PCA carried out using as variables the components labeled as significant by the Wilkoxon signed-ranks test ($\alpha$-pinene, 1,8-cineole, aromadendrene; $p \leq 0.05$).

As can be seen from Figure 5.2 below, the scatterplot obtained from the combination of Wilkoxon-signed ranks test and PCA (b) provides a better demonstration of the difference between the two ($T_0$ and $T_{24}$) leaf-oil groups compared to the scatterplot obtained from PCA alone (a). This is derived from the fact that in plot (b) a much greater variation in the data is interpreted by the first two principal components than that in plot (a) (96.6 to 58.6%). Selection of appropriate
**Figure 5.2.** 2-D Scatterplots obtained from the PCA of the hypothetical data set created for *E. camaldulensis* leaf-oil. a) Analysis by PCA only, using all oil-components as variables and b) PCA using as variables the components highlighted by the Wilkoxon signed-ranks test. Please refer to text for further explanation.
variables to be used in PCA reduces their number; a small sample size can then yield interpretable results even when small differences are present. If reduction of initial variables is not carried out then a large sample size or large differences in the groups compared (such as those encountered during chemotaxonomy studies) is required for the "extraction noise" in the data, derived from the insignificant variables, to be surpassed and differences demonstrated (Tabachnick and Fidell, 2001).

"Manual" selection of the variables to be used is not recommended, particularly for ecological studies, as minor components of the oils can also play important roles (e.g., Katoh and Croteau, 1998; Shu et al., 1998) and may be missed if not selected for PCA based on their small contribution to the leaf-oil.

There has been some debate in the literature on whether the data should be centred (i.e. subtract the mean from each variable) and autoscaled (i.e. divide by the standard deviation) (Hibbert, 1997) or just centred before PCA is carried out (Dunlop et al., 1995; 1999). Dunlop et al. (1995; 1999) has argued that for GC traces it may be preferable to only centre the data and not autoscale; this ensures that the major components of the oils contribute most to the PCA. This option may be used with satisfactory results in chemotaxonomy studies, where usually the major oil-components can adequately be used to distinguish between plants of different species. In ecological studies however, PCA of non-autoscaled data can produce very misleading outcomes as any changes in the levels of the minor oil-components will be ignored by the analysis. Autoscaling of the data ensures that all oil-components are seen as equivalent by PCA and in this study all PCA (including the analysis of the hypothetical data set, Figure 5.2) was carried out on centred and autoscaled data.
5.4 Conclusion

As for instrumental analysis techniques, data analysis and statistical techniques require modifications and/or development to suit the requirements of particular studies.

A novel (combination of inferential and descriptive statistical methods was employed for the analysis of data pre-treated according to the objectives of each experiment. This approach was found to be more efficient than individual methods alone as it allowed even small treatment-induced changes in the data to be demonstrated without the need for large sample sizes.
REFERENCES


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CHAPTER 6

THE EFFECTS OF MECHANICAL WOUNDING ON THE EMISSION OF PLANT VOLATILES

Although it is generally agreed that biotic elicitation yields a greater induction (Felton and Eichenseer, 1999 and references therein), wounding (an abiotic elicitor) may in some cases initiate plant responses similar to those activated by insect feeding (Hartley and Lawton, 1991).

Wounding is known to drastically alter the plant's pattern of gene expression and lead to a loss of cell-compartmentation thus exposing the cells to invading pathogens (Ayres, 1992).

This Chapter describes the wound-induced changes in the levels of terpenoids and lipoyxgenase (LOX)-derived products such as Z-3-hexenyl acetate. These were the only classes of volatiles detected to be present or produced by the leaves of Ocimum minimum, Salvia officinalis and Melaleuca alternifolia upon mechanical wounding.

PART 1: IMMEDIATE EFFECTS

6.1 Wounding-induced changes in essential oil concentration and composition

6.1.1 Materials and Methods

6.1.1.1 Plants

Potted, five-month old O. minimum, S. officinalis and M. alternifolia plants were used. The O. minimum and S. officinalis plants were purchased as seedlings from a local
nursery while the Australian Tea Tree Oil Research Institute (ATTORI) kindly provided the *M. alternifolia* plantlets. All plants were grown under field conditions and irrigated daily until used.

6.1.1.2 Headspace sampling

Part of a small branch (possessing approximately 3-4 pairs of leaves for *O. minimum* and *S. officinalis* or 3-4 small twigs for *M. alternifolia*) was placed in the sampling device described in Figure 2.1 (section 2.3.2, Chapter 2). After 30 minutes the headspace above the branch was sampled using a polydimethylsiloxane-coated fibre (10 minutes, 25 °C). Thermal desorption of the fibre in the injector of the GC and GC-MS system followed (220 °C, 10 min.). Three of the enclosed leaves were then cut longitudinally in half. The headspace was then monitored by SPME (10 min. exposure) for 70 minutes at 20-minute intervals. The wounded leaves remained attached to the plant at all times.

The whole procedure was repeated with unwounded leaves and the results were used to correct for depletion of the headspace volatiles due to the fibre and other headspace losses in the system (section 2.3.2.2, Chapter 2).

6.1.1.3 GC/GC-MS analysis

Identical equipment and parameters as those described in section 2.2.2.2.3 (Chapter 2) were used.

6.1.1.4 Quantitative determination of terpenoids in the vapour phase

The quantitation of terpenoids in the gas phase was achieved by utilising the SPME coating-gas partition coefficients ($K_{fg}$) calculated in Chapter 4. An example
(determination of concentration of 1,8-cineole above S. officinalis leaves) is given in Appendix 6.1.

6.1.2 Results-Discussion

Figure 6.1 shows the damage-induced changes in the concentration of essential oil in the headspace above M. alternifolia leaves. As it can be seen, a dramatic increase in the amount of all components occurs immediately upon wounding (0-10 min. post-wounding period) but levels return to unwounded (control) levels within a relatively short period of time (40-50 min. post-wounding period). Similar results were obtained for O. minimum and S. officinalis (Table 6.1).

![Diagram showing changes in detector response over post-wounding time.](image)

**Figure 6.1.** Changes in the headspace concentration of volatiles above M. alternifolia leaves as a result of wounding (mean ± standard error, n=3). Samples were adjusted to the same scale based on their unwounded (control) values. The immediate increase and then subsequent decrease in headspace concentration over time is evident.
The rapid release of essential oil in the atmosphere, as is demonstrated in Figure 6.1, is likely to be caused by the rupture of the leaf’s secretory cells as a result of the wounding process. In the case of *M. alternifolia* “intentional" release of oil through modified epidermal cells may also be possible considering the known localisation of oil in apoplastically isolated glands (List et. al., 1995). Experiments by Sachs et. al. (1990) determined the weekly loss through these cells to be equivalent to 3 % of the total oil content.

**Table 6.1.** Wounding-induced changes in the concentration of the headspace above *S. officinalis* L., *O. minimum* L. and *M. alternifolia* Cheel leaves for major representatives of the mono- and sesquiterpenoids (25 °C). Values (mean, n=3) are in nanograms within a headspace volume of 20 mL. An example of a calculation for one of these values can be found in Appendix 6.1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount in headspace, ng (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unwounded (control)</td>
<td>0-10 min. post-wounding</td>
<td>40-50 min. post-wounding</td>
<td></td>
</tr>
<tr>
<td><strong>S. officinalis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camphene</td>
<td>22.3 (5.6)</td>
<td>86.6 (7.9)</td>
<td>19.3 (3.5)</td>
<td></td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>66.3 (16.8)</td>
<td>216 (19.6)</td>
<td>92.5 (16.7)</td>
<td></td>
</tr>
<tr>
<td>α-thujone</td>
<td>260 (65.9)</td>
<td>654 (59.7)</td>
<td>369 (66.5)</td>
<td></td>
</tr>
<tr>
<td>β-thujone</td>
<td>24.0 (6.1)</td>
<td>62.4 (5.7)</td>
<td>36.1 (6.5)</td>
<td></td>
</tr>
<tr>
<td>Camphor</td>
<td>20.1 (5.1)</td>
<td>74.1 (6.8)</td>
<td>35.7 (6.3)</td>
<td></td>
</tr>
<tr>
<td>E-β-caryophyllene</td>
<td>0.7 (0.2)</td>
<td>1.6 (0.2)</td>
<td>0.9 (0.2)</td>
<td></td>
</tr>
<tr>
<td>α-humulene</td>
<td>1.2 (0.3)</td>
<td>2.2 (0.3)</td>
<td>1.5 (0.3)</td>
<td></td>
</tr>
<tr>
<td><strong>Totala (ng)</strong></td>
<td>395 (100)</td>
<td>1096 (100)</td>
<td>555 (100)</td>
<td></td>
</tr>
<tr>
<td><strong>O. minimum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>14.7 (9.6)</td>
<td>42.2 (8.9)</td>
<td>11.2 (7.1)</td>
<td></td>
</tr>
<tr>
<td>Linalool</td>
<td>21.0 (13.7)</td>
<td>64.3 (13.6)</td>
<td>19.5 (12.4)</td>
<td></td>
</tr>
<tr>
<td>Camphor</td>
<td>2.7 (1.8)</td>
<td>8.7 (1.8)</td>
<td>2.9 (1.8)</td>
<td></td>
</tr>
<tr>
<td>Eugenol</td>
<td>49.8 (32.5)</td>
<td>155 (32.7)</td>
<td>61.2 (38.9)</td>
<td></td>
</tr>
<tr>
<td>E-β-caryophyllene</td>
<td>8.0 (5.2)</td>
<td>26.1 (5.5)</td>
<td>9.2 (5.8)</td>
<td></td>
</tr>
<tr>
<td>E-α-bergamotene</td>
<td>34.1 (22.3)</td>
<td>102 (21.6)</td>
<td>32.3 (20.5)</td>
<td></td>
</tr>
<tr>
<td>Bicyclogermacrene</td>
<td>11.3 (7.4)</td>
<td>35.8 (7.6)</td>
<td>9.9 (6.3)</td>
<td></td>
</tr>
<tr>
<td>Δ-cadinene</td>
<td>11.6 (7.6)</td>
<td>39.5 (8.3)</td>
<td>11.3 (7.2)</td>
<td></td>
</tr>
<tr>
<td><strong>Totala (ng)</strong></td>
<td>153 (100)</td>
<td>474 (100)</td>
<td>157 (100)</td>
<td></td>
</tr>
</tbody>
</table>

(continued on next page)
The gradual decrease in oil concentration in the headspace above the wounded leaves (Figure 6.1, Table 6.1) is mostly due to the depletion of the ruptured oil-cavities' from their content.

The magnitude of the increase in the amount of terpenoids emitted by *M. alternifolia* leaves between the pre-wounding and 0-10 minutes post-wounding period (Table 6.1) is approximately 7 times greater than that observed for *O. minimum* and *S. officinalis* (30.5 to 464 compared with 153 to 474 and 395 to 1096 ng, respectively) (Table 6.1). This may reflect the fact that *M. alternifolia* possesses internal secretory structures (or cavities) (List et al., 1995) while *O. minimum* and *S. officinalis* have glandular trichomes (Croteau 1988; Werker et al., 1993). Thus, the difference in emission before and after wounding is greater in *M. alternifolia* than the other two species due to the (relatively) lower emission of terpenoids at the pre-wounding stage resulting from the embedding of the secretory structure within the leaf tissue.
A significant part of the previously-stored oil, when released by the damaged trichomes/cavities, comes in contact with leaf-tissue adjacent to the wound site causing rapid cell necrosis. This action may be "unintentional" but it can also mediate a simple but effective defense mechanism considering that many plants are known to invoke various protective measures (including rapid cell death or hypersensitive response\(^1\)) to "seal-off" damaged tissue from pathogens (e.g., Bate and Rothstein, 1998; Ward et al., 1991).

As Table 6.1 shows, there are no immediate significant wounding-induced changes in the essential oil \textit{composition} from the species examined. This is expected considering that studies on corn and cotton have showed that several hours are required for the \textit{de novo} biogenesis and release of wound-induced terpenoids in these crops (Paré and Tumlinson, 1997; Turlings \textit{et. al.}, 1995).

\(^1\) Further details about the hypersensitive reponse can be found in Chapter 8.
6.2 Lipoxygenase (LOX)-derived products

6.2.1 Introduction

Green leaves are known to produce C₆-C₉ compounds such as E-3-hexenol as a response to wounding (Charron and Cantliffe, 1995). These volatile alcohols, aldehydes and esters are collectively termed lipoxygenase (LOX)-derived products (Anderson, 1989). LOX products have been detected in a wide range of plants and in many species they form the major part of the plant's emissions (Charron and Cantliffe, 1995 and references therein).

The formation of LOX products is initiated by the hydrolysis of lipids by lipid acyl hydrolase (Charron and Cantliffe, 1995). Lipoxygenases (LOXs), which are nonheme iron containing dioxygenases, then catalyse the addition of molecular oxygen to the Z,Z,-1,4-pentadiene moiety of fatty acids such as linoleic (C₁₈:₂) and linolenic (C₁₈:₃) acids, resulting in the formation of fatty acid hydroperoxides (Anderson, 1989; Deng et al., 1993; Rosahl, 1996). The hydroperoxides are further converted, by lyases, into aldehydes such as hexanal and Z-3-hexenal; these aldehydes are then reduced to the corresponding alcohols by alcohol dehydrogenases (Anderson, 1989; Deng et al., 1993). Esterification of the most abundant alcohols such as Z- and E-3-hexenol with common metabolic carboxylic acids leads to the formation of esters such as E-3-hexenyl acetate (Ruther, 2000).

Previous studies have indicated that LOX-derived products (e.g., volatile alcohols and aldehydes, traumatin, jasmonic acid) may play a role in signal transduction of the wound response, as regulators of development and as antimicrobial substances in
host-pathogen interactions (Doehlert et al., 1993; Rosahl, 1996; Vaughn and Gardner, 1993).

6.2.2 Materials and methods

6.2.2.1 Plants

*O. minimum*, *S. officinalis* and *M. alternifolia* plants were used. These were physiologically similar to those described in section 6.1.1.1 above. *Prunus persica* L. Batch (Common peach) leaves (used for comparison) were obtained from a young tree purchased at a local nursery.

6.2.2.2 Headspace sampling

The level of emission of LOX-derived products in relation to post-wounding time was determined according to the procedure described in section 6.1.1.2 above. The absolute amount of LOX products emitted by *O. minimum*, *S. officinalis* and *M. alternifolia* leaves, as well as leaves from *P. persica* (for comparison), were determined by placing ~0.2 g of leaf material (cut in pieces) in a 0.35 mL glass insert inside a 2 mL vial (Supelco). The vial was sealed and the leaves were left to equilibrate for 1 hour before SPME was carried out for 1 hour using the 100 μm PDMS fibre (Supelco). GC and GC-MS analysis followed.

6.2.2.3 GC and GC-MS analysis

Identical equipment and parameters as those described in section 2.2.2.2.3 (Chapter 2) were used.

6.2.2.4 Quantitative determination of LOX products in the vapour phase

The quantitation of LOX products in the gas phase was achieved by experimentally determining SPME coating-gas partition coefficients (*K_{fr*}*) for the
compounds detected. The procedure was identical to that described in section 4.7.2 (Chapter 4) using commercially obtained Z-3-hexenol and Z-3-hexenyl acetate (Sigma-Aldrich). The calculation of the amount of analytes in the headspace was similar to that shown in Appendix 6.1. The results were expressed as amount of analyte released per mm of wound to enable comparison between the various species.

6.2.3 Results-Discussion

Z-3-hexenol and Z-3-hexenyl acetate (Figure 6.2) were the only LOX compounds detected in emissions from wounded *M. alternifolia, O. minimum,* and *S. officinalis* leaves. Other, commonly encountered LOX products, such as *E*-2-hexenol, *E*-3-hexenol and *E*-3-hexenyl acetate (Ruther, 2000) were not detected in the emissions of the species investigated.

![Chemical structures](Z-3-hexenol and Z-3-hexenyl acetate)

**Figure 6.2.** Structures of the LOX compounds detected in this study.

The wound-induced release of LOX-derived products for the three species of interest (*M. alternifolia, O. minimum, S. officinalis*) was found to be time dependent and followed a similar trend to that observed for the constitutive terpenoids (Figure 6.1, section 6.1.2).
FIGURE 6.3. Changes in the headspace concentration of Z-3-hexenol above *M. alternifolia* leaves as a result of wounding (mean ± standard error, n=3). Samples were adjusted to the same scale based on their unwounded (control) values. An identical trend was observed with Z-3-hexenyl acetate.

As can be seen from Figure 6.3, which shows the changes in headspace concentration of Z-3-hexenol above *M. alternifolia* leaves as an example, the production and release of the LOX compounds is initiated by leaf-tissue stress and damage. These volatiles reach their maximum level in the headspace between 20 and 30 post-wounding minutes and then gradually decrease to pre-wounding levels (Figure 6.3).
All species investigated exhibited identical behaviour (with that shown in Figure 6.3) in relation to the release of wound-induced LOX components. However, it was interesting to determine whether an inter-species variation about the amount of LOX products emitted was present, considering that Sekiya et al. (1983) found varying degrees of LOX activity in different species after surveying a wide range of non oil-bearing plants. In order to quantitatively investigate the level of variation (if any), the $K_{fg}$'s of Z-3-hexenol and Z-3-hexenyl acetate in relation to the 100 μm PDMS fibre were determined (Table 6.2).

**Table 6.2.** Experimentally determined $K_{fg}$'s for the LOX compounds released by the plant species investigated in this study. These values (mean ± sem, n=3) were determined using SPME and static HS (as per section 4.7.2, Chapter 4) on authentic standards prepared as described in section 4.2.2, Chapter 4.

<table>
<thead>
<tr>
<th>LOX products</th>
<th>Experimentally determined $K_{fg}$'s (100 μm PDMS at 25 °C) (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Z-3-hexenol</td>
<td>4.73x10⁹</td>
</tr>
<tr>
<td>Z-3-hexenyl acetate</td>
<td>9.64x10⁹</td>
</tr>
</tbody>
</table>

Using the $K_{fg}$'s from Table 6.2, the absolute amount of the two wound-induced LOX components emitted by the species of interest was determined (Table 6.3). The results were expressed as pg emitted per mm of wound to facilitate comparison between the species. Differences in leaf-anatomy and development, moisture, and size prohibited normalization of the data using other factors (e.g., weight).
Table 6.3. Absolute amounts of wound-induced LOX-derived products released by the three species of interest and *Prunus persica* for comparison (mean ± standard error of the mean, n=3). The length of wound inflicted in each species can be found in Appendix 6.2.

<table>
<thead>
<tr>
<th>Species</th>
<th>LOX-derived products detected (pg per mm of wound) (mean ± s.e.m, n=3)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z-3-hexenol</td>
<td>Z-3-hexenyl acetate</td>
</tr>
<tr>
<td><em>M. alternifolia</em></td>
<td>3.62±0.56</td>
<td>3.32±0.24</td>
</tr>
<tr>
<td><em>S. officinalis</em></td>
<td>3.89±0.42</td>
<td>3.76±1.07</td>
</tr>
<tr>
<td><em>O. minimum</em></td>
<td>4.02±0.99</td>
<td>1.99±0.10</td>
</tr>
<tr>
<td><em>P. prunus</em></td>
<td>7.70±1.66</td>
<td>21.60±1.94</td>
</tr>
</tbody>
</table>

As can be seen from Table 6.3, the three terpene-accumulating plants investigated emit approximately the same amount of the two LOX compounds; the only exception being *O. minimum* which emits slightly lower levels of Z-3-hexenyl acetate compared to *M. alternifolia* and *S. officinalis*.

Table 6.3 also shows that *Prunus persica* (common peach tree, a non oil-bearing plant randomly chosen for comparison) releases a significantly higher amount of the two LOX compounds (especially Z-3-hexenyl acetate) than any of the three essential oil-bearing plants used in this study. Although these results cannot be used for generalization due to the limited number of species used, they do suggest that oil-bearing plants may produce and utilize less amounts of LOX-derived compounds than non-terpene-accumulating species such as *P. persica*. Oil-bearing species are probably still employing LOX compounds for specific tasks but it is likely that they use terpenoids as their main protective and signaling substances. This view is supported by the fact that a greater amount of terpenoids than LOX compounds is emitted by terpene-accumulating plants when wounded (Table 6.4).
TABLE 6.4. Amounts (mean, n=3) of terpenoids and LOX products released by the three species of interest during the 0-10 min. post-wounding period (within a headspace volume of 20 mL). Values were determined as described in Appendix 6.1 using K<sub>6</sub>'s from Tables 4.4, 4.5 and 6.2.

<table>
<thead>
<tr>
<th>Species</th>
<th>Amount in headspace, ng (%)(mean, n=3)</th>
<th>LOX compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Terpenoids</td>
<td>LOX compounds</td>
</tr>
<tr>
<td>M. alternifolia</td>
<td>464 (89.6)</td>
<td>53.9 (10.4)</td>
</tr>
<tr>
<td>S. officinalis</td>
<td>1096 (97.9)</td>
<td>22.6 (2.1)</td>
</tr>
<tr>
<td>O. minimum</td>
<td>474 (97.9)</td>
<td>9.7 (2.1)</td>
</tr>
</tbody>
</table>

The utilization of terpenoids, for most day-to-day interactions between terpene-accumulating plants and their surrounding environment, appears to be a sensible approach considering the significant amount of energy, carbon and other resources required for the production of these essential oils (Gershenzon and Croteau, 1991). It would be very difficult and a waste of resources for these plants to maintain high levels of both terpenoids and LOX-derived compounds.

6.3 Immediate wound-induced changes: Conclusion

Rapid release of constitutive terpenoids and smaller amounts of LOX-derived compounds was observed to result from mechanical wounding of the terpene accumulating plants investigated. The release of these substances was found to peak between 0-30 post-wounding minutes and gradually return to pre-wounding levels within about 1 hour. All three oil-bearing plants tested exhibited this behaviour.
PART 2: LONGER-TERM EFFECTS

6.4 Wound-induced changes in leaf essential oil composition (over 48 hours)

6.4.1 Materials and Methods

6.4.1.1 Plants.

Five month old Ocimum minimum and Salvia officinalis plants were used. The plants were purchased as seedlings from a local nursery and were grown under field conditions (with daily irrigation) until required. Healthy, pest-free, two-year regrowth Melaleuca alternifolia trees were used (high terpnen-4-ol, low 1,8 cineole chemotype). The trees were part of a small plantation located at the Precinct of Horticulture, Faculty of Science, Technology and the Environment, University of Western Sydney Hawkesbury, New South Wales, Australia (Appendix 6.3).

6.4.1.2 Field conditions

The experiments were carried out during spring (temperature 21-26 °C, humidity 65-80 %). Sampling was performed at midday, only during full light conditions. The plants were irrigated daily and each plant was wounded only once.

6.4.1.3 Experimental protocol

M. alternifolia. Three leaves (of approximately the same age and size) were cut in half longitudinally at 0 hour (T₀), combined, weighed and immediately subjected to solvent extraction. After 24 hours the other half of the leaves (that had remained on the branch) was taken and treated similarly. The above process was carried out on eight different trees divided into two groups. The two groups were sampled one month apart in order to test the reproducibility of the results. The 48-hour treatment was carried out in the same way but this time the second half of each leaf was sampled 48 hours after hour zero. The whole procedure was carried out on
mature and flush growth (immature) *M. alternifolia* leaves. Different trees were used for each treatment and each leaf type. The 2\(^{nd}\) and 3\(^{rd}\) pair of leaves from the apex (yellowish-light green in colour) were used as flush growth.

*O. minimum* and *S. officinalis*. The process was similar to that described for *M. alternifolia* but in this case laterally-cut strips from three leaves (3\(^{rd}\)-4\(^{th}\) pair from the apex) were used.

6.4.1.4 Sample preparation

After sampling the leaf material (10-15 mg) was immersed in CH\(_2\)Cl\(_2\) (100 µL) spiked with 400 ppm octane (as an internal standard) for 24 hours at room temperature in the dark.

6.4.1.5 GC and GC-MS analysis

GC analysis was carried out using a HP 6890 gas chromatograph equipped with a flame ionisation detector and a HP 7673 GC/SFE auto-injector. The column used was BPX-5 (50 m length x 0.22 µm ID x 0.25 µm film thickness) (SGE Scientific, Melbourne, Australia). The injection volume was 2 µL, inlet temperature and pressure were 280 °C and 20 psi respectively, carrier gas was H\(_2\) (40 mL/min.), split ratio was 1:10, detector temperature at 280 °C, and the oven program was: initial temperature 60 °C for 5 min., increased to 180 °C at 4°C/min., final temperature maintained for 5 min.

GC-MS analysis conditions were as above with He as the carrier gas. The instrument used was a Varian 3800 gas chromatograph connected to a Varian 2000 ion trap detector (0.9 scans/sec, 20 µAmp). Compounds were identified by comparison of their mass spectra and retention indices (based on *n*-alkanes) with those of authentic standards (Fluka Chemicals, NSW, Australia) and by comparison with published data (Brophy et al., 1989; Adams, 1995).
6.4.1.6 Determination of moisture

$T_0$, $T_{24}$, and $T_{48}$ leaves (experimental design as described above, section 6.4.1.3) were placed in a glass petri-dish and dried for 10 days at 50 °C. Moisture was determined by the weighing of the leaves before and after drying. The experiment was performed three times.

6.4.1.7 Statistical analysis

The difference between the percentage contribution of each compound in the oil before and after wounding was tested for statistical significance using the Wilkoxon signed ranks test (paired samples) (Sall and Lehman, 1996). A correlation matrix was then built (based on the actual percentages) using as variables the compounds found to be statistically significant. PCA was then performed on the correlations and the eigenvalues obtained were used to visualize the relationship between objects (leaves) in two-dimensional score plots. The association between the significant variables (compounds) was examined based on their loading values on the first two principal components. Data from independent samples (e.g., comparison between treatments) was analysed as above; however in this case the Kruskal – Wallis test was applied (Sall and Lehman, 1996). The effect of wounding on the non-parametric measures of association between the major thujanes and $p$-menthanes based on their percentage contribution to the flush growth extracts was also investigated (Spearman's test) (Sall and Lehman, 1996). The software used for all data analyses was JMP-IN® (version 3 for Windows) (SAS Institute Inc., USA).
6.4.2 Results - Discussion

6.4.2.1 *Melaleuca alternifolia*

*a) Flush growth.* The statistical analysis showed a significant change in the percentage composition of several constitutive terpenoids between unwounded and wounded *M. alternifolia* flush growth leaves (Table 6.5). The 24 hour post-wounding results showed that the major *p*-menthanes (*α-* and *γ*-terpinene, terpinolene, terpinen-4-ol) increased in concentration at the expense of the thujanes (*Z*-sabinene hydrate, sabinene) (Table 6.5).

**Table 6.5.** Effect of mechanical damage on oil composition in flush growth of *M. alternifolia.* Selected components as detected after cold solvent extraction are presented. Asterisks indicate statistical significance (* at *p*<0.02; ** at *p*<0.01, Wilcoxon signed-ranks test).

<table>
<thead>
<tr>
<th>Components</th>
<th>Difference in percentage composition between unwounded and wounded leaves (mean±S.E,M) (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours after injury</td>
</tr>
<tr>
<td>α-thujene</td>
<td>-0.01±0.01</td>
</tr>
<tr>
<td>α-pinene</td>
<td>-0.03±0.03</td>
</tr>
<tr>
<td>sabinene</td>
<td>-0.44±0.23</td>
</tr>
<tr>
<td>cluster 1</td>
<td>-0.06±0.05</td>
</tr>
<tr>
<td>α-terpinene</td>
<td>0.34±0.06*</td>
</tr>
<tr>
<td>cluster 2</td>
<td>0.14±0.08</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>0.63±0.09*</td>
</tr>
<tr>
<td><em>E</em>-sabinene hydrate</td>
<td>0.04±0.11</td>
</tr>
<tr>
<td>terpinolene</td>
<td>0.29±0.04*</td>
</tr>
<tr>
<td><em>Z</em>-sabinene hydrate</td>
<td>-1.54±1.05</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>1.19±0.23*</td>
</tr>
<tr>
<td>α-terpineol</td>
<td>0.11±0.09</td>
</tr>
<tr>
<td>sesquiterpenes</td>
<td>-0.76±1.41</td>
</tr>
</tbody>
</table>

*cluster 1 includes *β*-pinene and myrcene
*cluster 2 includes *p*-cymene, limonene, *β*-phellandrene and 1,8-cineole.
The conversion of the thujane precursors to the more stable \( p \)-menthanes occurs naturally in \textit{M. alternifolia} flush growth as the leaves mature (Southwell and Stiff, 1989; Cornwell \textit{et al.}, 1995). During the aging process, \( Z \)-sabinene hydrate and to a lesser extent sabinene and \( E \)-sabinene hydrate are known to be implicated in the formation of terpinen-4-ol, terpinolene, \( \alpha \)- and \( \gamma \)-terpinene (Cornwell \textit{et al.}, 1995; Southwell, 1999).

\[ 
\text{HO}_1 \quad \text{1} \\
\text{2} \\
\text{3} \\
\text{4} \\
\text{5} \\
\text{6} \\
\text{7} \\
\text{8} \\
\]

**Figure 6.4.** Transformation of thujanes (\( E \)- and \( Z \)-sabinene hydrate (1), sabinene (2)) to \( p \)-menthane (terpinen-4-ol (5), terpinolene (6), \( \gamma \)- (7) and \( \alpha \)-terpinene (8)) monoterpenes in \textit{M. alternifolia} leaves via the thujyl (3) and \( \alpha \)-terpinyll (4) carbocations (Cornwell \textit{et al.}, 1995). The above transformations occur during the course of leaf development.

The results obtained from the mechanical wounding experiments confirmed the interdependence between thujanes and \( p \)-menthanes and demonstrated the approximate rate of conversion (Table 6.5, Figure 6.5).
Figure 6.5. Change (mean ± sem, n=8) in the level of thujanes and p-menthanes in relation to post-wounding time.

The correlations of the compounds of interest (thujanes and p-menthanes) before and after wounding were examined (Spearman’s test) to investigate whether mechanical wounding was responsible for any of the changes demonstrated above.

From Table 6.6 it can be seen that in unwounded flush growth significant linear correlations were obtained only between concentrations of compounds of the same skeletal group (with the exception of the relationship of Z-sabinene hydrate with α-terpinene). In contrast, in wounded leaves several significant linear correlations were observed between p-menthanes and their precursor thujanes. Sabinene and Z-sabinene hydrate appear to be strongly related to terpinen-4-ol and γ-terpinene whilst sabinene itself is also correlated with terpinolene. Also in wounded leaves α-terpinene was not linearly related with any of the major thujanes.
**Table 6.6.** Non-parametric correlation coefficients between the major thujanes and $p$-menthanes in *M. alternifolia* flush growth for unwounded (n=14) and wounded leaves (both 24 and 48 hours treatments, n=16). Asterisks indicates statistical significance (* at $p<0.05$; ** at $p<0.01$, Spearman's test).

<table>
<thead>
<tr>
<th>Variable by variable</th>
<th>Spearman's $\rho$</th>
<th>Unwounded leaves</th>
<th>Wounded leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-terpinene - sabinene</td>
<td>-0.2478</td>
<td>-0.2789</td>
<td></td>
</tr>
<tr>
<td>$\gamma$-terpinene - sabinene</td>
<td>-0.2156</td>
<td>-0.7574**</td>
<td></td>
</tr>
<tr>
<td>$\gamma$-terpinene - $\alpha$-terpinene</td>
<td>0.6619**</td>
<td>0.6966**</td>
<td></td>
</tr>
<tr>
<td>$E$-sabinene hydrate - sabinene</td>
<td>0.3633</td>
<td>0.0324</td>
<td></td>
</tr>
<tr>
<td>$E$-sabinene hydrate - $\alpha$-terpinene</td>
<td>0.0871</td>
<td>0.0496</td>
<td></td>
</tr>
<tr>
<td>$E$-sabinene hydrate - $\gamma$-terpinene</td>
<td>0.3917</td>
<td>0.1468</td>
<td></td>
</tr>
<tr>
<td>terpinolene - sabinene</td>
<td>-0.1597</td>
<td>-0.5403*</td>
<td></td>
</tr>
<tr>
<td>terpinolene - $\alpha$-terpinene</td>
<td>0.9319**</td>
<td>0.5583</td>
<td></td>
</tr>
<tr>
<td>terpinolene - $\gamma$-terpinene</td>
<td>0.6935**</td>
<td>0.8657**</td>
<td></td>
</tr>
<tr>
<td>terpinolene - $E$-sabinene hydrate</td>
<td>0.0000</td>
<td>0.4122</td>
<td></td>
</tr>
<tr>
<td>$Z$-sabinene hydrate - sabinene</td>
<td>0.5842*</td>
<td>0.7828**</td>
<td></td>
</tr>
<tr>
<td>$Z$-sabinene hydrate - $\alpha$-terpinene</td>
<td>-0.5957*</td>
<td>-0.5090</td>
<td></td>
</tr>
<tr>
<td>$Z$-sabinene hydrate - $\gamma$-terpinene</td>
<td>-0.3868</td>
<td>-0.7710**</td>
<td></td>
</tr>
<tr>
<td>$Z$-sabinene hydrate - $E$-sabinene hydrate</td>
<td>0.5233*</td>
<td>0.3291</td>
<td></td>
</tr>
<tr>
<td>$Z$-sabinene hydrate - terpinolene</td>
<td>-0.4286</td>
<td>-0.4293</td>
<td></td>
</tr>
<tr>
<td>terpinene-4-ol - sabinene</td>
<td>-0.1079</td>
<td>-0.7142**</td>
<td></td>
</tr>
<tr>
<td>terpinene-4-ol - $\alpha$-terpinene</td>
<td>0.8070**</td>
<td>0.6020*</td>
<td></td>
</tr>
<tr>
<td>terpinene-4-ol - $\gamma$-terpinene</td>
<td>0.9281**</td>
<td>0.9372**</td>
<td></td>
</tr>
<tr>
<td>terpinene-4-ol - $E$-sabinene hydrate</td>
<td>0.2923</td>
<td>0.3255</td>
<td></td>
</tr>
<tr>
<td>terpinene-4-ol - terpinolene</td>
<td>0.8574**</td>
<td>0.9407**</td>
<td></td>
</tr>
<tr>
<td>terpinene-4-ol - $Z$-sabinene hydrate</td>
<td>-0.4570</td>
<td>-0.6141*</td>
<td></td>
</tr>
</tbody>
</table>

The results presented above indicate that wounding has an effect distinctly different from the normal ontogenetical changes occurring during flush growth maturation.

It can only be speculated as to how leaf wounding possibly mediates this effect in *M. alternifolia* flush growth. Recent work has established that the thujanes to $p$-menthanes transformations are pH and water dependent (Southwell and Stiff, 1989; Cornwell 1999). Therefore it is possible that cell rupture alters pH and water levels within the leaf thus affecting the rate of chemical maturation in the flush growth and inducing the "abnormal" component-correlations seen in Table 6.6.
above. Experiments showed that water levels in flush growth are not greatly affected by wounding (at least within the 48-hour period) (Table 6.7) but this slight change may be adequate for the "catalysis" of the maturation process.

**Table 6.7.** Moisture content (%*, mean ± std, n=3*) for *M. alternifolia* flush growth in relation to mechanical wounding.

<table>
<thead>
<tr>
<th>Experiment type</th>
<th>Moisture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>‘Control’ (T₀)</td>
</tr>
<tr>
<td>24-hour treatment</td>
<td>59.7 ± 4.54</td>
</tr>
<tr>
<td>48-hour treatment</td>
<td>61.4 ± 3.95</td>
</tr>
</tbody>
</table>

Rapid chemical maturation of the flush growth may be interpreted as an attempt to reduce subsequent predation. The most damaging pest of *M. alternifolia*, the Pyrgo beetle (*Paropsisterna tigrina*) (Maddox, 1995; Treverrow, 1992), is known to feed exclusively on flush growth leaves and does not consume mature leaves even when it is starved to death (Maddox, 1995). Although other parameters such as leaf palatability may be responsible for this preference, the difference in oil composition between flush growth and mature leaves may also contribute. Plant-insect interactions are discussed in more detail in Chapter 8.

b) *Mature leaves.* Different (from each other) sets of oil constituents for each treatment are utilised to express the effect of wounding on *M. alternifolia* mature leaves (Table 6.8). α-Thujene, E- and Z-p-menth-2-en-1-ol and sesquiterpenes are involved in the 24-hour post-wounding response whilst α-thujene, cluster 2, terpinolene and Z-p-menth-2-en-1-ol are active during the 48-hour post-wounding response (Table 6.8). The monoterpenes α-thujene and Z-menth-2-en-1-ol are the only components that change significantly in both, 24- and 48-hour, post-wounding measurements (Table 6.8). The other terpenes that initially change their
concentration appear to revert back to their pre-wounding levels; a different set of monoterpenes is then used for the expression of the response.

**Table 6.8.** Effect of mechanical damage on oil composition in mature growth of *M. alternifolia*. Selected components as detected after cold solvent extraction are presented. Asterisks indicate statistical significance (* at *p*<0.05; ** at *p*<0.01, Wilcoxon signed-ranks test).

<table>
<thead>
<tr>
<th>Components</th>
<th>Difference in percentage composition between unwounded and wounded leaves (mean±S.E.M) (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours after injury</td>
</tr>
<tr>
<td>α-thujene</td>
<td>0.11±0.02**</td>
</tr>
<tr>
<td>α-pinene</td>
<td>0.02±0.03</td>
</tr>
<tr>
<td>β-pinene</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>myrcene</td>
<td>0.02±0.05</td>
</tr>
<tr>
<td>α-terpinene</td>
<td>-0.25±0.57</td>
</tr>
<tr>
<td>cluster 2 a</td>
<td>0.36±0.54</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>0.14±0.12</td>
</tr>
<tr>
<td>terpinolene</td>
<td>0.02±0.10</td>
</tr>
<tr>
<td><em>E</em>-p-menth-2-en-1-ol</td>
<td>0.10±0.01**</td>
</tr>
<tr>
<td><em>Z</em>-p-menth-2-en-1-ol</td>
<td>0.11±0.01**</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>-0.28±0.35</td>
</tr>
<tr>
<td>α-terpineol</td>
<td>0.05±0.03</td>
</tr>
<tr>
<td><em>E</em>-piperitol b</td>
<td>0.40±0.24</td>
</tr>
<tr>
<td>unknown</td>
<td>0.21±0.14</td>
</tr>
<tr>
<td>sesquiterpenes</td>
<td>-1.07±0.35*</td>
</tr>
</tbody>
</table>

* a cluster 2 includes *p*-cymene, limonene, β-phellandrene, 1,8-cineole
  b tentative identification

The information in Table 6.8 indicates that mature leaves, to a limited extent, are biosynthetically active during the post-wounding period so some oil constituents are synthesised at the expense of others. This view is also supported by the fact that certain terpenes (e.g., *E*- and *Z*-menth-2-en-1-ol) greatly increased their presence in the oil (> 60% relative increase) within 24 hours (Table 6.8).

The increase in monoterpene levels at the expense of the sesquiterpenes in the first 24 post-wounding hours is also very interesting. Direct biosynthetic interdependence between the two terpene groups is not likely given that they are
synthesized in different cellular compartments (Bohlmann, et al., 1998). However it is possible that wounding affects either the amount of substrate available or the terpenoid synthases responsible for the production of these oil constituents, as compared to normal biosynthesis (further details in Chapter 7).

It is possible that oil components other than those listed (Table 6.8) were affected by leaf wounding. However, only the components that showed a consistent behaviour across the different trees used were labelled as significant by the statistical analysis. This together with the fact that half the trees were sampled one month apart from the other half ensured the validity of the detected response. As described (Chapter 2), previous experiments also showed that the changes outlined above did not originate from the different oil composition that the two leaf halves might have possessed due to developmental or other factors. Instrumental errors were also found to be negligible (Chapter 2).

Principal components analysis (PCA) two-dimensional score plots were constructed in order to further elucidate the detected wounding response (Figure 6.6). Plots defined by the first two principal components in each case were sufficient for that purpose as it could explain most of the variation in the data (> 88.2 %). From Figure 6.6 it can be seen that the wounding response in each mature leaf (for both treatments) is independent of the pre-wounding levels of the particular compound expressing the response. Also, the magnitude of the response appears to be similar between replicates within the same treatment, again, despite the large inter-tree variation. Similar behaviour was observed in the case of flush growth (Figure 6.7).
Figure 6.6. PCA scatterplots of oil from *M. alternifolia* mature leaves on the first 2 principal components (unwounded leaves squared, wounded leaves not squared). Identical numbers indicate same leaf (before – after wounding). a) 24 hours after injury, b) 48 hours after injury. Individuals 5-7 were sampled one month after individuals 1-4 to test the reproducibility of the response.
Figure 6.7. PCA scatterplots of oil from *M. alternifolia* flush growth leaves on the first 2 principal components (unwounded leaves squared, wounded leaves not squared). Identical numbers indicate same leaf (before – after wounding). a) 24 hours after injury, b) 48 hours after injury. Individuals 5-8 were sampled one month after individuals 1-4 to test the reproducibility of the response.
It is likely that the different sets of compounds (α-thujene, E- and Z-p-menth-2-en-1-ol and sesquiterpenes during the first 24 post-wounding hours and α-thujene, cluster 2, terpinolene and Z-p-menth-2-en-1-ol during the second 24 post-wounding hours) involved in the post-wounding response serve distinct purposes. Differential timing in the production of defence oleoresin components has also been detected in the defence response of Grand fir (Abies grandis) (Steele et al., 1998). In that case the monoterpenes formed immediately after wounding act as insect toxins while their later production at solvent levels is implicated in wound sealing (Steele et al., 1998). A recent study has shown that the biosynthetic pathways operating in M. alternifolia leaves are active at different times during leaf ontogeny (Southwell and Russell, 2002). This demonstrates that M. alternifolia is capable of "differential timing" in the production of specific volatile oil components.

No significant differences were found when the results from the two post-wounding measurements were compared to each other (Kruskal-Wallis test, $p<0.05$). This is reflected on the PCA scatterplots shown in Figure 6.8, where no separation between the "control" and treated leaves is observed, for both flush and mature leaves, along any of the two axis used in each case. This was probably due to the high level of inter-tree variation observed when independent samples (leaves from different trees) were considered during the statistical analysis. It is likely that the small compositional changes (if any) were "masked" by the high inter-tree variability which can be seen in Table 2.2 (Chapter 2).
FIGURE 6.8. PCA scatterplots of oil from *M. alternifolia* on the first 2 principal components for the 24-hour and 48-hour treatments (*T*₂₄ leaves clear, *T*₄₈ leaves shadowed). a) Flush growth and b) mature leaves.
6.4.2.2 *O. minimum*

The statistical analysis of the essential oil composition obtained from *O. minimum* leaves highlighted a group of five compounds that changed their contribution to the essential oil as a result of mechanical wounding over the two-day period. As shown in Table 6.9, the concentrations of eugenol, linalool, camphor, myrcene and methyl eugenol in the oil changed significantly during the first 24 post-wounding hours. Eugenol and linalool were the compounds affected the most during that period. However, camphor was the only compound with significantly altered percent concentration during the second 24 post-wounding hours.

**Table 6.9.** Effect of mechanical damage on oil composition from *O. minimum* leaves. Constituents with a concentration greater than 0.2% in the oil are shown. Asterisks indicate statistical significance (* at p<0.05, ** at p<0.01, Wilkoxon signed-ranks test).

<table>
<thead>
<tr>
<th>Components</th>
<th>Difference in percentage composition between unwounded and wounded leaves (mean±S.E.M) (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours after injury</td>
</tr>
<tr>
<td>α-pinene</td>
<td>0.05±0.04</td>
</tr>
<tr>
<td>sabinene</td>
<td>0.06±0.02</td>
</tr>
<tr>
<td>β-pinene</td>
<td>0.13±0.05</td>
</tr>
<tr>
<td>myrcene</td>
<td>0.09±0.04*</td>
</tr>
<tr>
<td>limonene/p-cymene</td>
<td>0.02±0.02</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>0.78±0.37</td>
</tr>
<tr>
<td>E-sabinene hydrate</td>
<td>0.05±0.02</td>
</tr>
<tr>
<td>linalool</td>
<td>2.48±0.58**</td>
</tr>
<tr>
<td>camphor</td>
<td>-0.20±0.04**</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>-0.08±0.04</td>
</tr>
<tr>
<td>α-terpineol</td>
<td>0.01±0.02</td>
</tr>
<tr>
<td>bornyl acetate</td>
<td>0.05±0.08</td>
</tr>
<tr>
<td>eugenol</td>
<td>4.82±0.91**</td>
</tr>
<tr>
<td>methyl eugenol</td>
<td>0.35±0.09**</td>
</tr>
<tr>
<td>sesquiterpenes</td>
<td>0.60±0.50</td>
</tr>
</tbody>
</table>
These results indicate that in *O. minimum* most of the essential oil transformations after injury occur during the first 24 post-wounding hours. After this period the wounded leaves start to return slowly to their pre-wounding metabolic state. Similar behaviour has been reported thus far for other commercially important crops such as corn and cotton (Turlings and Tumlinson, 1992; Turlings et al., 1995).

Developmental processes such as maturation are known to have an impact on the oil-profile of terpene accumulating plants especially in herbaceous species and is possible that the small but statistically significant changes observed above were caused by such processes (Croteau, 1988; Southwell and Stiff, 1989). Figure 2.7 (Chapter 2) shows the ontogenetical changes occurring in *O. minimum* leaves for the terpenoids of interest. The only considerable biogenetic transformation observed in Figure 2.7 is the decrease in the concentration of eugenol as the leaf reaches full maturation. However, considering that the 3rd and 4th pair of leaves (from the apex) were used in this work it is evident (Figure 2.7) that this transformation does not account for the wounding response observed. All the other compounds of interest showed either negligible variation (considering that these transformations occur over a few months) or a trend that was opposite to that observed when the leaves were wounded. For example, camphor levels in *O. minimum* decline after wounding (Table 6.9) but the opposite is observed during the aging process of the plant (Figure 2.7, Chapter 2) at least until the leaf reaches full maturation. Studies on common sage (*S. officinalis* L.) showed that the key enzymes involved in the production of camphor in this species are at the highest levels during the period of leaf expansion (Croteau, 1988).
The different nature of the compounds involved in the wounding response suggest that they possibly serve distinct purposes. The acyclic terpenes linalool and myrcene are known to be induced on Lima beans, cotton and corn by the feeding of herbivores (Takabayashi et al., 1994; Turlings et al., 1995; Turlings and Tumlinson, 1992). If there is an analogous situation with *O. minimum*, it is possible that the role of these similar compounds during the wounding response (in *O. minimum*) is the protection of the wounded leaves from further damage. Similar function could be attributed to methyl eugenol considering its known insect antifeedant and deterrent properties (Ngoh et al., 1998). Increased levels of methyl eugenol in the leaves after wounding are likely to be associated with the observed loss of eugenol considering that these components are biosynthetically related (Lewinsohn et al., 2000). However, the amount of eugenol lost from the leaves upon wounding is much greater than the amount of methyl eugenol produced. Given that no other (known) volatile metabolites of eugenol were detected in *O. minimum* and that glycosidic-bound eugenol is known to be abundant in the Lamiaceae (Merks and Svendsen, 1989) it is possible that the unaccounted for eugenol is converted to a non-volatile derivative. A similar behaviour is likely to be exhibited by camphor and therefore accounts for the amount of the ketone lost during the post-wounding period. Work on common sage has already shown that camphor undergoes catabolism by conversion to 1,2-campholide (a lactone) and then to a β-D-glucoside-6-o-glucose ester of the corresponding hydroxy acid (Croteau, 1988). The ester is then transported to the roots where it is re-utilised by the plant (Croteau, 1988). In this case eugenol and camphor may be utilised during repairing processes operating in *O. minimum* wounded leaves.
The oil-compositional change caused by wounding was further elucidated by principal component analysis (PCA). This feature-reduction technique allowed the visualization of the response in a two-dimensional score plot (Figure 6.9). A plot defined by the first two principal components was sufficient for that purpose as it could explain most of the variation in the data (> 81.3%). As with *M. alternifolia* leaves, Figure 6.9 shows that the wounding response in each leaf (observed as a shift of the points mostly along the PC1 axis) is independent of the pre-wounding levels of the particular compound expressing the response. Also, the magnitude of the response (distance travelled) appears to be similar between replicates, despite the large inter-plant variation which is evident from the scattering of the positions of the control points on the plot. This behaviour was also observed with *M. alternifolia* leaves (Figures 6.6 and 6.7).
**Figure 6.9.** PCA scatterplot of oil-profiles from *O. minimum* leaves on the first 2 principal components extracted using the group of five compounds as variables (unwounded (T₀) leaves squared, wounded (T₂₄) leaves not squared). Identical numbers indicate same leaf (before – after wounding). Individuals 5-8 were sampled one month after individuals 1-4 to test the reproducibility of the response.
6.4.2.3 *S. officinalis*

The statistical analysis of the oil composition from *S. officinalis* leaves highlighted a group of five compounds that changed their contribution to the essential oil as a result of injury over the two-day period (Table 6.10). *E*-salvene was the only constituent that changed its contribution to the oil during the first 24 post-wounding hours. In contrast, the level of 4 different compounds (or groups of compounds) (*Z*-salvene, *α*-thujene, *E*-sabinene hydrate and sesquiterpenes) changed during the second 24 post-wounding hours (Table 6.10).

**Table 6.10.** Effect of mechanical damage on oil composition from *S. officinalis* leaves. Asterisks indicate statistical significance (* at *p*<0.05; ** at *p*<0.01, Wilkoxon signed-ranks test).

<table>
<thead>
<tr>
<th>Components</th>
<th>Difference in percentage composition between unwounded and wounded leaves (mean±S.E.M) (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After 24 hrs</td>
</tr>
<tr>
<td><em>E</em>-salvene</td>
<td>-0.36±0.14*</td>
</tr>
<tr>
<td><em>Z</em>-salvene</td>
<td>0.24±0.43</td>
</tr>
<tr>
<td><em>α</em>-thujene</td>
<td>-0.02±0.04</td>
</tr>
<tr>
<td><em>α</em>-pinene</td>
<td>-0.31±0.22</td>
</tr>
<tr>
<td>camphene</td>
<td>-0.23±0.15</td>
</tr>
<tr>
<td>cluster 1 a</td>
<td>-0.39±0.22</td>
</tr>
<tr>
<td><em>p</em>-cymene/limonene</td>
<td>-0.07±0.07</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>-0.61±0.70</td>
</tr>
<tr>
<td><em>γ</em>-terpinene</td>
<td>-0.25±0.10</td>
</tr>
<tr>
<td><em>E</em>-sabinene hydrate</td>
<td>0.00±0.03</td>
</tr>
<tr>
<td><em>α</em>-thujone</td>
<td>-0.15±2.01</td>
</tr>
<tr>
<td><em>β</em>-thujone</td>
<td>-0.09±0.23</td>
</tr>
<tr>
<td>camphor</td>
<td>-0.16±0.52</td>
</tr>
<tr>
<td>pinocamphene</td>
<td>-0.03±0.03</td>
</tr>
<tr>
<td>borneol</td>
<td>-0.05±0.05</td>
</tr>
<tr>
<td>bornyl acetate</td>
<td>0.02±0.02</td>
</tr>
<tr>
<td>sesquiterpenes</td>
<td>-0.03±0.04</td>
</tr>
</tbody>
</table>

*cluster 1 includes *β*-pinene and myrcene
Experiments with *S. officinalis* leaves showed that the biogenetic transformations (Figure 2.6, Chapter 2) for the compounds of interest follow exact opposite trends with those seen in Table 6.10. This indicates that the detected changes are not due to ontogeny or other developmental processes.

The results presented in Table 6.10 demonstrate that in *S. officinalis* most of the wounding-induced essential oil transformations occur during the second 24 post-wounding hours. This behaviour is unlike that observed with *O. minimum* and *M. alternifolia* as those plants exhibit most changes during the first 24 post-wounding hours (Zabarás *et al.*, 2002; Zabarás and Wyllie, 2001; sections 6.4.2.1 and 6.4.2.2 here).

The detected change caused by wounding during the second 24 post-wounding hours was further elucidated by principal component analysis (PCA). Figure 6.10 shows that the wounding response in each leaf (observed as a shift of the points mostly along the PC1 axis) is independent of the pre-wounding levels of the particular compound expressing the response. Also, the magnitude of the response (distance between T₀ and T₄₈ leaves) appears to be similar between replicates, despite the large inter-plant variation which is evident from the scattering of the positions of the control points on the plot. Identical observations were made during the investigation of the wounding response of *O. minimum* and *M. alternifolia* (Zabarás *et al.*, 2002; Zabarás and Wyllie, 2001; sections 6.4.2.1 and 6.4.2.2).
PC 1 (81.6%)
($\alpha$-thujene and E-sabinene hydrate vs E-salvene and sesquiterpenes)

Figure 6.10. PCA scatterplot of oil-profiles from S. officinalis leaves on the first 2 principal components extracted using the group of four compounds as variables (unwounded ($T_0$) leaves squared, wounded ($T_{24}$) leaves not squared). Identical numbers indicate same leaf (before – after wounding). Individuals 5-8 were sampled one month after individuals 1-4 to test the reproducibility of the response.

6.4.2.4 Comparison of the response between the three species examined

The wounding response in each species was found to be independent of the pre-wounding levels of the particular compound expressing the response. Also, the magnitude of the response was similar between replicates, despite the large inter-plant variation. These aspects and the small in magnitude compositional changes of the essential oil appear to be common parameters of the wounding response produced by the terpene accumulating plants investigated. This may reflect the similarity in biosynthetic "tools" (i.e. enzymes, substrates) and pathways that must be activated in order for their terpenoid constituents to be produced.
6.4.2.5 Comparison of the response between oil- and non-oil producing plants

The oil-profile differences demonstrated above between unwounded and wounded *M. alternifolia*, *O. minimum* and *S. officinalis* leaves are small in magnitude especially when compared with published results obtained from non-terpene accumulating plants (Paré and Tumlinson, 1997; Turlings et al., 1995; Turlings and Tumlinson, 1992). It is possible that an elicitor may be required for a greater response to be induced. Studies on cabbage and corn seedlings have shown that the wound-response in these plants is much greater when an elicitor (for example enzymes found in the herbivore’s saliva) is present rather than the response obtained by simple mechanical damage (Coleman et al., 1997; Turlings et al., 1993). This hypothesis in relation to terpene accumulating plants will be tested in Chapter 8.

Also, it is possible that inducible defences in oil-bearing plants are limited due to the biosynthetic constraints faced by fully expanded leaves. Section 6.5 below will investigate the validity of this hypothesis using changes in leaf-essential oil as a guide. Wound-induced changes in leaf enzymatic activity will be investigated in Chapter 7.
6.5 The essential oil composition of leaves before wounding and emergent leaves 6 months later

6.5.1 Introduction

Studies on members of the Lamiaceae such as *Mentha piperita* (peppermint), *Salvia officinalis* and *Ocimum basilicum* (sweet basil), have shown that the activity of their cells specialised for terpene biosynthesis peaks very early in leaf-development and then gradually declines to zero as leaves mature (Croteau, 1988; Fahn, 1979; List et al., 1995; Maffei et al., 1989; McConkey et al., 2000; Werker et al., 1993). After their synthesis, the terpenoids are sequestered out of the cytoplasm and cell wall and are enclosed into the subcuticular space of the trichomes (Venkatachalam et al., 1984; Werker et al., 1993).

There is still controversy on whether chemical changes, such as monoterpene catabolism or turnover\(^1\), can occur while the secreted material is present within the subcuticular space (Croteau 1988; Gershenzon et al., 1993; List et al., 1995; Werker et al., 1993). These biosynthetic and physiological limitations however, suggest that wounding-induced changes in terpenoids should be sought not only in leaves present at the time of damage but also in leaves initiated and developed after damage has occurred (Gershenzon and Croteau, 1991). Leaves developed after damage may exhibit damage-induced changes in essential oil composition and concentration that other leaves, present on the plant during the damage, cannot express due to the constraints described above.

---

\(^1\) Monoterpene turnover is discussed further in Chapter 9.
It is important to test the validity of this hypothesis as the information obtained may be used to explain the small magnitude of the wound-induced changes observed for the species examined. In addition, if such a hypothesis is not tested, a characterisation of the behaviour of any oil-bearing plant under stress remains incomplete as there is a great risk of damage-induced responses passing undetected; this could lead to inaccurate conclusions being formed about the species studied.

This section describes an attempt to determine wound-induced changes in the terpenoids of *M. alternifolia* leaves initiated after wounding. Different leaves from the same plants, sampled before wounding, were used for comparison.

### 6.5.2 Materials and Methods

#### 6.5.2.1 Plant sampling

Two-year regrowth *M. alternifolia* trees (terpinen-4-ol chemotype) were used (part of a small plantation located at the University of Western Sydney, Hawkesbury Campus, New South Wales, Australia) (Appendix 6.3). Control samples (3 mature leaves per sample) were collected during Autumn 2000 (April), the trees were then wounded by cutting half of all their remaining leaves with scissors. In Spring 2000 (October) samples from the first flush growth observed were taken. Eight different trees were sampled.

#### 6.5.2.2 Solvent extraction

After sampling the leaf material (10-25 mg) was subjected to solvent extraction (section 2.2.2.2.2).
6.5.2.3 GC and GC-MS analysis

GC/GC-MS analysis carried out according to the parameters in section 2.2.2.2.3).

6.5.2.4 Data processing and statistical analysis

Data was processed using HP Chemstation® software (version A 3.0.1, Hewlett Packard, Melbourne Australia). The peak areas of the integrated gas-chromatograms were used to express the percentage contribution of each monoterpenoid (and of the sesquiterpenoids measured as a group) in the oil. The Kruskal-Wallis test (Sall and Lehman, 1996) was then applied to determine significant differences between the two (before and after) datasets.

6.5.3 Results-Discussion

The effect of mechanical damage on oil composition of *M. alternifolia* leaves initiated and developed after damage is shown in Table 6.11. Damage was carried out on other leaves present initially on the plants. The damaged plants were left for 6 months; then newly emerged flush leaves were used to determine any difference in essential oil composition between them and the leaves present on the plants initially. As can be seen, there is a large difference in the relative amount of thujanes (sabinene, *E*- and *Z*-sabinene hydrate) and *p*-menthanes (*α*-* and *γ*-*terpinene, terpinene-4-ol, *α*-terpineol, terpinolene) present in the oil of the leaves before and after damage. This simply reflects the different age of the leaves at each sampling and is not a result of the wounding process. The leaves used for the “before” values had matured (and thus were rich in *p*-menthanes) in contrast to the flush growth leaves used for the “after” values (rich in thujanes). The observed changes in the levels of *E*- and *Z*- *p*-menth-2-en-1-ol as well as *E*-piperitol, are also likely to originate from
the different age of the leaves used; the two \( p \)-menthenols were found to be more abundant in flush growth rather than the mature \( M. \) alternifolia leaves used in this study (Table 6.12). The opposite trend was seen for \( E \)-piperitol (Table 6.12).

**Table 6.11.** Effect of mechanical damage on oil composition of \( M. \) alternifolia leaves initiated after damage (mean ± standard error of the mean, \( n=8 \)). Components as detected after cold solvent extraction are presented. Asterisks indicate statistical significance between the before and after damage values (* at \( p<0.05 \); ** at \( p<0.01 \), Kruskal–Wallis test).

<table>
<thead>
<tr>
<th>Components</th>
<th>Percentage composition (mean±S.E,M) (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaves already present on the plant (sampled before damage)</td>
</tr>
<tr>
<td>( \alpha )-thujene</td>
<td>0.55±0.02</td>
</tr>
<tr>
<td>( \alpha )-pinene</td>
<td>2.14±0.07</td>
</tr>
<tr>
<td>sabinene **</td>
<td>nd</td>
</tr>
<tr>
<td>cluster 1 (^a)</td>
<td>0.62±0.02</td>
</tr>
<tr>
<td>( \alpha )-terpinene **</td>
<td>0.47±0.01</td>
</tr>
<tr>
<td>cluster 2 (^b)</td>
<td>9.30±0.41</td>
</tr>
<tr>
<td>( \gamma )-terpinene **</td>
<td>15.8±0.35</td>
</tr>
<tr>
<td>( E )-sabinene hydrate **</td>
<td>nd</td>
</tr>
<tr>
<td>terpinolene **</td>
<td>1.22±0.06</td>
</tr>
<tr>
<td>( Z )-sabinene hydrate **</td>
<td>nd</td>
</tr>
<tr>
<td>( E )-p-menth-2-en-1-ol **</td>
<td>nd</td>
</tr>
<tr>
<td>( Z )-p-menth-2-en-1-ol **</td>
<td>nd</td>
</tr>
<tr>
<td>terpinen-4-ol **</td>
<td>38.5±0.76</td>
</tr>
<tr>
<td>( \alpha )-terpineneol **</td>
<td>3.39±0.09</td>
</tr>
<tr>
<td>( E )-piperitol **</td>
<td>2.64±0.16</td>
</tr>
<tr>
<td>unknown</td>
<td>0.66±0.04</td>
</tr>
<tr>
<td>sesquiterpenes</td>
<td>22.8±1.57</td>
</tr>
</tbody>
</table>

\(^a\) cluster 1 includes \( \beta \)-pinene and myrcene

\(^b\) cluster 2 includes \( p \)-cymene, limonene, \( \beta \)-phellandrene and 1,8-cineole

\(^c\) tentative identification
TABLE 6.12. Difference in relative levels of E-piperitol, E- and Z- p-menth-2-en-1-ol between (unwounded) mature and flush M. alternifolia leaves (mean ± standard error of the mean, n=8). Asterisks indicate statistical significance (at p<0.05, Kruskal-Wallis test).

<table>
<thead>
<tr>
<th>Components</th>
<th>Percentage composition (mean±S.E,M) (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mature leaves</td>
</tr>
<tr>
<td>E- p-menth-2-en-1-ol*</td>
<td>0.63±0.01</td>
</tr>
<tr>
<td>Z- p-menth-2-en-1-ol*</td>
<td>0.79±0.03</td>
</tr>
<tr>
<td>E-piperitol</td>
<td>1.42±0.04</td>
</tr>
</tbody>
</table>

Statistically significant changes were also detected for clusters 1 (β-pinene, myrcene) and 2 (p-cymene, limonene, β-phellandrene, 1,8-cineole). Flush growth leaves are expected to have lesser amount of cluster 2 components than mature leaves considering that p-cymene is the dehydrogenation product of p-menthane hydrocarbons (Southwell, 1988; Zabaras and Wyllie, 2002; Chapter 4). The level of 1,8-cineole, the other major component of the cluster, is known to remain unchanged over the maturation process (Southwell and Stiff, 1990).

According to Southwell and Stiff (1989), cluster 1 components β-pinene and myrcene are known not to vary significantly during the course of leaf-development. Although not as notable as the changes occurring with other oil constituents, Table 6.13 shows that (at least for the trees used here) a statistically significant decrease in these components occurs as M. alternifolia leaves mature. Consequently, even though the observed difference between mature and flush leaves is about three times smaller than that shown (for cluster 1) in Table 6.11 above, these results indicate that leaf ontogeny rather than wounding is the cause of the changes detected in the levels of β-pinene and myrcene.
TABLE 6.13. Difference in relative levels of β-pinene and myrcene between (unwounded) mature and flush *M. alternifolia* leaves (mean ± standard error of the mean, n=8). Asterisks indicate statistical significance (at *p*<0.01, Kruskal-Wallis test).

<table>
<thead>
<tr>
<th>Components</th>
<th>Percentage composition (mean±S.E,M) (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mature leaves</td>
</tr>
<tr>
<td>β-pinene</td>
<td>0.63±0.01</td>
</tr>
<tr>
<td>myrcene</td>
<td>0.79±0.03</td>
</tr>
<tr>
<td>Total</td>
<td>1.42±0.04</td>
</tr>
</tbody>
</table>

Mechanical wounding did not affect the composition of the oil produced by *M. alternifolia* leaves initiated after damage. The only changes seen were those consistent with the ongoing leaf ontogeny operating in *M. alternifolia*. Thus, it appears that a long-term wound-induced response is not activated in this species. These results also suggest that leaf biosynthetic constraints are not responsible for the limited inducible defences observed for the oil-bearing plants examined. This issue is investigated further in Chapter 7, which describes an attempt to detect wound-induced changes in leaf terpene synthase activity for the three oil-bearing species studied.
6.6 Wound-induced changes in essential oil concentrations (over 48 hours)

6.6.1 Materials and Methods

All materials, methods and equipment were identical to those described in section 2.4.1. Leaf-oil concentration was calculated based on the internal standard (400 ppm octane in CH₂Cl₂) and was expressed per sample dry weight (more details in Chapters 3 and 5).

6.6.2 Results and Discussion

6.6.2.1 M. alternifolia

Figure 6.11 shows the change in oil concentration in both mature and flush growth M. alternifolia leaves caused by wounding. The observed change was similar for both leaf types during the first 24 hours but differed markedly at 48 hours after injury. After this time the oil concentration of the mature leaves appears to have returned to its pre-wounding level. In contrast, the flush growth leaves show a continuing loss as post-wounding time elapses (Figure 6.11) although it is possible that some of this loss is relative, reflecting the increasing weights of leaf structural features due to the ongoing ontogeny of the flush growth.

The values in Figure 6.11 are expressed on a dry weight basis due to the substantial variations that can occur with moisture content (Murtagh and Smith, 1996). The leaf-moisture content for both, mature and flush growth, M. alternifolia leaves in relation to the mechanical wounding protocol was determined from appropriate leaf-drying experiments (values in Appendix 6.2).

As can be seen from Figure 6.11, the absolute oil-concentration values as determined here are about 30 times lower than the usual oil yields reported for M.
*alternifolia* (Murtagh and Etherington, 1990). This is likely to reflect the low efficiency of solvent extraction compared to the usual steam- and vacuum-distillation techniques used to obtain oil samples for commercial purposes.

![Bar chart showing changes in oil concentration of M. alternifolia leaves as a result of mechanical damage (mean ± standard error of the mean, n=8). Identical letters indicate statistical significance between values (p<0.01, Wilcoxon signed ranks test).](image)

**Figure 6.11.** Changes in oil concentration of *M. alternifolia* leaves as a result of mechanical damage (mean ± standard error of the mean, n=8). Identical letters indicate statistical significance between values (p<0.01, Wilcoxon signed ranks test).

Results from headspace experiments indicate that the initial post-wounding oil loss is due to the volatilization of the oil constituents into the atmosphere (Figure 6.1, section 6.1.2). From an ecological perspective, this increased emission of volatiles may be a preliminary response by the leaf to deter further predation by insect herbivores.
The recovery of the oil concentration observed after 48 hours for the mature leaves supports the view derived from the compositional changes observed (section 6.4.1.2) that biosynthetic processes, although somewhat limited due to physiological constraints, are active in *M. alternifolia* mature leaves during the post-wounding period and as a result any oil losses caused by wounding are replenished.

### 6.6.2.2 *O. minimum* and *S. officinalis*

No significant differences (Wilcoxon signed ranks test, *p*<0.05) between wounded and unwounded *O. minimum* leaves in relation to oil concentration were detected for both 24- and 48-hour post-wounding periods (Figure 6.12). Similar results were obtained for *S. officinalis* (Figure 6.13).

![Graph showing oil concentration over time](image)

**Figure 6.12.** No changes (mean ± standard error of the mean, n=8) in oil concentration of *O. minimum* leaves as a result of mechanical damage were detected (*p*<0.05, Wilcoxon signed ranks test).
**Figure 6.13.** No changes (mean ± standard error of the mean, n=8) in oil concentration of *S. officinalis* leaves as a result of mechanical damage were detected (*p*<0.05, Wilkoxon signed ranks test).

The fact that no "long term" changes in essential oil concentration from these two herbaceous species were detected suggests that these plants are possibly capable of replenishing their initial oil losses caused by gland rupture (section 6.1.2) before the 24-hour post-wounding sampling. In this respect these species exhibit a faster response than that shown by *M. alternifolia.*
6.7 Long-term wound-induced changes: Conclusion

Mechanical wounding was found to induce small compositional changes in the oil from *M. alternifolia*, *O. minimum* and *S. officinalis* leaves. Leaf-tissue damage stimulated increased production of different terpenoids in each species at the expense of others. The wounding-induced response was found to have similar characteristics across all three species such as independence of the pre-wounding oil composition and the pre-wounding levels of the particular components involved in the response.

Wounding-induced changes in the total oil concentration were detected only for *M alternifolia* leaves. In this species, a wounding-induced decrease in the oil concentration of both mature and flush growth leaves was demonstrated. In contrast to the flush growth, the mature leaves returned to their pre-wounding oil concentration 48 hours after damage.

Experiments showed that wounding of the plants did not affect the composition of oil from *M. alternifolia* leaves emergent after damage.
APPENDICES

Appendix 6.1. Calculation of the concentration of 1,8-cineole in the
headspace above S. officinalis leaves (the calculation of the 0-10 post-
wounding min. value shown in Table 6.1 is presented).

Mean (n=3) peak area in control (1st fibre exposure): 305x10^3 pA
Mean (n=3) peak area in 0-10 post-wounding min. (2nd fibre exposure): 846x10^3 pA

A separate experiment showed the loss of analyte due to headspace depletion and
other losses through the system to be 47.3% for 1,8-cineole after the 1st fibre
exposure (Figure 2.6, section 2.5.2). Thus, corrected, the mean peak area for the 0-10
post-wounding minutes becomes:

\[ \frac{[846x10^3 + (305x10^3 \times 47.3)]}{100} = 990x10^3 \text{ pA} \]

Based on the external calibration curve for \( \alpha \)-pinene (assuming the relative response
factor is 1) the peak area for 1,8-cineole corresponds to:

\[ \frac{(400 \times 990x10^3)}{760x10^3} = 521 \text{ ng} \]

The coating volume of the fibre used (100 \( \mu \)m PDMS) is known to be: 6.60x10^-4 mL
(Schäfer et al., 1995). Therefore, the concentration of analyte absorbed on the fibre
will equal:

\[ C_{\text{fibre}} = 521 \text{ ng} / 6.60x10^{-4} \text{ mL} = 789x10^3 \text{ ngmL}^{-1} \]

The calculated \( K_{fg} \) for 1,8-cineole is 7.37x10^4 (Table 4.4, section 4.7.3). Thus, the
concentration of analyte in the headspace will be:

\[ C_{\text{headspace}} = C_{\text{fibre}} / K_{fg} = 789x10^3 \text{ ngmL}^{-1} / 7.37x10^4 = 10.8 \text{ ngmL}^{-1} \]

The volume of the headspace was 20 mL. Thus, the actual amount of 1,8-cineole in
the headspace was:

\[ \text{mass} = C_{\text{headspace}} \times V_{\text{headspace}} = 10.8 \text{ ngmL}^{-1} \times 20 \text{ mL} = 216 \text{ ng} \]
Appendix 6.2. Length of wound (mean ± s.e.m, n=3) inflicted in each species for the quantitative determination of wound-induced LOX compounds.

<table>
<thead>
<tr>
<th>Species</th>
<th>Length of wound (mm) (mean ± sem, n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. alternifolia</td>
<td>6.0±0.0(^a)</td>
</tr>
<tr>
<td>S. officinalis</td>
<td>21.0±0.5</td>
</tr>
<tr>
<td>O. minimum</td>
<td>16.0±0.0</td>
</tr>
<tr>
<td>P. prunus</td>
<td>20.5±0.6</td>
</tr>
</tbody>
</table>

\(^a\) The value 6.0 was obtained from the sum of wounds on 5 M. alternifolia leaves (5 x 1.2 mm each)

Appendix 6.3. Location of the University of Western Sydney, Hawkesbury Campus, NSW, Australia.
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CHAPTER 7

THE EFFECTS OF MECHANICAL WOUNDING ON MONOTERPENOID BIOGENESIS

7.1 Introduction

7.1.1 Terpenoid biosynthesis

Isopentenyl diphosphate (IPP) is the universal precursor of all isoprenoids (Gershenzon and Croteau 1993; McCaskill and Croteau, 1999). In plants IPP biosynthesis can occur via two distinct pathways: the mevalonate pathway which operates in the cytosol (Spurgeon and Porter, 1983; Gershenzon and Croteau 1993) and the recently discovered deoxyxylulose 5-phosphate (DXP) pathway which occurs in the plastids (Eisenreich et al., 1997; Lichtenthaler et al., 1997; Rohmer, 1999) (Figure 7.1). The two IPP pools are distinct to one another. Higher-order terpenoid "building-blocks" such as geranyl diphosphate (GPP; C\textsubscript{10}) and farnesyl diphosphate (FPP; C\textsubscript{15}) are produced as result of the action of various prenyltransferases (e.g., geranyl diphosphate synthase) on IPP (McGarvey and Croteau, 1995) (Figure 7.1).

GPP is assumed to be the universal precursor of monoterpenes (Croteau, 1987). Monoterpene synthases (termed cyclases) are able to catalyse both, the isomerisation of GPP to linalyl pyrophosphate (LPP) (due to steric constraints from the E-2,3 double bond GPP cannot cyclise directly) and the subsequent cyclisation of LPP to form cyclic monoterpenes through the α-terpinyl cation (Croteau and Cane, 1985; McConkey et al., 2000; Wise et al., 1998) (Figure 7.2). Acyclic monoterpenes are obtained from the action of synthases on either GPP or LPP before the formation of the α-terpinyl cation (Wise et al., 1998) (Figure 7.2).
**FIGURE 7.1.** Pathways for terpenoid biosynthesis in plants. The unboxed section (IPP to FPP) is common to both pathways, although distinct isomerases and phenyltransferases are operating in each compartment (Bohlmann et al., 1999; McGarvey and Croteau, 1995).
**Figure 7.2.** Conversion of GPP to various cyclic and acyclic monoterpenes (e.g., Bohlmann et al., 1998; Pichersky et al., 1995; Wise et al., 1998).
7.1.2 Characteristics of monoterpen synthases

Most monoterpen synthases, although purified and characterised from different sources (i.e angiosperms, gymnosperms, bryophytes), appear to possess similar characteristics (Adam et al., 1996; Croteau and Karp, 1977; Phillips et al., 1999). They are operationally soluble enzymes with molecular weights between 50,000-100,000 (Croteau, 1987). Studies mostly on cyclases (but also on linalool synthase, an acyclic synthase (Pichersky et al., 1995)) have shown these enzymes to be hydrophobic and to possess relatively low pI values (Croteau, 1987; Croteau and Cane, 1985). Monoterpen synthases exhibit a requirement for a divalent metal ion as a cofactor, usually Mg$^{2+}$ or Mn$^{2+}$ (Bohlmann et al., 1998). The pH optimum for these enzymes is usually between 6.0-7.0 and sharp pH curves (half- maximum velocity values within a half unit deviation from the optimum) are common (Bohlmann et al., 1998; Croteau, 1987).

With the exception of linalool synthase (Pichersky et al., 1995), most other monoterpen synthases can utilise geranyl- (GPP), neryl- (NPP) and linalyl diphosphates (LPP) as acyclic precursors (Croteau, 1987; Croteau and Cane, 1985). GPP however, is considered to be the natural substrate as no free intermediates are formed during its isomerisation and cyclisation (Bohlmann et al., 1998).

7.1.3 Wound-inducible synthases

Several studies have shown that conifers, such as Abies, Picea and Pinus species, respond to wounding by an accelerated de novo biosynthesis of oleoresin (Berryman, 1972; Lewinsohn et al., 1991; Miller et al., 1986; Raffa and Berryman, 1982; Steele et al., 1998). In Grand Fir (Abies grandis) saplings this wound response was found to consist of both an evident increase of constitutive monoterpen cyclase activity and the appearance of new cyclisation activity (Gijzen et al., 1991; 1992);
(-)-pinene synthase (producing α- and β-isomers) was one of the inducible enzymes isolated (Lewinsohn et al., 1992). Subsequent experiments revealed a considerable inter-tree variation in both, the induced-activity levels and the distribution of the products formed (Katoh and Croteau, 1998).

An attempt to determine the effects of mechanical wounding on the leaf (monoterpene)-synthase levels of *M. alternifolia* and *O. minimum*, and also, to investigate further any detected synthase-activity, is described below. Results from these experiments could provide information related to the origin of the wound-induced changes described previously (Chapter 6).

### 7.2 Materials and Methods

#### 7.2.1 Plants

Leaves obtained from *M. alternifolia* and *O. minimum* plants identical to those described in section 2.2.2.2.1 were used.

#### 7.2.2 Wounding protocol

Leaves from several plants were cut in half longitudinally at 0 hour (*T₀*). After 24 hours the other half of the leaves (that had remained on the branch) was taken. The *T₀* and *T₂₄* leaves were then subjected to the procedure described below (section 7.2.3).

#### 7.2.3 Enzyme extraction

The collected leaf material (~1g) was subjected to an identical procedure to that described in section 4.8.2.7. The resulting crude enzyme extract was assayed for activity (section 7.2.4) and, when required, was purified further by fast-protein liquid chromatography (FPLC) (section 7.2.5).
7.2.4 Enzyme assay and HS-SPME conditions

A manual SPME holder (Supelco, USA) equipped with a 1 cm-long polydimethylsiloxane (PDMS)-coated fiber (Supelco, USA) was used. GPP (2.74 mM) (20 μL) was mixed with assay buffer (50 mM Tris, 1 mM MnCl₂, 10 mM MgCl₂, 10 % glycerol, adjusted to pH 7.2 with HCl) (980 μL) in a 2 mL screw-top glass vial (Agilent Technologies, USA). The total volume of the GPP-buffer mixture was kept 1 mL to allow the full length of the fiber to be exposed to the headspace of the mixture within the vial. The mixture was left to equilibrate for 15 minutes at 32 °C and then the fiber was exposed to the headspace of the mixture for a further 15 minutes (32 °C). Thermal desorption (220 °C for 10 minutes) of the fiber in the split/splitless injection port of the GC-MS system followed (section 7.2.6). Before use the fiber was conditioned according to the manufacturer's instructions.

Assay controls that were carried out included incubations with enzyme extract but no GPP and incubations with GPP but boiled (120 °C for 30 min) enzyme extracts. All chemicals were obtained from Sigma (NSW, Australia).

7.2.5 Enzyme purification by FPLC

A three-phase purification strategy was employed. This is common in cases where not much is known about the protein of interest (Janson and Rydén, 1998). A flow-chart of the overall process is shown in Figure 7.3. An ÄKTA™ FPLC was used equipped with a Frac-950 fraction collector. The system was controlled via the UNICORN™ (ver. 3.11) software. All FPLC columns and accessories were from Amersham-Pharmacia Biotech.
7.2.5.1 Ion exchange chromatography (IEC)

The column used was a Hi-Trap Q HP (5 mL bed volume of Q-Sepharose) which is an anion exchange column. Before use the column was pre-equilibrated (x 5 column volumes) with assay buffer (section 7.2.4). The crude enzyme extract was injected and the bound fraction was eluted with a gradient (0-100%) of a high salt buffer (1M KCl). The bound fraction was then desalted through a Hi-Trap Desalting column (Sephadex G-25 Superfine bed), assayed and passed through a MonoQ HR 5/5 column (1 mL bed volume). This column was used because although its characteristics are similar to those of Hi-Trap Q, it is known to provide better
resolution (Anonymous, 1999). The bound fraction was then eluted with a gradient of 1M KCl. A small part (1 mL) of the bound fraction was desalted and assayed. The remaining part was concentrated by centrifugation (20,000 g at 4 °C) to about 2 mL and subjected to hydrophobic interaction chromatography (HIC).

7.2.5.2 Hydrophobic interaction chromatography (HIC)

A Resource PHE (1 mL bed volume, phenyl hydrophobic ligands phase) column was used. The concentrated high-salt fraction from above was passed through the pre-equilibrated (with high salt buffer) column using a reverse phase gradient this time (i.e. concentration of salt from 100-0%). After assaying, the fraction of interest (unbound) was subjected to gel filtration.

7.2.5.3 Gel filtration (GF)

100 µL of the desalted unbound fraction from HIC were passed through a Superose 12HR 10/30 column and eluted isocratically (no salt buffer). The retention volume for the peak showing activity was used to estimate molecular size against a calibration table constructed using 4 lyophilised protein standards. The proteins (Ribonuclease A 13.7 kDa, BSA 67 kDa, Aldolase 158 kDa, Catalase 232 kDa) (Amersham Pharmacia Biotech) were reconstituted with (assay) buffer to a 10 mgmL⁻¹ concentration and mixed. 100 µL of the mixture was the passed through the Superose 12HR 10/30 column.

7.2.5.4 GC-MS analysis

The instrument used was a Varian 3800 gas chromatograph connected to a Varian 2000 ion trap detector (0.9 scanssec⁻¹, 20 µA current) operating in the electron ionization mode. The column used was CP-Sil 8CB (30 m length x 0.25 µm ID x 0.25 µm film thickness) (Chrompack, USA). The inlet temperature and pressure were 220 °C and 10 psi respectively, carrier gas was He, split ratio was 1:20, detector
temperature at 260 °C, and the oven program was: initial temperature 60 °C, increased to 180 °C at 6 °C/min. Identity of peaks was confirmed by injection of authentic standards (Fluka Chemicals, NSW, Australia).

7.3 Results and Discussion

7.3.1 Comparison of synthase activity between wounded and unwounded leaves

The products formed during the incubation of enzyme extracts with GPP, as detected by HS-SPME, were used to determine wound-induced changes in leaf synthase activity; studies on conifers have shown that both cyclase activity and oleoresin formation increase upon stem wounding or attack by mountain beetles (Lewinsohn, et al., 1991; Miller et al., 1986).

As Figures 7.4 and 7.5 show, there are no notable differences in the chromatograms (obtained after incubation with GPP and HS-SPME analysis) from the wounded and unwounded (control) cell-free extracts for both M. alternifolia and O. minimum. Assuming that there was no loss of activity during the enzyme extraction process, these results indicate that wounding has no or very little (undetectable by the extraction, assay and analysis procedures followed here) effect on the synthase activity in M. alternifolia and O. minimum leaves over the time-frame tested.

Studies on conifers have shown that a significant wound-induced increase in cyclase activity occurs only in species that lack high levels of constitutive oleoresin such as Abies spp.; the cyclase activity of species with well-developed resin duct systems such as Pinus spp. was not affected by wounding (Lewinsohn et al., 1991). This may explain the lack of wound-induced synthase activity observed for M. alternifolia and O. minimum.
Figure 7.4. IT-GC-MS analysis of the products generated from the GPP assay of the cell-free extracts from a) wounded and b) unwounded *M. alternifolia* leaves. Terpenoids identified include myrcene (2), limonene/E-β-oicimene (3), Z-β-oicimene (4), terpinolene (5), linalool (6), terpinen-4-ol (extract contaminant) (7). Unknown non-terpenoids (1, 8) were also detected.
**Figure 7.5.** IT-GC-MS analysis of the products generated from the GPP assay of the cell-free extracts from a) wounded and b) unwounded *O. minimum* leaves. Terpenoids identified include myrcene (2), limonene/E-β-ocimene (3), Z-β-ocimene (4), linalool (5), geraniol (6). Unknown non-terpenoids (1, 7) were also detected.
7.3.2 Investigation of the assay-generated terpenoid products

Although no differences in enzymatic activity between wounded and unwounded leaves were observed, it was of interest to investigate further the obtained GPP-assay products (Figures 7.4 and 7.5). In contrast to S. officinalis (e.g., Gamblie et al., 1984; Wise et al., 1998), none of the synthases assumed to be operating in O. minimum and M. alternifolia leaves (based on their essential oil composition) have been isolated and characterised.

The nature of the products obtained from both O. minimum and M. alternifolia cell-free extract assays (Figures 7.4 and 7.5) indicated that the non-enzymatic solvolysis of GPP was responsible for their formation. Apart from hydrogen ions (acid catalysed hydrolysis of GPP was described in Chapter 4), bivalent metal ions such as Mn$^{+2}$ and Mg$^{+2}$ are also known to enhance the hydrolysis of GPP by neutralising the negative charge of the phosphate moiety (Brems and Rilling, 1977; Vial et al., 1981). However, results from assays carried out without cell-free extracts and with "boiled" cell-free extracts (Figure 7.6) showed that, under these conditions, the non-enzymatic solvolysis of GPP was negligible and could not account for the products generated during the GGP assays (Figures 7.4 and 7.5; GC traces from assays without GPP are shown in Appendix 7.1).

This indicated that enzymatic activity was responsible for the formation of the detected products. The fact that linalool was the major product formed during the assays was interesting, especially for M. alternifolia, since only a trace amount of this monoterpenic alcohol is present in the essential oil produced from this species (Brophy et al., 1989). Linalool is known to be one of the major components of the essential oil from O. minimum leaves (Martins et al., 1999; Zabaras and Wyllie, 2001).
**Figure 7.6.** IT-GC-MS analysis of the products generated from the incubation of a) buffer + GPP, b) boiled *O. minimum* cell-free extract + GPP and c) boiled *M. alternifolia* cell-free extract + GPP. Peaks: myrcene (1), linalool (2). Scale used is the same as that in Figures 7.4 and 7.5 to assist comparison.
The isolation and purification protocol described in Materials and Methods (section 7.2.5) was used in order to investigate the detected activity further. Typical examples of the GC-MS traces obtained by HS-SPME analysis of the fractions observed to produce volatiles during GPP-assays are shown in Figures 7.7 and 7.8; the results for the complete isolation/purification sequence for both species are given in Figures 7.9 and 7.10 (extracts from *M. alternifolia* and *O. minimum*, respectively). As can be seen, the profile of the GC-MS traces changes as the active fraction is passed through the different purification steps. This, and the fact that volatiles were not detected from all fractions (Appendix 7.2), support the view that the observed volatiles were generated by enzymatic activity and they were not solvolysis products or other chemical artefacts.

The protein of interest did not bind to the hydrophobic (Resource PHE) column. This indicates that the protein has no or very little surface hydrophobicity (not many hydrophobic groups exposed). The molecular weight of the protein was determined to be about 64 kDa by gel filtration chromatography based on a calibration curve constructed using 4 authentic standards (Ribonuclease A, BSA, Aldolase and Catalase). These characteristics are in agreement with those exhibited by most monoterpene synthases (Croteau and Cane, 1985, and references cited therein) although the linalool synthase isolated from *Clarkia breweri* flowers was slightly larger (76±3 kDa, Pichersky *et al.*, 1995).
**Figure 7.7.** IT-GC-MS analysis of the products generated from the GPP assay of the active fractions from *M. alternifolia* cell-free leaf-extracts: a) bound fraction from the Hi-Trap Q column, b) bound fraction from the MonoQ HR 5/5 column and c) unbound fraction from the PHE HIC column. Terpenoids identified include myrcene (3), limonene/E-β-ocimene (4), Z-β-ocimene (5), linalool (6). Unknown non-terpenoids (1) and benzene derivatives (2) were also detected. Broken arrows indicate the purification sequence.
**FIGURE 7.8.** IT-GC-MS analysis of the products generated from the GPP assay of the active fractions from *O. minimum* cell-free leaf-extracts: a) bound fraction from the Hi-Trap Q column and b) bound fraction from the MonoQ HR 5/5 column. Terpenoids identified include myrcene (2), limonene, E-β-ocimene and Z-β-ocimene (3), linalool (4). Unknown non-terpenoids (1, 5, 6) were also detected. Broken arrows indicate the purification sequence.
<table>
<thead>
<tr>
<th>Isolation/purification stage</th>
<th>Products detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>Linalool (major), myrcene limonene, $E$- and $Z$-ocimenes (minor).</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Hi-Trap Q column (combined fractions 12-17 only active)</td>
<td>Myrcene (major), lesser amounts of linalool, limonene, $E$- and $Z$-ocimetes.</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>MonoQ column (combined fractions 5-9 only active)</td>
<td>Linalool and myrcene (major), limonene, $E$- and $Z$-ocimenes, benzene compounds (minor).</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe-HIC column (fraction 7 only active)</td>
<td>Linalool</td>
</tr>
</tbody>
</table>

**Figure 7.9.** Summary of results obtained after each purification step from the crude cell-free extract of *M. alterifolia* leaves.
<table>
<thead>
<tr>
<th>Isolation/purification stage</th>
<th>Products detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>Linalool (major), myrcene (minor), traces of limonene, E- and Z- β-ocimenes.</td>
</tr>
<tr>
<td>Hi-Trap Q column (combined fractions 15-16 only active)</td>
<td>Linalool (major), myrcene (minor), traces of limonene, E- and Z-β-ocimenes, terpinolene and an unknown non-terpenoid.</td>
</tr>
<tr>
<td>MonoQ column (combined fractions 7-9 only active)</td>
<td>Linalool (major), unknown non-terpenoid (minor), myrcene (trace).</td>
</tr>
<tr>
<td>Superose HR column (combined fractions 11-12 only active)</td>
<td>Linalool (major). Estimated size using gel filtration calibration: 63.7 kDa</td>
</tr>
</tbody>
</table>

**FIGURE 7.10.** Summary of results obtained after each purification step from the crude cell-free extract of *O. minimum* leaves.
If, as the evidence suggests, there is an enzyme present in *M. alternifolia* that generates linalool as the major product, then it is worth questioning the existence of this monoterpenic alcohol only as a trace in the essential oil of this species. Linalool is believed to be a key intermediate in the biogenesis of *p*-menthane terpenes in citrus plants (Attaway and Buslig, 1968; Potty and Bruemmer, 1970). To test whether linalool serves a similar role in *M. alternifolia* leaves, and thus does not accumulate in the leaves' secretory tissues, experiments were carried out where cell-free extracts (from both *O. minimum* and *M alternifolia*) were incubated with linalool (100 µM) as the substrate. The results obtained are shown in Figure 7.11. As can be seen, a range of terpenoids and other compounds was detected, including myrcene, limonene and decanol (tentative, mass spectrum is given in Appendix 7.3); these components were not observed in the results from the "control" experiments (using "boiled" cell-free extracts, Appendix 7.4).

However, for linalool to be useful in terpenoid biosynthesis it must be converted to linalyl pyrophosphate by the action of a kinase enzyme (Figure 7.12; Erman, 1985). The presence of such an enzyme has been demonstrated in orange cell-free extracts, where linalool is phosphorylated by the kinase in the presence of ATP (Potty and Bruemmer, 1970). To check whether such a kinase was present in the cell-free extracts from *M. alternifolia* leaves, assays were carried out in which linalool was used as the substrate for the partially purified (after the MonoQ 5/5 HR column) protein (at a concentration equivalent to that of GPP) in the presence of ATP (concentrations used were between 1mM-50µM). The results obtained (Figure 7.13) were identical between the "control (no ATP)" and ATP assay (for all ATP concentrations), thus, suggesting that no kinase was present in the cell-free extract from *M. alternifolia* leaves.
**Figure 7.11.** IT-GC-MS analysis of the products generated from the linalool assay of the cell-free leaf-extracts from a) *O. minimum* leaves and b) *M. alternifolia* leaves. Terpenoids identified include α-pinene (1), β-pinene (2), myrcene (3), limonene, E-β-ocimene (4). Peaks (6) and (7) exhibit a mass spectrum similar to dihydrocarvone (Appendix 7.5), while peaks 8 and 9 appear as menthone isomers (Appendix 7.6). Non-terpenoids (decanol (5), unknown (10)) were also detected.
**Figure 7.12.** Diagram outlining the pathways for the biogenesis of terpenoids from GPP (modified from Erman, 1985). The broken arrow indicates the part investigated by the ATP experiments (conversion of linalool to LPP).
**Figure 7.13.** IT-GC-MS analysis of the products generated from the linalool assay of the partially purified protein from *M. alternifolia* leaves: a) in the absence of ATP and b) in the presence of 100 μM ATP. Peaks: unknown (1), 1,8-cineole (2, extract contaminant), linalool (3), unknown (4).
Based on the evidence described above, no conclusion about the role of linalool in relation to \( p \)-menthane biogenesis in \textit{M. alternifolia} and \textit{O. minimum} leaves can be drawn.

Another hypothesis, that explains the results described above, is that the isolated enzyme is a crippled monoterpen synthase or a synthase that behaves abnormally because it is outside its normal environment; as a result it produces mainly linalool due to its inability to cyclise further the intermediate products of the initial ionisation and rearrangement of GPP. An enzyme (3\( R \)-linalool synthase) of similar characteristics was isolated from the leaves of \textit{Artemisia annua}, a fragrant herb that produces an essential oil with no detectable levels of linalool (Jia \textit{et al.}, 1999).

\textbf{7.4 Conclusion}

Wounding had no discernable effect on the synthase activity in \textit{M. alternifolia} and \textit{O. minimum} leaves. The results obtained highlighted the presence of an enzyme, that produced linalool as the major product, in the cell-free extracts from both \textit{M.alternifolia} and \textit{O. minimum}. An attempt to establish the role of linalool in the biogenesis of terpenoids in these species did not yield conclusive evidence.
Appendix 7.1. Results from assays where no GPP was added to the incubation mixture.

Figure A7.1.1. IT-GC-MS analysis of the products generated from the "control" assay (no GPP) of the cell-free extracts from a) *O. minimum* and b) *M. alternifolia* wounded leaves. Traces from unwounded leaves were identical.
Appendix 7.2. Examples of IT-GC-MS traces of fractions were no activity was detected.

Figure A7.2.1. Examples of IT-GC-MS traces of fractions were no activity was detected (obtained from GPP assaying): a) bound (no.: 11) fraction of *O. minimum* extract from the Mono Q HR 5/5 column and b) bound fraction (no.: 19-21) of *M. alternifolia* extract from the Hi-Trap Q column.
Appendix 7.3. Mass spectrum of the compound tentatively identified as decanol.

Figure A7.3.1. Mass spectrum of the compound tentatively identified as decanol a), and comparison with that of decanol b) (Adams, 1995).
Appendix 7.4. Examples of IT-GC-MS traces from assays of "boiled" cell-free extracts using linalool as a substrate.

Figure A7.4.1. Examples of IT-GC-MS traces from assays of "boiled" cell-free extracts using linalool as the substrate: a) extract from O. minimum leaves and b) extract from M. alternifolia leaves. Peaks: linalool (1) and "menthone-like"components (2, 3).
Appendix 7.5. Mass spectra of the "dihydrocarvone-like" compounds.

Figure A7.5.1. Mass spectra of compounds 6 (a), 7 (b) and comparison with that of E-dihydrocarvone (c) (Adams, 1995).
Appendix 7.6. Mass spectrum of the compounds believed to menthone isomers.

Figure A7.6.1. Mass spectra of compounds 8 (a), 9 (b) and comparison with that of menthone (c) (Adams, 1995).
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Gambliel H, Croteau R. Pinene cyclases I and II: Two enzymes from sage (Salvia officinalis) which catalyse stereospecific cyclisations of geranyl pyrophosphate to monoterpene olefins of opposite configuration. J. Biol. Chem. 1984, 259, 740-748.


CHAPTER 8

THE EFFECTS OF HERBIVORY AND PATHOGEN ATTACK ON THE EMISSION OF PLANT VOLATILES

8.1 Introduction

Previous studies using non oil-bearing plants have shown that the wound-response in these plants is much greater when an elicitor is present as compared to the response obtained by simple mechanical damage (Coleman et al., 1997; Turlings et al., 1993). Experiments described in the Chapter will test this hypothesis in relation to terpene accumulating plants using herbivores (Ctenoplosia spp. caterpillars), chemicals (jasmonic acid) and a plant pathogen (Botrytis cinerea) as elicitors.

8.1.1 Biochemical pathways / mechanisms of response

The first described and best understood pathway of plant induced-response was that mediated by salicylic acid and was induced by a variety of pathogens (Karban and Kuć, 1999). Activation of this pathway results in the production of putative defence compounds such as chitinases and peroxidases that protect the plant against the pathogen (Hammerschmidt and Nicholson, 1999). The nature of the translocational signal in this pathway is still undetermined (Karban and Kuć, 1999).

Herbivory is known to activate another signalling pathway mediated by jasmonic acid. Jasmonic acid is a 12-carbon regulator derived from the oxygenation

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1 There are several terms that are used to describe induced plant defences against herbivores and pathogens such as systemic acquired resistance (SAR), induced systemic resistance (ISR) and induced resistance (IR). Some authors have tried to distinguish the terms (e.g., van Loon, 1998) while others believe that these terms are synonymous (Kloeper et al., 1992). Here for clarity these terms are avoided and the pathways are defined based on the substance believed to be involved in them.
of linolenic acid by 13-lipoxygenase (13-LOX); the resultant 13-hydroperoxide is then cyclised to 12-oxo-phytodienoic acid (OPDA) which is further metabolised to jasmonic acid (Liechti and Farmer, 2002) (Figure 8.1).

\[ \text{Linolenic acid} \xrightarrow{13-LOX} \text{12-oxo-phytodienoic acid (OPDA)} \xrightarrow{OPDA reductase 3} \text{Jasmonic acid} \]

**Figure 8.1.** Biosynthesis of jasmonic acid (Liechti and Farmer, 2002; Turner *et al.*, 2002).

The jasmonic acid-mediated pathway, sometimes referred to as the octadecanoic pathway (Farmer and Ryan, 1992), utilises systemin (a small polypeptide hormone) as the translocational signal that activates the formation of
jasmonates and other factors that induce resistance (Bergey et al., 1996; Wasternack and Parthier, 1997) (Figure 8.2).

Plants also possess other, not so well understood signalling pathways that are activated upon herbivore and/or pathogen attack. The plant hormone ethylene is known to be involved in one of those pathways although its exact role is not clear (Lawton et al., 1993).

From what is known so far there is no doubt that these pathways interact with one another (e.g., Felton et al., 1999) (Figure 8.2). Jasmonic acid, although found to mediate anti-herbivore responses in most plants, has also been observed to mediate responses against pathogens (van Loon et al., 1998). Salicylic acid can also induce resistance to some herbivores in addition to pathogens (Hardie et al., 1994).

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**Figure 8.2.** Major defence signalling pathways in plants (modified from Staswick and Lehman, 1999). Broken arrows indicate possible interactions between the pathways. JA stands for jasmonic acid while SA represents salicylic acid.
8.1.2 Insect-plant interactions and the effect of herbivory on the emission of plant volatiles

Numerous studies have shown that mechanical wounding can only partially mimic herbivory (e.g., Mattiacci et al., 1995; de Moraes et al., 1998; Stout et al., 1998). Plants have the ability to distinguish between the two processes; they can also differentiate herbivory caused by chewing insects against that caused by sucking insects (Felton et al., 1994). Qualitative and/or quantitative differences in elicitors in the (oral) secretions of the various herbivores (such as caterpillars) are likely to be the "clues" the plants detect and adjust their response accordingly (Felton and Eichenseer, 1999).

As described earlier (section 8.1.1), leaf herbivory induces the activation of the jasmonic acid and/or salicylic acid mediated pathway resulting in the production of defensive phytochemicals. Although not much is known on how the herbivores "deal" with the newly-formed wound-induced compounds, there is considerable information related to the behaviour of some insects against host constitutive terpenoids. Sawflies feeding on eucalypts are known to remove foliage-terpenoids by sequestering them in thoracic sacs; the stored terpenoids are then used for their own protection (Morrow et al., 1976). Surfactant-producing bacteria were isolated from the gut of Eucalyptus-feeding sawflies (Ohmart et al., 1988); the purpose of these surfactants is likely to be the reduction of precipitation of dietary proteins caused by plant tannins (Martin and Martin, 1984). Absorption of eucalyptus terpenoids by chrysomelids (Ohmart and Larsson, 1989; Southwell et al., 1995) has also been reported. Pine bark beetles possess pathways with which they detoxify monoterpenic terpenoids (Pierce et al., 1987) a strategy also used by the Tea tree Pyrgo beetle which converts leaf-derived 1,8-cineole to the non-toxic 2β-hydroxycineole (Southwell et al., 1995).
Field-collected (whilst feeding on *O. minimum* L. plants) moth larvae, tentatively identified as *Ctenoplusia spp.* (Figure 8.3), were used as the herbivores.

![Ctenoplusia spp moth larvae](image)

**Figure 8.3.** *Ctenoplusia spp* moth larvae (Herbison-Evans and Crossley, 2001) used during the herbivore-related experiments.

8.1.3 Pathogen-plant interactions and the effect of pathogen attack on the emission of plant volatiles

Plants resist pathogen infection through physical and chemical defenses that may be constitutive or induced after pathogen attack (Moerschbacher and Mendgen, 2000). After reaching and recognising a host plant, a pathogen penetrates into the host tissue via natural openings, wounds or as is the case for fungal pathogens, directly through the epidermal cell wall using cutinases (Moerschbacher and Mendgen, 2000).

Pathogen infection activates a cascade of salicylic acid (mostly) mediated events that result in the production of defensive phytochemicals (section 8.1.1). In many cases this induced defense is associated with a hypersensitive response (HR) and oxidative burst in which plant cells around the site of infection die and either kill the pathogen (Gilchrist, 1998) or reduce its growth due to the lack of nutrients.
(Govrin and Levine, 2000). Damage is thus minimised to the cells dead from the HR and the pathogen invasion is unsuccessful. HR is not effective against pathogens that require dead cells for their growth (termed necrotrophs) (Govrin and Levine, 2000). 

Among plant pathogens, *Botrytis cinerea* Pers.:Fr. exhibits one of the widest host range specificity infecting fruits and plant tissue (Derckel et al., 1999). This necrotrophic fungus is the causal agent of grey mold and is known to infect sweet basil (*Ocimum basilicum*) (Garibaldi et al., 1997; Sharabani et al., 1999).

Studies have shown lipoxygenase (LOX)-derived compounds (Hamilton-Kemp et al., 1992) and several monoterpenoids (Tsao and Zhou, 2000) to be effective *B. cinerea* growth inhibitors; thymol and carvacrol were the only monoterpenoids found to be fungicidal (Tsao and Zhou, 2000). Another study found the essential oil from *S. officinalis*, its oxygenated fraction and camphor (i.e. one of its major components) to be fungicidal against *B. cinerea* (Carta et al., 1996); the same formulations were only fungistatic (inhibiting *B. cinerea* mycelium growth) when tested for *in vivo* activity on tomato plants (Moretti et al., 1998).

### 8.2 Materials and Methods

#### 8.2.1 Determination of herbivory-induced changes

Field-collected caterpillars (collected whilst feeding on *Ocimum minimum* L. plants) were used. One caterpillar was placed on a healthy single leaf of a healthy single *O. minimum* or *Salvia officinalis* plant (section 6.1.1.1) and was allowed to feed overnight (10-12 hours). The following day a small part of the chewed leaf was cut (section 6.4.1.3) and subjected to solvent extraction (section 6.4.1.4) followed by GC and GC-MS analysis (section 6.4.1.5). A similar part from the other (undamaged) leaf within the same leaf-pair was also sampled for comparison purposes.
8.2.2 Determination of terpenoids in caterpillar frass

The collected caterpillars (group of 9) were kept in 70 mL sterile plastic containers and maintained at room temperature with normal daylight. The caterpillars were fed fresh *O. minimum* or *S. officinalis* leaves provided daily. Frass collection started at least 24 hours after they were feeding on the species of interest. The collected frass (~0.1 g) was subjected to solvent extraction (section 6.4.1.4) followed by GC and GC-MS analysis (section 6.4.1.5) using conditions identical to those used for plant tissue. Parts of leaves fed to the caterpillars were retained and analysed similarly.

8.2.3 Jasmonic acid treatment

*M. alternifolia* (section 6.4.1.1) and *Fragaria ananassa* L. (strawberry, obtained form a local nursery) plants were used. Sixteen small twigs (for *M. alternifolia*) or 16 stems with 1-2 leaves each (for *F. ananassa*) were cut under water (to prevent loss of tension). Eight of the cut twigs/stems were placed immediately in glass vials (room temperature, natural light conditions) containing 10 mL of 1 mM jasmonic acid\(^2\) (Sigma Chemicals, NSW, Australia) in 1% acetone solution\(^3\). The remaining eight twigs/stems were placed in identical vials containing only 10 mL acetone solution (no jasmonic acid) (controls). Leaf-parts from all twigs/stems were cut (section 6.4.1.3, *F. ananassa* as per *O. minimum*) immediately after placement in the vials (T\(_0\)) and after 24 hours (T\(_{24}\)). All T\(_0\) and T\(_{24}\) samples were subjected to solvent extraction (section 6.4.1.4) followed by GC and GC-MS analysis (6.4.1.5).

\(^2\) Based on Dicke et al., 1999.
\(^3\) The use of acetone was necessary to enhance the solubility of the jasmonic acid.
8.2.4 *B. cinerea* treatment

8.2.4.1 *B. cinerea* cultures and conidial suspensions

The *B. cinerea* isolate (no.: 115.1) used in this study was generously provided by Dr. L. Legendre (University of Western Sydney, Australia). The isolate was maintained on potato dextrose agar at 21 °C with a 16/8 hour photoperiod (Derckel et al., 1999). Liquid cultures were made by adding 2x10^6 conidia into 150 mL of the synthetic medium containing 0.1% (w/v) polygalacturonic acid (Sigma Chemicals, NSW, Australia) and 20 gL^-1 glucose. Shaking (160 rpm at 21 °C) under neon tubes followed (16/8 hours photoperiod). After three weeks conidial suspensions (1x10^5 conidia/mL) were made using water and glucose (20 gL^-1). Aliquots (5 μL) of these suspensions were used to infect the plants.

8.2.4.2 Plants used

*M. alternifolia, S. officinalis* and *O. minimum* potted plants identical to those described in section 6.1.1.1 were used.

8.2.4.3 Experimental protocol

The potted plants were placed (one at a time) inside the Perspex-chamber system described in section 2.3.2.2 (Figure 2.4). The conditions inside the chamber (high humidity, 18-21 °C) were favourable for *B. cinerea* growth (Sharabani et al., 1999). Air was supplied at 70 mLmin^-1 with natural daylight. Immediately after their placement in the chamber the plants were inoculated with *B. cinerea* conidial suspensions as follows: Using a micropipette (Gilson Inc., USA) 5 μL aliquots of the conidial suspension were placed onto 3 different locations of a single leaf. During the aliquot placement the outer leaf-tissue epidermis was gently ruptured with the tip of the micropipette to assist *B. cinerea* invasion. Three leaves of each plant were inoculated. Leaf samples were taken immediately after inoculation (T₀) (section
6.4.1.3) and 10 days after inoculation ($T_{10d}$). Samples from neighbouring (uninfected) leaves were also taken at $T_{10d}$. The process was repeated for three inoculated and three non-inoculated plants for each species. All samples were subjected to solvent extraction (section 6.4.1.4) followed by GC and GC-MS analysis (section 6.4.1.5).

8.2.4.4 Principal component analysis

Performed as described in section 6.4.1.7.

8.3 Results and Discussion

8.3.1 Effects of herbivory and jasmonic acid treatment on the emission of plant volatiles

8.3.1.1 The effect of herbivory

It was not possible to determine the effect of caterpillar-feeding on the composition of the oil produced by $O.\ minimum$ and $S.\ officinalis$ leaves. The use of real herbivores to induce leaf-damage and thus possible activation of the plants' defensive mechanism meant that the leaf-division technique to obtain "control" values (section 2.4) could not be employed in this case; the caterpillars often moved from the leaf they were placed and started feeding on other leaves of the same plant. For this reason, undamaged leaves of the same pair (with the caterpillar-damaged leaves) were employed to obtain control values. Although inter-leaf variations and ontogenetical differences between such leaves had been demonstrated (sections 2.2.2 and 2.5, respectively) it was hoped that possible herbivory-induced changes would be greater than these variables.

However, as can be seen on Table 8.1 very large variations for all components were observed when the oil compositional differences between the
caterpillar-damaged and undamaged leaves were compared across all replicates (indicated by the high RSD values, Table 8.1).

### Table 8.1. Difference in essential oil composition between caterpillar-damaged and undamaged leaves (of the same pair) for *O. minimum* and *S. officinalis.*

The averaged relative differences (%) (mean ± standard error of the mean, SEM) for each terpenoid between leaves from eight pairs together with the relative standard deviation (RSD) are given. ND stands for not detected.

<table>
<thead>
<tr>
<th>Components</th>
<th>Difference in percentage composition between damaged and undamaged leaves (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>O. minimum</em></td>
</tr>
<tr>
<td></td>
<td>Mean±SEM</td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Z-salvene</td>
<td>ND</td>
</tr>
<tr>
<td>E-salvene</td>
<td>ND</td>
</tr>
<tr>
<td>α-thujene</td>
<td>ND</td>
</tr>
<tr>
<td>α-pinene</td>
<td>0.08±0.04</td>
</tr>
<tr>
<td>camphene</td>
<td>ND</td>
</tr>
<tr>
<td>cluster 1[^a^]</td>
<td>0.17±0.22</td>
</tr>
<tr>
<td>α-terpinene</td>
<td>ND</td>
</tr>
<tr>
<td>cluster 2[^b^]</td>
<td>0.39±0.59</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>-0.14±0.09</td>
</tr>
<tr>
<td>E-sabinene hydrate</td>
<td>-0.09±0.124</td>
</tr>
<tr>
<td>terpinolene</td>
<td>0.07±0.02</td>
</tr>
<tr>
<td>farnesol</td>
<td>0.13±0.77</td>
</tr>
<tr>
<td>Z-sabinene hydrate</td>
<td>0.01±0.06</td>
</tr>
<tr>
<td>α-thujone</td>
<td>ND</td>
</tr>
<tr>
<td>β-thujone</td>
<td>ND</td>
</tr>
<tr>
<td>camphor</td>
<td>-0.14±0.08</td>
</tr>
<tr>
<td>pinocamphene</td>
<td>ND</td>
</tr>
<tr>
<td>endo-borneol</td>
<td>ND</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>0.07±0.11</td>
</tr>
<tr>
<td>α-terpinolene</td>
<td>0.02±0.06</td>
</tr>
<tr>
<td>bornyl acetate</td>
<td>-0.03±0.03</td>
</tr>
<tr>
<td>eugenol</td>
<td>1.16±0.89</td>
</tr>
<tr>
<td>methyl eugenol</td>
<td>0.18±0.09</td>
</tr>
<tr>
<td>sesquiterpenes</td>
<td>-0.54±1.32</td>
</tr>
</tbody>
</table>

[^a^] cluster 1 includes sabinene, β-pinene and myrcene
[^b^] cluster 2 includes p-cymene, (*M. alternifolia* and *S. officinalis* only), limonene, β-phellandrene (*M. alternifolia* only) and 1,8-cineole
Inter-leaf variation and ontogenetical differences between the caterpillar-
damaged and undamaged (control) leaves are likely to be responsible for the large
variations observed in Table 1; herbivory-induced changes (if any) are smaller than
the observed variation and cannot be detected.

Problems with standardisation of experiments when using real herbivores
have also been reported by other researchers (e.g., Turlings et al., 1993). An
approach used to eliminate such problems is the application (after isolation) of the
eliciting substance on mechanically damaged leaves in order to mimic herbivory
(Turlings et al., 1993). A similar approach (use of jasmonic acid as an elicitor) was
employed here (section 8.3.1.3).

8.3.1.2 Fate of caterpillar-ingested terpenoids

The caterpillars used for the herbivory experiments described above refused
to feed on M. alternifolia leaves (flush growth and mature) even when they were
starved to death. M. alternifolia leaf-recognition by the caterpillars was based on
smell rather than taste; this was concluded after observing the herbivores exhibiting
clear signs of discomfort immediately after the insertion of M. alternifolia leaves into
their container. In contrast to M. alternifolia leaves, the caterpillars readily consumed
O. minimum and S. officinalis leaves. Although factors such leaf palatability,
phenolics, moisture and nitrogen levels could be responsible for this behaviour
(Morrow and Fox, 1980; Rhoades, 1979), it is also possible that caterpillars could not
detoxify or metabolise the terpenoids found in M. alternifolia leaves.

An investigation into the fate of the ingested terpenoids was carried out in
order to test the validity of the above hypothesis. A comparison of the results
obtained from the analysis of leaves fed to the caterpillars and of freshly collected
excreta indicated that these herbivores excrete the majority of ingested terpenoids
unaltered (Figure 8.4); few only terpenoids were found to be present in reduced amount in the frass in comparison to the leaf-tissue (Table 8.2).

**Figure 8.4.** GC-FID traces of a) caterpillar-frass collected following their feeding on *O. minimum* leaves and b) *O. minimum* leaves used as food source for the caterpillars. Please refer to Table 8.2 for the identity of the peaks.
**Table 8.2.** Relative amount of terpenoids present in the caterpillar-derived frass compared to that found on the *O. minimum* and *S. officinalis* leaves used as the food source. Peak numbers refer to Figures 8.4 and 8.5.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Compound Name</th>
<th>Relative amount$^b$ (mean±SEM, n=3)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>O. minimum</strong></td>
<td><strong>S. officinalis</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Frass</td>
<td>Leaves</td>
<td>Frass</td>
</tr>
<tr>
<td>10</td>
<td>α-thujene</td>
<td>trace</td>
<td>trace</td>
<td>5.82±1.31</td>
</tr>
<tr>
<td>1</td>
<td>α-pinene</td>
<td>3.75±1.45</td>
<td>4.25±1.55</td>
<td>28.3±9.46</td>
</tr>
<tr>
<td>11</td>
<td>camphene</td>
<td>-</td>
<td>-</td>
<td>15.6±5.47</td>
</tr>
<tr>
<td>2</td>
<td>cluster$^b$</td>
<td>9.75±1.45</td>
<td>10.2±1.30</td>
<td>34.7±12.3</td>
</tr>
<tr>
<td>12</td>
<td>limonene</td>
<td>trace</td>
<td>trace</td>
<td>4.34±1.31</td>
</tr>
<tr>
<td>3</td>
<td>1,8-cineole</td>
<td>66.7±16.1</td>
<td>94.6±6.20</td>
<td>8.05±2.13</td>
</tr>
<tr>
<td>4</td>
<td>linalool</td>
<td>32.9±11.2</td>
<td>105±11.2</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>α-thujone</td>
<td>-</td>
<td>-</td>
<td>107±21.3</td>
</tr>
<tr>
<td>14</td>
<td>β-thujone</td>
<td>-</td>
<td>-</td>
<td>8.62±1.21</td>
</tr>
<tr>
<td>15</td>
<td>camphor</td>
<td>-</td>
<td>-</td>
<td>20.2±3.25</td>
</tr>
<tr>
<td>5</td>
<td>hydrocarbons$^c$</td>
<td>72.3±8.61</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>eugenol</td>
<td>144±9.60</td>
<td>716±136</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>E-β-caryophyllene</td>
<td>100±0.00</td>
<td>100±0.00</td>
<td>47.5±7.14</td>
</tr>
<tr>
<td>16</td>
<td>α-humulene</td>
<td>trace</td>
<td>trace</td>
<td>100±0.00</td>
</tr>
<tr>
<td>8</td>
<td>δ-cadinene$^c$</td>
<td>29.2±1.50</td>
<td>22.3±1.45</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>unknown (MW 204)</td>
<td>47.4±5.91</td>
<td>38.9±5.40</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ expressed as % relative to E-β-caryophyllene (for *O. minimum*) or α-humulene (for *S. officinalis*)

$^b$ Includes sabinene, β-pinene and myrcene

$^c$ tentative identification

As can be seen from Table 8.2 the terpenoids found in reduced amount in the frass as compared to the leaves (1,8-cineole, linalool and eugenol for *O. minimum*; 1,8-cineole, α-thujone and camphor for *S. officinalis*) are oxygenated compounds known to possess wide-spectrum fungicidal and insecticidal properties (Asha et al., 2001; Lee-Sangkyun et al., 1997; Maga et al., 2000; Obeng-Ofori et al., 1997). The purpose of the unaccounted amount of these terpenoids can only be speculated; examples from other studies suggest that the "missing" terpenoids (or their derivatives) may be utilised by the insects for their own benefit (Morrow et al., 1976; Pierce et al., 1987) or simply detoxified (Southwell et al., 1995). Apart from a few hydrocarbons found in the excreta derived from *O. minimum* leaves (peak 5 in Figure...
8.4) no other volatiles were detected in the analysis of the caterpillar-frass (Figures 8.4 and 8.5).

**Figure 8.5.** Traces of caterpillar-frass collected following their feeding on *S. officinalis* leaves. a) RIC and b) m/z 69+93 (characteristic of terpenes). Please refer to Table 8.2 for the identity of the peaks.
Several isomers of mono- and dihydroxy-1,8-cineole have been found in the frass excreted by various insects fed on cineole-rich leaves (e.g., Southwell et al. 1995). However, no such compounds were detected in the frass of the caterpillars used in this study.

The ability of caterpillars to deal with such a diverse range of volatiles found in *O. minimum* and *S. officinalis* suggests that there is no specificity in the way they process terpenoids; a similar conclusion was reached by Morrow and Fox (1980) for a range of eucalyptus herbivores and Southwell et al. (1995) for a known pest of *M. alternifolia* (the Pyrgo beetle, *Paropsisterna tigrina*). Thus, although leaf terpenoids seem to be important as semiochemicals, it is not likely that they play a major part in the rejection of *M. alternifolia* leaves by the caterpillars used here.

Studies on marsupial folivore-*Eucalyptus* interactions have also shown that terpenoids are used by the folivores as a cue to levels of toxic compounds (diformylphloroglucinols) in the leaves rather than acting as toxins themselves (Lawler et al., 1999).

### 8.3.1.3 Effects of jasmonic acid treatment

Jasmonic acid treatment was carried out on *M. alternifolia* and *Fragaria ananassa* (strawberry) plants. *F. ananassa* plants emit only trace amounts of terpenoids. They were subjected to jasmonic acid treatment to facilitate comparison of their response to that produced by *M. alternifolia*; possible differences between the response shown by non-terpene accumulating plants and oil-bearing plants could then be detected.

Aqueous jasmonic acid was fed to cut stems/twigs rather than applied on the surface of the leaves to ensure its absorption by the plant parts; also, this approach required lesser amount of the regulator substance compared to leaf-spraying.
Twenty-four hours after treatment all plants (for both species) had taken-up some of the jasmonic solution (although the volume varied between individuals within the same species). Overall, *F. ananasa* plants absorbed more jasmonic acid solution than *M. alternifolia* plants.

The results obtained for the untreated (control) and jasmonic acid-treated plants for both *M. alternifolia* and *F. ananasa* are shown in Tables 8.3 and 8.4, respectively.

**Table 8.3.** Effect of jasmonic acid-treatment on oil composition in mature growth of *M. alternifolia*. Selected components as detected after cold solvent extraction are presented. With the exception of terpinen-4-ol (control plants) none of the differences (*T*24 - *T*0) shown for both control and treated plants found to be statistically significant (at *p* < 0.05, Wilcoxon signed-ranks test).

<table>
<thead>
<tr>
<th>Components</th>
<th>Difference in percentage composition between <em>T</em>0 and <em>T</em>24 leaf-parts (mean±S.E.M) (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated (control) plant-parts (<em>T</em>24-<em>T</em>0)</td>
</tr>
<tr>
<td>α-thujene</td>
<td>0.57±0.61</td>
</tr>
<tr>
<td>α-pinene</td>
<td>1.06±1.57</td>
</tr>
<tr>
<td>sabinene</td>
<td>-0.72±0.37</td>
</tr>
<tr>
<td>β-pinene</td>
<td>-0.12±0.10</td>
</tr>
<tr>
<td>myrcene</td>
<td>-0.22±0.13</td>
</tr>
<tr>
<td>α-terpinene</td>
<td>-2.44±0.92</td>
</tr>
<tr>
<td>cluster 2 <em>a</em></td>
<td>-0.29±0.47</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>-0.71±0.47</td>
</tr>
<tr>
<td>E-sabinene hydrate</td>
<td>-0.36±0.44</td>
</tr>
<tr>
<td>terpinolene</td>
<td>-1.09±0.42</td>
</tr>
<tr>
<td>Z-sabinene hydrate</td>
<td>-1.74±1.84</td>
</tr>
<tr>
<td>E-p-menth-2-en-1-ol</td>
<td>-0.45±0.27</td>
</tr>
<tr>
<td>Z-p-menth-2-en-1-ol</td>
<td>-0.44±0.24</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>6.78±3.16 <em>a</em></td>
</tr>
<tr>
<td>α-terpineol</td>
<td>2.00±0.62</td>
</tr>
<tr>
<td>E-piperitol <em>b</em></td>
<td>-0.10±0.11</td>
</tr>
<tr>
<td>unknown</td>
<td>-0.23±0.17</td>
</tr>
<tr>
<td>sesquiterpenes</td>
<td>-1.50±1.28</td>
</tr>
</tbody>
</table>

*a* cluster 2 includes *p*-cymene, limonene, β-phellandrene, 1,8-cineole

*b* tentative identification
Table 8.4. Effect of jasmonic acid-treatment on profile of terpenoids and other volatiles emitted by F. amansa leaves. None of the differences (T24 - T0) shown for both control and treated plants found to be statistically significant (at p<0.05, Wilkoxon signed-ranks test).

<table>
<thead>
<tr>
<th>Components</th>
<th>Difference in percentage composition between T0 and T24 leaf-parts (mean±S.E.M) (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated (control) plant-parts</td>
</tr>
<tr>
<td>α-terpinene</td>
<td>-1.46±0.47</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>-1.31±1.25</td>
</tr>
<tr>
<td>linalool</td>
<td>4.10±3.33</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>0.74±0.35</td>
</tr>
<tr>
<td>α-terpineol</td>
<td>0.35±0.51</td>
</tr>
<tr>
<td>sesquiterpenes</td>
<td>5.00±3.67</td>
</tr>
</tbody>
</table>

*a tentative identification

As can be seen from Table 8.3 there is a significant increase in the level of terpinen-4-ol in the leaves of the untreated (control) M. alternifolia plants during the 24 post-wounding (detached twigs) hours. This increase may be a wounding-induced effect especially when none of the other p-menthans exhibit a similar trend; in contrast, their levels decrease. These results suggest that the terpinen-4-ol increase is independent from the chemical transformations known to occur over time in M. alternifolia leaves.

A comparison of the essential oil changes detected in the untreated (control) M. alternifolia leaves and those seen in the jasmonic acid-treated plants indicates that jasmonic acid treatment (under the conditions used here) does not have large effects on the essential oil profile of M. alternifolia leaves. The only notable difference between the two sets of results (Table 8.3) is the increase in the level of Z-sabinene hydrate in the treated leaves compared with the slight decrease of the same component observed for the untreated leaves. Normally the levels of Z-sabinene hydrate in M. alternifolia leaves are known to decrease over time as it is converted...
(together with the E- isomer and sabinene) to terpinen-4-ol and other p-menthane components (Southwell and Stiff, 1989).

Wounding-induced effects (increases in the levels of linalool and sesquiterpenes) were also detected in the profile of terpenoids produced by the F. ananasa leaves (Table 8.4). No significant differences were observed between untreated (control) and jasmonic acid-treated leaves in relation to terpenoid emission. Although studies have shown that jasmonic acid treatment induces defensive pathways in many non-terpene accumulating plants (e.g., Karban and Kuć, 1999 and references therein; Meiners and Hilker, 2000) it is possible that not all species employ the same elicitors.

Another explanation may be that activation of the jasmonic acid-induced pathway results in the production of non-volatile compounds rather than terpenoids or other volatiles.

8.3.2 Effects of pathogen attack on the emission of plant volatiles

O. minimum was found to be the most vulnerable to B. cinerea infection of all three plant species used as 6 out of the 9 leaves exhibited visible necrotic lesions 10 days after inoculation (Table 8.5). All O. minimum plants tested died within a month of leaf-inoculation as the fungus gradually invaded the whole plant. In contrast, M. alternifolia plants appeared to be completely resistant to B. cinerea whilst S. officinalis plants were able to survive (without subsequent visible effects) the invasion of the fungus in some of their leaves (Table 8.5).
TABLE 8.5. Susceptibility of the three species towards infection by *B. cinerea*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Leaves infected (%)</th>
<th>Mycelium/conidial growth&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Survival of infected plant&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. minimum</em></td>
<td>6 of 9 (66)</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td><em>S. officinalis</em></td>
<td>2 of 9 (22)</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td><em>M. alternifolia</em></td>
<td>0 of 9 (0)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Refers to whether or not mycelium and/or conidial growth was observed on the necrotic lesion.

<sup>b</sup> Plants exhibiting necrotic lesions were considered to be infected.

The differences between the oil compositions at T<sub>0</sub> and T<sub>10d</sub> for both control and infected leaves are given in Tables 8.6 for *O. minimum* and 8.7 for *S. officinalis*.

TABLE 8.6. Differences (mean ± standard error of the mean, n=6) in essential oil composition between T<sub>0</sub> and T<sub>10d</sub> values for control and *B. cinerea* infected *O. minimum* leaves.

<table>
<thead>
<tr>
<th>Components</th>
<th>Difference in percentage composition between T&lt;sub&gt;0&lt;/sub&gt; and T&lt;sub&gt;10d&lt;/sub&gt; (T&lt;sub&gt;10d&lt;/sub&gt;-T&lt;sub&gt;0&lt;/sub&gt;) (mean±S.E.M) (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control leaves</td>
</tr>
<tr>
<td>α-pinene</td>
<td>-0.17±0.04</td>
</tr>
<tr>
<td>sabinene</td>
<td>-0.15±0.05</td>
</tr>
<tr>
<td>β-pinene</td>
<td>-0.14±0.04</td>
</tr>
<tr>
<td>myrcene</td>
<td>-0.07±0.05</td>
</tr>
<tr>
<td>limonene/p-cymene</td>
<td>-0.12±0.07</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>-0.78±0.37</td>
</tr>
<tr>
<td>E-sabinene hydrate</td>
<td>-0.05±0.02</td>
</tr>
<tr>
<td>linalool</td>
<td>0.29±0.01</td>
</tr>
<tr>
<td>camphor</td>
<td>-0.12±0.04</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>0.00±0.02</td>
</tr>
<tr>
<td>α-terpineol</td>
<td>-0.12±0.04</td>
</tr>
<tr>
<td>bornyl acetate</td>
<td>0.02±0.02</td>
</tr>
<tr>
<td>eugenol</td>
<td>5.73±1.50</td>
</tr>
<tr>
<td>methyl eugenol</td>
<td>-0.08±0.03</td>
</tr>
<tr>
<td>sesquiterpenes</td>
<td>-3.89±0.74</td>
</tr>
</tbody>
</table>
Table 8.7. Differences (mean, n=2) in essential oil composition between \( T_0 \) and \( T_{10d} \) values for control and \( B. \) cinerea infected \( S. \) officinalis leaves.

<table>
<thead>
<tr>
<th>Components</th>
<th>Difference in percentage composition between ( T_0 ) and ( T_{10d} ) (( T_{10d} - T_0 ) (mean) (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control leaves</td>
</tr>
<tr>
<td>( E )-salvene</td>
<td>0.01</td>
</tr>
<tr>
<td>( Z )-salvene</td>
<td>0.18</td>
</tr>
<tr>
<td>( \alpha )-thujene</td>
<td>-0.26</td>
</tr>
<tr>
<td>( \alpha )-pinene</td>
<td>0.11</td>
</tr>
<tr>
<td>camphene</td>
<td>0.02</td>
</tr>
<tr>
<td>cluster 1(^a)</td>
<td>-0.78</td>
</tr>
<tr>
<td>( p )-cymene/limonene</td>
<td>0.21</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>0.89</td>
</tr>
<tr>
<td>( \gamma )-terpinene</td>
<td>-0.16</td>
</tr>
<tr>
<td>( E )-sabinene hydrate</td>
<td>0.01</td>
</tr>
<tr>
<td>( \beta )-thujone</td>
<td>0.66</td>
</tr>
<tr>
<td>camphor</td>
<td>-0.79</td>
</tr>
<tr>
<td>pinocamphene</td>
<td>-0.02</td>
</tr>
<tr>
<td>borneol</td>
<td>0.05</td>
</tr>
<tr>
<td>bornyl acetate</td>
<td>-0.19</td>
</tr>
<tr>
<td>sesquiterpenes</td>
<td>0.28</td>
</tr>
</tbody>
</table>

\(^a\) cluster 1 includes \( \beta \)-pinene and myrcene

As it can be seen from the Tables above, there are no notable changes in the essential oil profiles of the infected leaves of both species that are not observed in their respective control leaves. The differences observed between \( T_0 \) and \( T_{10d} \) values (e.g., increase in eugenol levels in \( O. \) minimum leaves) are known to arise due to developmental changes (section 2.5, Chapter 2).

To determine whether leaf-biosynthetic constraints (also examined in section 6.5, Chapter 6) were responsible for the absence of infection-induced changes, the essential-oil profile of \( S. \) officinalis leaves obtained before fungus-infection was compared to that of emergent leaves (from the same plant) 2 months post-infection (Table 8.8). This was carried out only with \( S. \) officinalis as \( O. \)
minimum plants did not survive B. cinerea infection under the fungus-favourable conditions used here.

**Table 8.8.** Effect of B. cinerea infection on oil composition of S. officinalis leaves initiated after damage (mean ± standard error of the mean, n=8). Asterisks indicate statistical significance between the before and after damage values (* at p<0.05; ** at p<0.01, Kruskal–Wallis test).

<table>
<thead>
<tr>
<th>Components</th>
<th>Percentage composition (mean±S.E,M) (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaves already present on the plant (sampled before infection)</td>
</tr>
<tr>
<td>α-thujene*</td>
<td>0.62±0.06</td>
</tr>
<tr>
<td>α-pinene</td>
<td>2.47±0.21</td>
</tr>
<tr>
<td>camphene</td>
<td>1.70±0.18</td>
</tr>
<tr>
<td>cluster 1*</td>
<td>6.11±0.61</td>
</tr>
<tr>
<td>p-cymene/limonene**</td>
<td>1.06±0.12</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>3.85±0.40</td>
</tr>
<tr>
<td>γ-terpinene**</td>
<td>0.61±0.03</td>
</tr>
<tr>
<td>α-thujone</td>
<td>37.6±3.06</td>
</tr>
<tr>
<td>β-thujone</td>
<td>2.77±0.19</td>
</tr>
<tr>
<td>camphor**</td>
<td>8.79±1.04</td>
</tr>
<tr>
<td>pinocamphene</td>
<td>0.54±0.04</td>
</tr>
<tr>
<td>borneol</td>
<td>0.40±0.08</td>
</tr>
<tr>
<td>terpinen-4-ol</td>
<td>0.33±0.02</td>
</tr>
<tr>
<td>sesquiterpenes*</td>
<td>30.9±3.72</td>
</tr>
</tbody>
</table>

* cluster 1 includes β-pinene and myrcene

As it can be seen from Table 8.8 there are differences between the essential oil profiles of S. officinalis leaves obtained before fungus infection and those emerged after infection; camphor (-6.32%) and sesquiterpenes (+11.3%) are the compounds exhibiting the greatest changes. However, these differences are consistent with the ontogenetical changes occurring in S. officinalis leaves (section 2.5, Chapter 2). Thus, the differences demonstrated above simply illustrate the dissimilar ages (emergent leaves the younger) of the two leaf-groups.
There are many physical/morphological factors that determine whether a fungal or bacterial plant-pathogen will successfully invade a host cell (Moerschbacher and Mendgen, 2000). To check whether differences in constitutive terpenoids could also contribute to fungal resistance, PCA was performed on the oil profiles of *S. officinalis* leaves inoculated with *B. cinerea*. The major oil components found on *S. officinalis* leaves (1,8-cineole, α- and β-thujone, camphor, sesquiterpenes) were used as variables. The plot constructed from the scores of the PCA (Figure 8.6) clearly distinguishes the infection-resistant and susceptible leaves based on camphor and sesquiterpene levels.

![PCA scatterplots of oil from *B. cinerea*-inoculated *S. officinalis* leaves on the first 2 principal components.](image)

**Figure 8.6.** PCA scatterplots of oil from *B. cinerea*-inoculated *S. officinalis* leaves on the first 2 principal components.
PCA suggests that low camphor and sesquiterpene levels may increase the susceptibility of *S. officinalis* leaves to *B. cinerea* infection whilst the levels of 1,8-cineole, α- and β-thujone do not have an effect. This theory is in agreement with the evidence provided by Carta *et al.* (1996) according to which camphor and the oxygenated fraction (including sesquiterpenoids) of *S. officinalis* oil were fungicidal against *B. cinerea* at 1.75 and 1.85 gL⁻¹, respectively. The same study found that 1,8-cineole, α- and β-thujone had no effect on the test organism (Carta *et al.*, 1996).

### 8.4 Conclusion

Herbivory, pathogen attack, and jasmonic acid elicitation did not induce major changes in the essential oil profile of the plants tested. However, evidence suggested that constitutive terpenoids play important roles in the interactions between plants and their herbivores / pathogens.
REFERENCES


CHAPTER 9

GENERAL DISCUSSION AND CONCLUSION

9.1 General discussion

9.1.1 Overview

Previous efforts have shown that stress-induction of secondary metabolites that exhibit changes in their levels depending on plant and/or leaf age are difficult to detect (e.g., induction of leaf-alkaloids) (Constabel, 1999). This study, using the appropriate design, methodology and analysis, produced evidence of damage-induced changes in the leaf-chemistry of the terpene-accumulating species examined despite the interference of factors such as terpenoid-ontogenetical changes.

However, despite the application of eliciting organisms/chemicals and the use of experimental designs that eliminated biosynthetic constraints, the detected induced-changes were small and not as distinctive as those reported for many non oil-bearing species (Chapter 1 and references therein).

Although small in magnitude, the observed changes may be economically and ecologically significant. For example, it may be possible to increase oil yields during the commercial harvesting of \textit{M. Alternifolia} by mechanically wounding the leaves prior to distillation. Commercial potential may also be associated with the differential resistance/susceptibility of \textit{S. officinalis} leaves to \textit{B. cinerea}. From an ecological perspective, small effects are not always unimportant as even minute gains in fitness can be vital contributions to future generations (e.g., Bruin \textit{et al.}, 1995).
9.1.2 Metabolic costs and plant defense

The presence of limited induced-defenses in terpene-accumulating plants is likely to be associated with the high levels of constitutive terpenoids they possess. Accumulation of terpenoids is an "expensive" process; metabolic costs for their manufacture and storage were estimated to be the highest of most other primary and secondary metabolites (Gershenzon, 1994). Thus, plants that synthesise and store terpenoids invest large amounts of resources early in their lifetime for that purpose. As a consequence they have very little resources to commit for the production of additional defensive compounds if required at a latter stage of their development.

A modest stress-induction of phenolics (also known to be "costly" secondary metabolites; Gershenzon, 1994) has been reported for trees that are known to possess high concentrations of phenolics such as trembling aspen (Clausen et al., 1989).

The results obtained here suggest that the metabolic-cost theory also applies in the case of the limited induction of terpenoids. For example, all the wounding-induced changes observed in Chapter 6 were associated with constitutive terpenoids; "new" compounds (not normally present in the leaves) were not formed. With this practice the plants do not utilise great amounts of resources as they employ enzymes, biogenetic pathways and other processes (e.g., ontogeny) that are already in place to express their response.

This approach appears even more sensible when considering that essential oil constituents can have multiple ecological effects within a community depending on their concentration (e.g., Langenheim, 1994 and references therein). Thus, from a defensive perspective, an essential-oil blend provides a general broad-spectrum protection; if selectivity is then required it is achieved (although to a limited extend) by altering the dosage of the appropriate constituent in the blend.
Another cost-effective response mechanism that appears to be employed by damaged terpene-accumulated plants is the catabolism of certain terpenoids (e.g., camphor in *O. minimum*) for the generation of extra-amount of others (e.g., linalool, in *O. minimum*) if required (section 6.4.2.2). Evidence for the existence of such a turnover has also been reported in several studies that utilised detached foliage/parts from terpene-accumulating plants (e.g., Banthorpe and Ekundayo, 1976; Croteau and Loomis, 1972). In line with cost-effectiveness, terpene-turnover was not observed in unwounded plants of several species including *S. officinalis* and *M. alternifolia* (Gershenzon *et al*., 1993).

### 9.1.3 Plant growth and chemical defense

The growth-differentiation hypothesis (Coley *et al*., 1985) has been used regularly to explain differences in the defensive behaviours between plant species (e.g., Loughrin *et al*., 1994). According to this hypothesis, slow-growing plants prefer constitutive defenses because although they are expensive during construction, their cost is independent of leaf lifetime (Edwards, 1992). Induced defenses are employed by fast-growing plants such as annuals because this strategy allows them, in periods of non-attack, to allocate all their energy towards biomass-growth (Edwards, 1992).

According to Murtagh and Baker (1994) the growth-differentiation hypothesis does not apply to *M. alternifolia* as they found no correlation between oil concentration and plant growth rate. The authors did not provide any alternative theory but it may be possible that the risk of discovery by herbivores is the major factor influencing the type of defense employed by plants; species with high risk of herbivory prefer constitutive, broad spectrum defenses whilst species usually at risk
only by specialised herbivores employ highly-specific, induced defenses (Feeny, 1976).

9.1.4 Future work

The outcomes of this study highlighted the need for further investigations focusing on determining whether mechanical wounding/stressing of terpene accumulating leaves could result in increased oil yields during commercial harvesting of species such as *M. alternifolia*. If the small changes demonstrated here were to be induced during harvesting, then substantial increases in oil yield would be observed.

Future investigations should be directed towards the possible stress-induced defense mechanisms of essential oil-bearing plants expressed by non-volatile secondary metabolites such as proteins and phenolics. Results from such studies combined with the outcomes of this investigation would provide a complete picture of the chemical defenses employed by terpene-accumulating plants.

Besides the scientific gains resulting from the better understanding of the chemical ecology of such species, commercial exploitation of induced-defenses expressed by non-volatiles and especially proteins may also be possible. In contrast to the complexity of the biogenetic pathways of most other defensive phytochemicals, proteins also have the advantage of being encoded by a single gene which after isolation can be readily used to genetically engineer plants for enhanced pest resistance (Boulter, 1993; Constabel, 1999). At the moment genetic engineering of most other secondary metabolites remains impractical (Constabel, 1999).
9.2 General conclusion

The effect of biotic stress-factors (mechanical wounding, herbivory, pathogen attack, jasmonic acid elicitation) on the production of volatiles by the leaves of three economically important terpene-accumulating plants was determined following the planning of an appropriate experimental design and the development of non-destructive methodologies.

Stress-induced changes in emission of constitutive terpenoids and lipoxygenase-derived products by M. alternifolia, S. officinalis and O. minimum leaves were demonstrated. Although the detected changes were small in magnitude they may be significant from an ecological as well as an economic viewpoint.

The results suggest that the limited resources of terpene-accumulating plants, resulting from their large investment in constitutive defense early in their lifetime, may be the major constraints of their induced defense.
REFERENCES


