COMPARING INDUCED RESPONSES OF *MEDICAGO TRUNCATULA* TO BIOTIC CHALLENGES:

COMMON THEMES, VARYING PATTERNS

Dissertation

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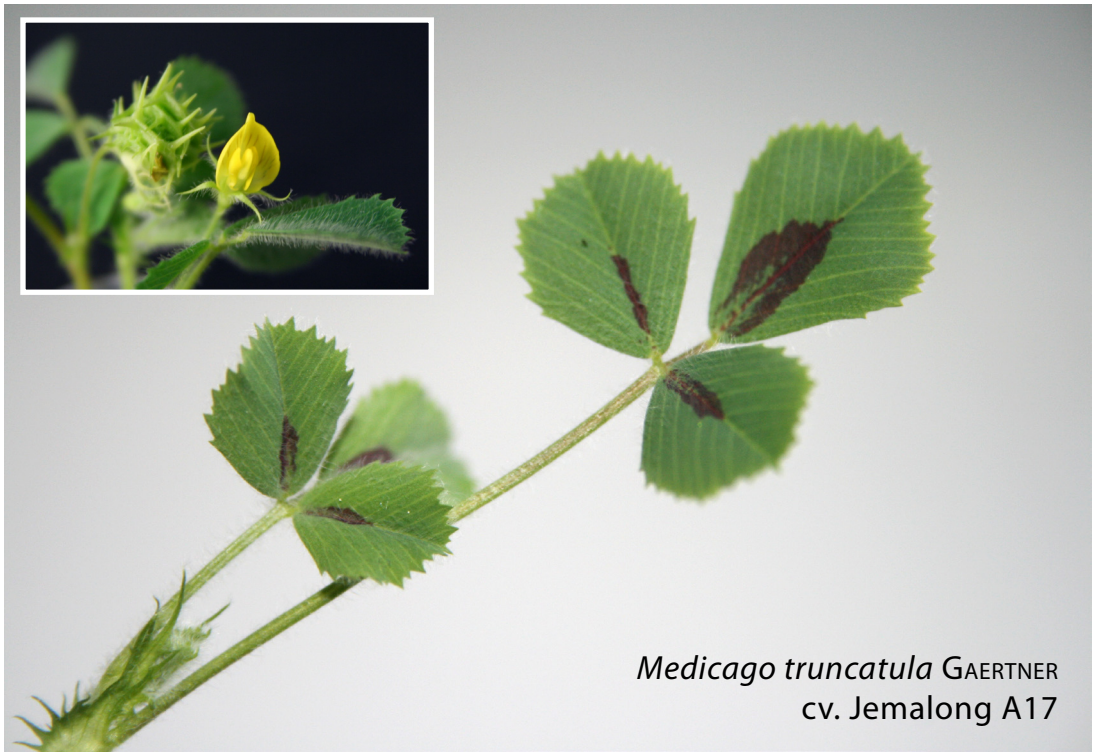


Medicago truncatula GAERTNER
cv. Jemalong A17

Contents

Abbreviations

acetyl-SA	acetylsalicylic acid
ADH	alcohol dehydrogenase
ALA	alamethicin
AM	arbuscular mycorrhiza / arbuscular mycorrhizal
ANOVA	analysis of variance
AOC	allene oxide cyclase
AOS	allene oxide synthase
BA2H	benzoic-acid-2-hydroxylase
CH4	*N,N',N'',N'''*-tetraacetylchitotetraose
DAB	3,3'-diaminobenzidine
DAF-2 DA	4,5-diaminofluorescein diacetate
DMNT	4,8-dimethyl-1,3,7-nonatriene
DMSO	dimethylsulfoxide
DXR	1-deoxy-D-xylulose 5-phosphate reductoisomerase
DXS	1-deoxy-D-xylulose 5-phosphate synthase
ER	endoplasmic reticulum
GC	gas chromatography
GLV	green leaf volatile
HR	hypersensitive response
ICS	isochorismate synthase
IPL	isochorismate pyruvate lyase
IS	internal standard
ISR	induced systemic resistance
JA	jasmonic acid
LCO	lipochitooligosaccharide
LDA	linear discriminant analysis
LOX	lipoxygenase
MAPK	mitogen-activated protein kinase
MeJA	methyl jasmonate
MEP	2-*C*-methyl-D-erythritol 4-phosphate
MeSA	methyl salicylate
misc.	miscellaneous
MS	mass spectrometry
MVA	mevalonic acid
NBT	nitroblue tetrazolium
NMDS	non-metric multidimensional scaling
NO	nitric oxide
Nod-factor	nodulation-factor

NOS	nitric oxide synthase
NR	nitrate reductase
OPDA	12-oxo-phytodienoic acid
PAL	phenylalanine ammonia lyase
PAMP	pathogen-associated molecular pattern
PR-protein	pathogenesis-related protein
RI	retention index
RNI	reactive nitrogen intermediates
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic acquired resistance
TMTT	3*E*,7*E*-4,8,12-trimethyltrideca-1,3,7,11-tetraene
VOC	volatile organic compound

Zusammenfassung

In der vorliegenden Arbeit wurden Abwehrantworten und Reaktionen der Modellpflanze *Medicago truncatula* Gaertner (gestutzter Schneckenklee, Fabaceae) auf verschiedene biotische Reize verglichen. Aus der Vielzahl der möglichen Vergleichsparameter wurden Mechanismen der direkten und indirekten Verteidigung gewählt, sowie Komponenten der Signaltransduktion. Besonderes Augenmerk wurde auf die Emission von Duftstoffen gerichtet, die von *M. truncatula* in hoher Vielfalt freigesetzt werden. Dabei wurden über 90 Substanzen detektiert, die in Reaktion auf verschiedene Stimuli differenziell emittiert werden.

Ein Vergleich der Abwehr gegen Herbivoren mit unterschiedlichem Fraßverhalten zeigte, daß kauende Arthropoden (Raupen der Gattung *Spodoptera*) und stechend-saugende Herbivoren (Spinnmilben, *Tetranychus urticae* Koch) deutlich unterschiedliche Abwehrreaktionen hervorrufen. Die Muster der emittierten Duftstoffe waren eindeutig abweichend. Die Freisetzung dieser Substanzen stellt eine aktive Form der indirekten Verteidigung dar, wie durch Verhaltensstudien (mittels Y-Olfaktometer) an Raubmilben (*Phytoseiulus persimilis* Athias-Henriot) belegt wurde. Allerdings diskriminierten die Räuber nicht zwischen den unterschiedlichen Duftmustern; sie wurden von spinnmilben- wie raupeninduziertem Duft gleichermaßen angelockt. Auch phytohormonelle Antworten zeigten klare Kontraste. Während in Reaktion auf Raupenfraß nur Jasmonsäure (JA) in nennenswertem Maße akkumulierte, scheint die Verteidigung gegen Spinnmilben durch JA und Salicylsäure (SA) kontrolliert zu werden. Beide Herbivoren induzierten die lokale Deposition von phenolischen Substanzen und eine Anreicherung von reaktiven Sauerstoffspezies (reactive oxygen species, ROS) an der Verwundungsstelle. Diese Reaktionen werden hauptsächlich mit der Abwehr von Pathogenen in Verbindung gebracht. Tatsächlich konnte eine vergleichbare Reaktion auch durch β-Glukane aus der Zellwand des phytopathogenen Oomyceten *Phytophtora sojae* Kaufmann & Gerdemann hervorgerufen werden, was auf eine Überschneidung der Abwehr gegen Fraßfeinde und Pathogene hinweist.

Auch die Zusammensetzung der Duftstoffe, die nach Behandlung mit dem Glukan-Elicitor freigesetzt wurde, ähnelte stark dem durch Raupen hervorgerufenen Muster. Daraufhin wurden weitere mikrobielle Oligosaccharide auf ihre Fähigkeit getestet, vergleichbare Reaktionen auszulösen. Neben den bereits erwähnten β-Glukanen wurden auch *N,N',N'',N'''*-Tetraacetylchitotetraose (CH4, ein Bestandteil der pilzlichen Zellwand) und zwei Nod-Faktoren (von denen nur einer biologische Aktivität hinsichtlich der Induktion einer Nodulierungsantwort in *M. truncatula* aufweist) verwendet, um ihren jeweiligen Einfluß auf pflanzliche Verteidigungsmechanismen zu untersuchen. Auch in diesem Fall konnten distinkte Duftmuster in Reaktion auf die unterschiedlichen Elicitoren gemessen werden. Nur CH4 löste keine derartige Antwort aus. Ein Konzentrationsanstieg von JA konnte nach Behandlung mit den Oligosacchariden aus Pathogenen detektiert werden, nicht aber nach Kontakt mit Nod-Faktoren. Abschneiden der Pflanzen führte zu einem starken Anstieg des SA-Gehaltes, der durch Zugabe keines der getesteten Elicitoren weiter gesteigert werden konnte. Insgesamt wurde keine Korrelation zwischen Duftstoffemission und phytohormonellen Änderungen festgestellt. Überproduktion von ROS wurde durch alle getesteten Signalstoffe verursacht, außer durch alleinige mechanische Verwundung. Interessanterweise riefen alle Substanzen, die zu erhöhter Duftemission führten, auch die Akkumulierung von Stickoxid (NO) hervor.

Des Weiteren wurde, um einen Einblick in multiple biotische Interaktionen zu erlangen, der Einfluß von Mykorrhizierung mit *Glomus intraradices* Schenck & Smith auf herbivorieinduzierte Duftfreisetzung untersucht. Obwohl die Auswirkungen der Symbiose auf diese Abwehrantwort nicht sehr auffällig waren, konnten sie mittels multivariater statistischer Methoden veranschaulicht werden. Es war auch möglich, anhand des Duftmusters Vorhersagen über den physiologischen Status der jeweiligen Pflanze zu treffen. In dieser Teilstudie wurden zwei verschiedene Kultivare von *M. truncatula* verwendet (cv. Jemalong A17 und ein handelsübliches Gemisch aus cv. Paraggio und cv. Jemalong). Erstaunlicherweise waren die auf Mykorrhizierung zurückzuführenden Effekte in den beiden Genotypen genau entgegengesetzt. Während die Pflanzen des Kultivargemisches dazu tendierten, bestimmte Substanzen in höherem Maße freizusetzen, zeigten mykorrhizierte Pflanzen des cv. Jemalong A17 reduzierte Emission von raupeninduziertem Duft. Diese Wirkungen betrafen allerdings nur Komponenten, die ohnehin nur in geringen Mengen oder sogar nur in Spuren freigesetzt wurden. Es wurden aber auch zwei bislang unidentifizierte Substanzen gefunden, die in etwas größeren Mengen emittiert wurden und deren Freisetzung sich in beiden Kultivaren durch Mykorrhizierung vergleichbar änderte.

In Anbetracht der Vielfalt der von *M. truncatula* emittierten Duftstoffe und der hohen Spezifität der resultierenden Muster, wurden weitere Elicitoren biotischen (Alamethicin) und abiotischen (Kupfersulfat und Coronalon) Ursprungs, sowie die exogene Applikation von JA und SA auf ihr duftinduzierendes Potential hin getestet. Wieder konnten distinkte Duftmuster gemessen werden. Jede der getesteten Substanzen induzierte dabei sehr spezifische und klar unterscheidbare Duftmuster.

Des Weiteren zeigte sich, daß multivariate statistische Methoden geeignete Hilfsmittel zur Visualisierung und Klassifizierung von Duftmustern darstellen. Diese Verfahren könnten es ermöglichen, in Zukunft Signalkaskaden und Effekte auf andere Organismen mit spezifischen Duftmustern in Verbindung zu bringen, anstatt mit einzelnen Komponenten aus komplexen Mischungen.

Insgesamt scheinen sich die Antworten von *M. truncatula* auf biotische Stimuli in Hinsicht auf die qualitativen Merkmale stark zu ähneln. Trotzdem zeigten sich in der quantitativen und zeitlichen Entwicklung dieser Reaktionen große Unterschiede. Die induzierten Antworten dieser Pflanze in biotischen Interaktionen können daher als Variationen eines gemeinsamen Themas interpretiert werden. Obwohl die verwendeten Komponenten oft ähnlich waren, entstand durch diese leichten Variationen ein spezifisches Reaktionsmuster auf jeden der getesteten Reize. Außerdem konnten deutliche Unterschiede zwischen den herbivorieinduzierten Duftmustern verschiedener Kultivare festgestellt werden. Zusammen mit der Tatsache, daß einige der gemessenen Reaktionen in Widerspruch zu anderen in der Literatur belegten Studien stehen, weist dies auf hohe Abhängigkeit bestimmter Antworten von dem genetischen Hintergrund der Pflanze hin. Es scheint daher eindeutig sinnvoll, biotische Interaktionen anhand einer Modellart zu untersuchen, um prinzipielle Überlappungen und Unterschiede festzustellen. Allerdings zeigen die oben erwähnten Disparitäten die Notwendigkeit auf, diese grundlegenden Studien durch Vergleiche verschiedener Genotypen einer Art und anderer Spezies zu ergänzen.

Summary

The present study aimed to compare the responses of the model legume *Medicago truncatula* Gaertn. to different biotic stimuli. It concentrated on direct and indirect defence reactions as well as some components of signal transduction, and thereby attempted to establish a model system for studying multiple biotic interactions. Special focus was placed on the emission of volatile organic compounds (VOCs) that proved to be released in high diversity, with more than 90 compounds being differentially emitted in response to varied stimuli.

A comparison of the responses of *M. truncatula* to herbivores with different modes of feeding revealed that chewing arthropods (lepidopteran larvae of the genus *Spodoptera*) and cell-content feeders (spider mites, *Tetranychus urticae* Koch) induced clearly distinct defence reactions. The patterns of VOCs emitted after infestation differed strongly. The release of VOCs represents an active means of indirect defence in this instance, as ascertained by behavioural studies of predatory mites (*Phytoseiulus persimilis* Athias-Henriot) using Y-tube olfactometer tests. The attraction of predators was not specific for the attacking herbivore though, as predatory mites were equally attracted by VOC blends induced by host and non-host organisms. Also the responses in terms of phytohormonal changes differed depending on the type of herbivore. While in the defence against *Spodoptera* spp. only jasmonic acid (JA) was found to accumulate in appreciable amounts, defence against spider mites seems to be mediated by both JA and salicylic acid (SA). Both phytophages induced the local deposition of phenolic compounds and the accumulation of reactive oxygen species (ROS), presumably as modes of direct defence. These reactions, mainly linked to defence against pathogens, were also detected in reaction to β-glucans from the cell wall of the phytopathogenic oomycete *Phytophthora sojae* Kaufmann & Gerdemann, and thus indicate overlapping defence responses to herbivores and pathogens.

The VOCs emitted in response to the latter also exhibited remarkable similarity to the blends detected after caterpillar feeding. Consequently, additional microbial oligosaccharides were tested for their potential to induce similar responses. Besides the aforementioned β-glucans, *N,N',N'',N'''*-tetraacetylchitotetraose (CH4, a component of the fungal cell wall), and two Nod-factors (of which only one induces the nodulation response in *M. truncatula*) were used to compare their respective impact on defensive traits. Again, distinct VOC emission patterns were recorded for each of the compounds tested, with only CH4 being inactive in inducing VOC release. JA accumulated only after induction with the pathogen-derived elicitors, but not in response to Nod-factors. Cutting the plants proved to strongly increase SA levels, which could not be further enhanced by adding any of the oligosaccharides. Thus, no correlation between VOC emission and phytohormonal changes can be stated. The overproduction of ROS could be detected in response to all signalling compounds tested, but not after mechanical damage alone. Interestingly, the capability of the elicitors to induce nitric oxide (NO) accumulation correlated with the induction of VOC emission.

A step towards the investigation of multiple interactions was taken by assessing the impact of mycorrhization with *Glomus intraradices* Schenck & Smith on herbivore-induced VOC emission. Though the impact of the symbiotic state was not massive, it was traceable with multivariate statistical methods and even proved to be predictable to some extent. Strikingly, the effect on two different cultivars of *M. truncatula* (cv. Jemalong A17 and a commercially available mixture of cv. Paraggio and cv. Jemalong) was exactly the opposite: the cultivar mixture tended to emit

higher amounts of certain compounds, cv. Jemalong A17 exhibited reduced emissions. This effect, however, solely concerned substances emitted in minor amounts. But also the release of two so far unidentified compounds changed with mycorrhization and exhibited comparable alterations in both cultivars. Both substances were emitted in amounts notably higher than the other compounds affected.

Furthermore, in view of the multitude of VOCs emitted by *M. truncatula* and the high specificity of the respective patterns, several elicitors from biotic (alamethicin) and abiotic origin (copper sulphate and coronalon) along with exogenously applied JA and SA were tested for their potential to induce distinct VOC blends. Again, statistically distinguishable patterns of differential emission could be ascertained.

Finally, VOC emission patterns could be visualised and classified using multivariate statistical methods. Using this approach, it might be possible to link signalling events and observed effects on other organisms to specific VOC patterns rather than to particular components of a blend.

Altogether, data gathered on biotic interactions indicate that the response to different stimuli is often similar with respect to qualitative traits, though in terms of quantitative and temporal development, reactions showed clear distinctions. Responses to biotic stimuli thus seem to be made up of slight variations of a common theme in *M. truncatula*. Taken together, these variations in single parameters produce unique patterns of responses to distinct biotic stimuli. The recorded responses seem to be in part highly species specific, with respect to numerous contrasting studies found in the literature. Even between distinct cultivars, considerable differences were found in this study concerning herbivore-induced VOC emissions. Though it is clearly useful to compare diverse biotic interactions using one model plant in order to elucidate overlaps and distinctions, intraspecific as well as interspecific comparisons are essential.

1 General introduction

1.1 Biotic interactions

Just like all living organisms, plants are continuously confronted with a diverse range of challenges from their environment. These can be either abiotic, referring to any deviance of the physical or chemical environment that is adverse to the plant's needs and integrity, or biotic. The latter means the interactions with other organisms, which can be beneficial or detrimental by their nature. As plants are very restricted in their motility, they cannot react by fleeing. But they are not merely patient sufferers, as they make us of an arsenal of physico-chemical opportunities to actively influence and participate in biotic interactions. In this complex net of interactions, all kinds of organisms are involved, ranging from bacteria to mammals; and of course this also includes interactions between plants, be it within one species or between different species (Figure 1-1).
Bacteria can be deleterious to plants as pathogens, or beneficial as symbionts, as for example nitrogen-fixing rhizobia. The same holds true for fungi, which as pathogens can oppose plants, but also can enter into close association with plants as in the example of mycorrhizal fungi. Likewise, the interactions with animals are diverse. On the one hand, plants as primary producers are consumed by a wide range of herbivores, including amongst others nematodes, arthropods, molluscs, and of course mammals. On the other hand, plants take advantage of animals, using them as pollinators, seed dispersers, defenders, and even as supplementation for their nutrition in the case of carnivorous plants.
As varied as those interactions are, as long could be any enumeration. In the following sections the emphasis will be placed on only some of them, namely interactions with herbivorous arthropods, and bacterial and fungal pathogens as well as symbionts.

1.1.1 Classification of plant defence mechanisms

Defence mechanisms of plants can generally be classified as constitutive and induced. Examples of constitutive defences are thorns or prickles that may deter bigger herbivores, massive cell walls and cuticles that hinder the penetration of bacteria or fungi, and trichomes that can constitute a mechanical barrier against smaller herbivores, and can also carry secretory structures, producing and storing antimicrobial compounds, feeding deterrents, or toxins.
In contrast to constitutive defences, induced mechanisms are activated only in situations of actual threat. In the following, the former will be neglected in favour of the treatise of induced defences.
Another separation allows defence responses to be classified as direct and indirect lines of action. The first is targeted directly at the offending organism. Such mechanisms include the induced accumulation of toxins and feeding deterrents, fortification of cell walls, and the hypersensitive reaction. The term "indirect defences" is mainly connected to the defence against herbivores. It refers to any opportunity the plant has to manipulate the behaviour of predators or parasitoids of an herbivore that increases protection of the plant. The mechanisms of indirect defence include the offer of accommodation to carnivores, as for example by domatia, and the offer of alternative food sources, like extrafloral and floral nectar or food bodies (Sitte *et al.*, 2002). With the emission

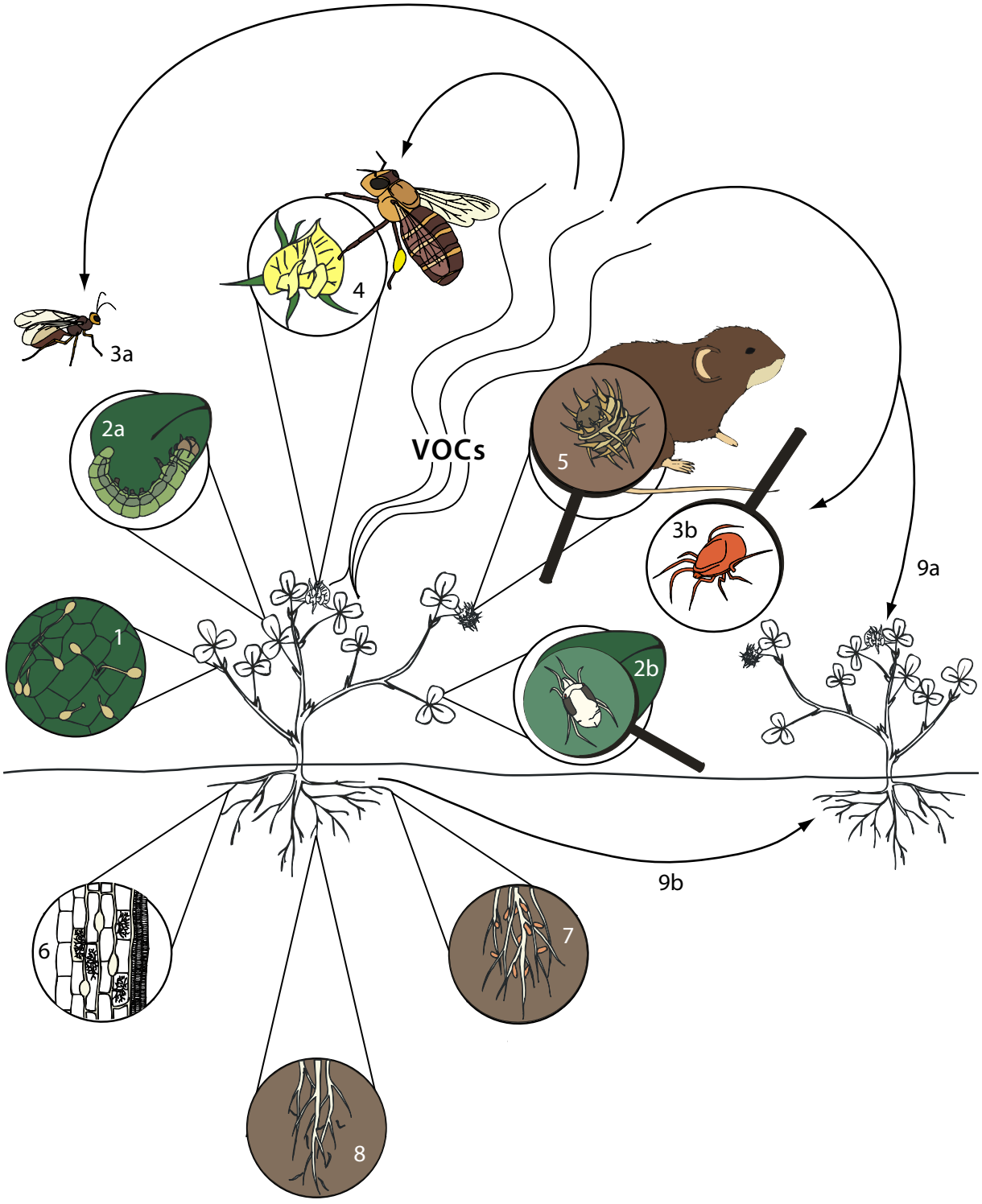


Figure 1-1 Selected biotic interactions involving plants. 1, infection with pathogenic fungi; 2, herbivore attack; 2a, by chewing insects, e.g. lepidopteran larvae; 2b, by cell content feeders, e.g. spider mites; 3, attraction of parasitoids or predators by herbivore-induced volatiles; 3a, parasitoid wasps attracted to caterpillar-damaged plants; 3b, predatory mites attracted to spider mite-infested plants; 4, attraction of pollinators by floral volatiles and nectar; 5, animals may act as seed dispersers; 6, plant roots are often colonised by symbiotic arbuscular mycorrhizal fungi; 7, legumes can also enter into symbiosis with rhizobia, resulting in nodulation of the roots; 8, belowground herbivory, e.g. by nematodes.

of VOCs (volatile organic compounds) the plant provides information for the location of food resources to predators or parasitoids (Dicke & Van Poecke, 2002; Kessler & Baldwin, 2002). Direct and indirect defences are represented in both constitutive and induced defence mechanisms.
Finally, an alternative way to cope with various stresses is by developing tolerance. Instead of investing in defences, sources can be allocated to compensatory growth, thus conferring tolerance.

1.2 Induced defences against herbivores

Regarding direct defences of a plant against phytophagous attack, the first step usually consists of physical factors an herbivore has to overcome in order to reach its food source. In plants such defence mechanisms are represented by trichomes, thorns or prickles, lignification, and the overall plant habitus that might be unfavourable for certain herbivores. The production of toxins is a widespread strategy of plants to reduce herbivory. The effects produced by these chemically diverse substances comprise membrane disruption, inhibition of transport or signal transduction, impaired metabolism, and even disruption of hormonal control of development (Gatehouse, 2002). Another way of coping with herbivore attack is the production of digestibility reducers. These include tannins, resins, proteinase inhibitors, and silica (Price *et al.*, 1980; Sitte *et al.*, 2002). By reducing the digestive efficiency of the herbivore, they delay its development; also lowered resistance to disease and reduced fecundity can be consequences of poor nutritional quality (Price *et al.*, 1980). Most of the above-mentioned strategies can be either constitutive or inducible. In the case of chemical defence, the compounds used in both strategies are often the same within one given plant species. Many forms of induced defence are not restricted to local responses at the wounding site, but can be detected systemically throughout the plant.
As defined above, indirect defences are mechanisms a plant employs to attract predators or parasitoids of an attacking herbivore. It can indirectly reduce feeding pressure by eliminating the herbivores with natural enemies. By offering nutrients, plants can achieve a loose mutualism between themselves and certain carnivores. Secretions of nectar by extrafloral or floral nectaries as well as the offer of food bodies are well established examples for rewards given to potential protectors of the plant (Sitte *et al.*, 2002; Heil *et al.*, 2004). Furthermore, plants can provide housing for carnivores by certain anatomical structures such as domatia or hollow thorns (Sitte *et al.*, 2002; Romero & Benson, 2005). Finally, with the emission of VOCs plants make valuable information available to predators and parasitoids about the location of potential food sources.

1.3 Defence mechanisms against pathogens

Plants are antagonised by a vast variety of pathogens, including fungi, bacteria, and viruses. They can enter a plant either by direct penetration, through natural openings, such as stomata or lenticels, or through wounds (Agrios, 1978). For successful infection pathogens need to evade or overcome the plant's defences. In order to do so they can employ different strategies, as for example the production of toxins, the synthesis of phytohormones or analogous substances to

manipulate the physiology of the host, or the synthesis of suppressors of plant defence responses and hydrolytic enzymes (Sitte *et al.*, 2002).

Plants counter these offences with multifaceted defence reactions. Basically, host and non-host interactions can be distinguished. The first is race-specific and occurs only in comparatively few cases between selective pathogens and their respective hosts (Dangl & Jones, 2001; Rathjen & Moffett, 2003). As any extensive description of this broad topic would be far beyond the scope of this introduction, the following will focus only on mechanisms of non-host resistance.

As most plant pathogens have a rather limited range of potential hosts in which they can cause disease, most interactions are non-specific. Non-host disease resistance substantially overlaps with mechanisms of host resistance, though the attacking pathogen is not specifically recognised with regard to gene-for-gene interaction (Thordal-Christensen, 2003; Halim *et al.*, 2006). Instead, recognition of non-host pathogens is mediated by general elicitors of plant defence. These compounds consist of breakdown products of the cell wall or membrane of either of the interacting organisms, resulting from lytic processes at the infection site. Structures like oligogalacturonic acid from the plant primary cell wall, flagellin fragments, chitin and glucan oligomers, fungal sterols, and glycopeptid fragments can have elicitor activity (Ebel & Mithöfer, 1998; Sitte *et al.*, 2002). In many aspects, those molecules share features of pathogen-associated molecular patterns (PAMPs), which are important for non-self recognition in the animal immune system (Nürnberger *et al.*, 2004). The chemical structures of microbial elicitors are of considerable diversity, though the cellular effects caused by them share some similarities. The plant can react to those challenges with structural and chemical induced defences. Induced structural barriers include the formation of callose, a β-1,3-glucan, at the penetration site of the pathogen, fortification of the cell walls, and lignification. The chemical part of induced defences consists amongst others of the accumulation of phytoalexins, many of which are terpenoids or phenylpropanoids and are produced *de novo* in response to pathogen attack. Furthermore, the fast synthesis of toxins, especially phenolics, and pathogenesis-related proteins (PR-proteins), the oxidative burst, and programmed cell death are hallmarks of induced defences against pathogens (Ebel & Mithöfer, 1998).

Besides this armoury of local responses, plants also mount systemic defence in response to pathogen infection. After immediate local defence responses, systemic acquired resistance (SAR) develops. It implicates the accumulation of salicylic acid throughout the plant as well as the expression of PR-proteins, and renders the plant more resistant to subsequent infections (Glazebrook, 2001). Though it is agreed that there has to be a systemic signal that is mobile but not species specific, as demonstrated by grafting experiments, the nature of this signal remains elusive (Durrant & Dong, 2004).

The events illustrated above bear some striking analogies to processes related to innate immunity in animals, where the first action to control infection is non-specific (Menezes & Jared, 2002; Jones & Takemoto, 2004). First, the pathogen has to overcome preformed barriers, be they the cuticles or cell walls of plants, or the skin or mucous membranes of animals. Chemical barriers such as antimicrobial secondary metabolites or peptides are mirrored by biochemical and physiological barriers in animals, such as acidity of skin and stomach, body temperature, production of lysozyme, etc. If those preformed defences fail to confine pathogen invasion, the next defensive system is activated via the recognition of PAMPs. PAMPs are structures that are crucial for non-self recognition, not present in the potential host, and essential for microbial fitness (Nürnberger *et al.*, 2004). Upon the recognition of those general elicitors, plants activate different inducible

defences. Just to mention some examples, the generation of reactive oxygen species (ROS) during the hypersensitive response (HR) in plants exhibits many similarities to the oxidative burst in mammalian neutrophils (Cohn *et al.*, 2001). The same holds true for the involvement of second messengers as signals that are conserved among most eukaryotes, including increased intracellular calcium levels, generation of nitric oxide (NO), and mitogen-activated protein kinase (MAPK) cascades (Nürnberger *et al.*, 2004). The only conspicuous difference between immunity in plants and animals is the lack of an adaptive immune system in the former. A comparison of some analogies between plant and animal immunity is summarised in Table 1-1. Those similarities prompted agreement regarding the term "plant innate immunity". It comprises both host and non-host resistance (Nürnberger & Kemmerling, 2006).

Table 1-1 Comparison of plant defence mechanisms and the animal immune system.

		Plants	**Vertebrates**	
Preformed defences	Physical	Cell wall Wax layer Bark	Skin Mucous membranes	Physical
	Chemical	Secondary metabolites (terpenoids, phenolic compounds, alkaloids …) Anti-microbial enzymes	Temperature Low pH Lysozyme	Biochemical / physiological
Induced defences	Recognition	**PAMPs (pathogen-associated molecular patterns)**		Recognition
	Pathogen	Chitin-fragments, glucans, flagellin, peptides …	Lipopolysaccharides, peptidogycans, bacterial DNA, dsRNA …	Pathogen
	Host	Toll-family members Intracellular protein kinases	Toll-family members Intracellular protein kinases	Host
	Signal transduction	Ca^{2+}, NO, ROS, SA, JA …	ROS, NO, cytokines, chemokines	Signal transduction
	Defence response	HR, PR-proteins, phytoalexins, etc.	Necrosis, apoptosis, inflammation, phagocytosis	Defence response
	-	-	antigen-specific T and B-lymphocytes	Adaptive Immunity

1.4 Symbiotic interactions

1.4.1 Mycorrhiza

Mycorrhization is an enormously widespread symbiosis between roots of terrestrial plants and fungi belonging to the Glomeromycota; about 90 % of land plants are colonised by mycorrhizal fungi. The evolution of this symbiosis is assumed to date back at least 450 – 500 million years, where it may have aided plants during the colonisation of the land by supporting the uptake of mineral nutrients and water. This notion is substantiated by the fact that certain bryophytes and pteridophytes are still capable of forming mycorrhizal symbiosis (Strack *et al.*, 2003). Today, the predominant hosts, however, are angiosperms; but also some gymnosperms, lycopods, and

the already mentioned mosses and ferns are able to enter into symbiosis with mycorrhizal fungi, whereas only few plant families withstand invasion by these symbionts. Although, for example, Brassicaceae, Cyperaceae, and Amaranthaceae do not enter into symbiosis with arbuscular mycorrhizal (AM) fungi, the latter have developed an obligate symbiotic life cycle (Sitte *et al.*, 2002). Among several types of mycorrhiza, arbuscular mycorrhiza is predominant, occurring in about 80 % of mycorrhizal plants (Strack *et al.*, 2003); it is a form of endomycorrhiza, where the fungus enters the root cortex and also builds haustoria in the cells. In contrast, in ectomycorrhiza the fungus establishes a coat of hyphae around the root; though the mycelium also enters the root cortex, no haustoria are formed in plant cells (Sitte *et al.*, 2002).
In brief, colonisation of the roots by AM fungi commences with the germination of fungal spores, which is stimulated by plant root exudates and volatiles (Harrison, 2005); this is followed by only poorly understood signalling events leading to directed growth of the hyphae to the plant roots. The hyphae subsequently enter the roots, penetrate cortical cells, and finally build highly branched haustoria inside the cells, called arbuscules. These structures remain separated from the cytoplasm of the plant by the plasma membrane of the plant cell, building a periarbuscular membrane similar to the structures housing rhizobia. A net of hyphae outside of the plant root produced by the fungus greatly enhances the contact surface to the soil, thereby facilitating the uptake of nutrients and water. Thus, the fungus supports the plant in supplying the needs in mineral nutrients, mainly phosphate, and water. Conversely, the plant provides the fungus with sugars. As for the improved nutritional status of the plant, one of the most obvious effects of the symbiosis is enhanced growth and resistance against pathogenic fungi and nematodes (cf. Chapter 4).

1.4.2 Nodulation

Plants by themselves are not able to fix dinitrogen (N_2) and to reduce it to ammonium. This capability is restricted to some eubacteria and cyanobacteria, and depends on the enzyme nitrogenase. Fabaceae, however, are able to enter into symbiosis with rhizobia. The term rhizobia refers to gram-negative bacteria belonging to the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*. These bacteria are not obligate symbionts, but usually do not fix nitrogen under non-symbiotic conditions. All three subfamilies of the Fabaceae, namely Papilionoideae, Mimosoideae and Caesalpinioideae, include genera that are able to establish symbioses with rhizobia; however, the most primitive of them, Caesalpinioideae, contains comparatively many genera that do not nodulate, which indicates that this symbiosis developed quite late during legume evolution (Van Rhijn & Vanderleyden, 1995). Hence, this mutualism is considerably younger compared to mycorrhization, having only evolved as late as about 58 million years ago (Kistner & Parniske, 2002; Sprent, 2007).
The events leading to this association start with chemotactic attraction of free living rhizobia, caused by flavonoids secreted by the root. As a consequence, bacteria attach to the root surface and get entrapped by curling of young growing root hairs. Starting from a local lesion in the plant root cell wall, bacteria can enter into the root hair through a plant-derived infection thread, which can be figured as inwardly directed, reverse tip growth of the root hair. At the same time, cortical cells start to divide and form a nodule primordium (Van Rhijn & Vanderleyden, 1995; Esseling & Emons, 2004). The infection thread reaches this primordium, releasing the bacteria that always remain surrounded by the plant plasma membrane, which in this state is called the peribacteroid

membrane. Finally, after the bacteria have arrived in the nodule primordium, they undergo changes in shape and cell wall structure, and develop into so-called bacteroids that do not divide anymore but start to fix dinitrogen (Sitte *et al.*, 2002) in a microaerobic environment, as nitrogenase is highly sensitive to oxygen. Low oxygen concentration is maintained by leghemoglobin, an oxygen-binding plant protein, along with bacterial respiration, making a relevant sink for oxygen (Buchanan *et al.*, 2000).
To achieve such a close association, a lot of preliminaries have to occur. One comparatively well studied part of the interaction is the primary recognition of the symbiotic partners. Upon the perception of root flavonoids, rhizobia activate a range of Nod-genes, many of which are responsible for the biosynthesis of Nodulation-factors (Nod-factors). These signalling compounds confer a major proportion of host specificity and selectivity, though the degree of host selectivity varies considerably depending on the rhizobial strain (Van Rhijn & Vanderleyden, 1995). Nod-factors are usually composed of an oligomeric chitin backbone (with three to five units), with a fatty acid chain attached to it by an amide bond, and can additionally carry several modifications (for a comprehensive overview, see D'Haeze & Holsters, 2002). The structure of the fatty acid chain and other substituents is crucial for the host specificity (D'Haeze & Holsters, 2002; Limpens & Bisseling, 2003). Up to now, however, the subsequent events, including reception of the signal, its transduction, and evasion of plant defence responses, remain unclear.

To establish symbiosis with both AM fungi and rhizobia, the successful suppression of the plant's defence responses is a crucial step. Like all microorganisms, they carry PAMPs. This term can be somewhat misleading, as substances summarised therein do not occur only in pathogens but in all bacteria and fungi, including plant symbionts such as mycorrhizal fungi and rhizobia. These structures are recognised irrespective of whether the organism detected is beneficial or harmful for plant health. Thus, plant defences have to be suppressed if a functional symbiosis is to be established. The mechanisms underlying this process remain elusive (Mithöfer, 2002). However, the containment of the symbionts within parts of the plant may play a central role: in the case of nodules, the release of bacteroids from the symbiosom leads to an immediate activation of defences in the plant.

1.5 Signal components mediating induced defence responses

1.5.1 Reactive oxygen species and nitric oxide

Reactive oxygen species (ROS) is the collective term for different highly reactive forms of oxygen, including amongst others superoxide anions ($O_2^{\cdot-}$), hydroperoxyl radicals ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$) (Vranová *et al.*, 2002). In healthy cells they are produced as products of normal metabolism, e.g. in mitochondria, chloroplasts, and peroxisomes, and highly effective antioxidant systems care for the maintenance of an equilibrium in order to prevent oxidative damage to the cell. Many biotic stresses not only disturb this equilibrium but also induce excessive production of ROS as active signal component.
The so-called oxidative burst, the rapid accumulation of ROS, is one of the earliest reactions induced by pathogen attack (Lamb & Dixon, 1997). The generation of ROS in this context is biphasic. First,

a rapid but transient accumulation of ROS occurs that seems to be a non-specific reaction. In the second phase, the oxidative burst is prolonged and more pronounced (Lamb & Dixon, 1997).
The main enzyme responsible for the enhanced production in the course of the oxidative burst is a NADPH-oxidase, localised in the plasma membrane (Vranová *et al.*, 2002), and generating the short-lived $O_2^{\cdot-}$. Also cell wall-bound peroxidases and amine oxidases may contribute to the enhanced production of ROS (Bolwell, 1999). The major reactive species accumulating is H_2O_2, which is produced from $O_2^{\cdot-}$ either by spontaneous or enzyme-mediated dismutation. It is comparatively long-lived, and able to diffuse through biological membranes. ROS can have several functions in the plant's defence against pathogens. They may function as direct antimicrobial agents, contribute to structural defences via oxidative cross-linking of cell wall components, and constitute components of intra- and intercellular signal transduction (Lamb & Dixon, 1997).

In animals, the pivotal role of NO in diverse physiological processes is a comparatively well understood phenomenon. It is involved in blood pressure regulation, neurotransmission, immune regulation, and in numerous pathological conditions (Valko *et al.*, 2007). It also plays a role in oxygen-dependent defence mechanisms in the case of the respiratory burst in macrophages.
For the sake of simplicity, the term NO here collectively refers to the nitrosyl radical ($NO^{\cdot}$), the nitroxyl (NO^{-}), and the nitrosonium ion (NO^{+}), as suggested by Nathan (2004). Reactive nitrogen intermediates (RNI) additionally include nitrite and higher oxides of nitrogen, *S*-nitrosothiols, peroxynitrite, and dinitrosyl-iron complexes (Nathan, 2004).
In plants, comparable importance of NO has been recognised during the past few years. It participates in processes of plant growth and development, regulation of stomatal conductance, and reactions to abiotic and biotic stresses (reviewed by Neill *et al.*, 2003). Though its significance is well documented by now, its biosynthetic pathways remain elusive. Several sources exist or are suspected to exist in plants. Nitric oxide synthase (NOS)-activity has unequivocally been detected in plants in response to diverse stimuli. For the respective enzyme, this is not the case. Another source of NO in plants is nitrate reductase (NR). Normally, it converts nitrate to nitrite. But it is also able to further reduce nitrite to NO (Wendehenne *et al.*, 2004). However, nitrate is a competitive inhibitor of nitrite reduction, which makes it rather unlikely that NR would produce appreciable amounts of NO under normal physiological conditions (Crawford, 2006). NO production may also take place via non-enzymatic mechanisms. Nitrite can be reduced to NO at low pH in the presence of reducing phenolic compounds as has been detected in the apoplast of barley aleurone layers (Bethke *et al.*, 2004); furthermore, nitrogen dioxide may be converted to NO, catalysed by carotenoids (Neill *et al.*, 2003). As for the action of NO, several ways to interfere with signal transduction are conceivable (Neill *et al.*, 2003). Some major modifications of biomolecules produced by reactive nitrogen species are nitrosylation or nitrosation of heme iron or peptides, nitration of fatty acids and peptides, oxidation of peptides and bases of DNA as well as the deamination of DNA bases (Nathan, 2004). Only recently were *S*-nitrosylated proteins detected in *Arabidopsis thaliana*, providing the first evidence for NO-dependent protein modification in plants (Lindermayr *et al.*, 2005).

By their nature, ROS and RNI are ideal candidates for influencing diverse signalling pathways. As such ubiquitous molecules *per se* are not very likely to be specific signals by themselves, they may exert effects by modifying proteins and thereby their respective activity. This certainly could

include transcription factors, thus linking ROS and RNI generation with changes in gene expression. The modification of other biomolecules can create pools of bioactive oxides of nitrogen, as in the case of nitrated fatty acids (Nathan, 2004; Schopfer *et al.*, 2005; cf. Chapter 3).

1.5.2 The jasmonic acid pathway

Oxylipins are bioactive compounds that are produced from polyunsaturated fatty acids via oxidative processes. One of their most prominent representatives is jasmonic acid (JA); it is synthesised from linolenic acid via the 13-lipoxygenase (LOX) pathway. In this process, the fatty acid is oxygenated by LOX. The resulting 13-hydroperoxylinolenic acid is converted by allene oxide synthase and allene oxide cyclase into 12-OPDA (12-oxo-phytodienoic acid). It is then reduced by 12-OPDA reductase, with three rounds of subsequent β-oxidations finally yielding jasmonic acid (Figure 1-2) (Howe & Schilmiller, 2002). Two branches in the biosynthetic pathway can also lead to the production of VOCs. Some green leaf volatiles can be produced from 13-hydroperoxylinolenic acid via the action of hydroperoxy lyase, and methylation of JA yields the volatile methyl jasmonate, which can be found in emitted VOC blends.

JA and related compounds are involved in signal transduction in responses to biotic and abiotic stresses and development (Creelman & Mullet, 1995; Creelman & Mullet, 1997; Turner *et al.*, 2002). Mutants impaired either in the biosynthesis or in the perception of JA are severely compromised in their defence against insect attack and some pathogenic soil fungi (Halim *et al.*, 2006).

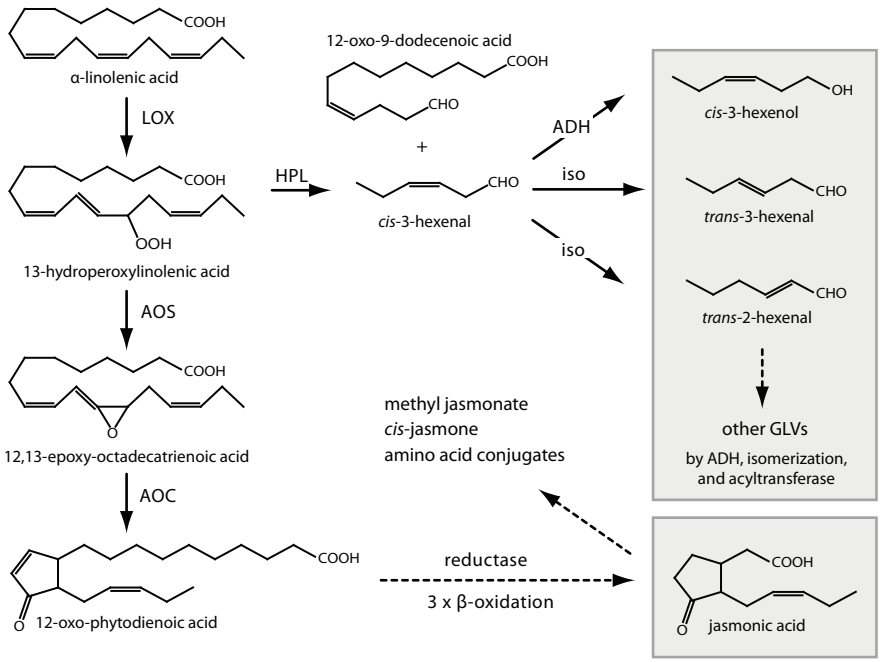


Figure 1-2 Biosynthesis of jasmonic acid and fatty acid-derived green leaf volatiles. Dashed lines indicate multiple biosynthetic steps. Abbreviations: LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; ADH, alcohol dehydrogenase; iso, isomerization; GLVs, green leaf volatiles.

1.5.3 The salicylic acid pathway

Though not irrevocably proven to date, two biosynthetic pathways seem to exist in plants. Salicylic acid (SA) synthesis might start as a branch of the shikimic acid pathway. After the conversion of shikimate to chorismate, synthesis can presumably proceed via two different pathways. Chorismate can be converted to phenylalanine and from this, amongst others by the action of phenylalanine ammonia lyase and benzoic-acid-2-hydroxylase, SA is produced. Alternatively, chorismate can be converted to isochorismate and this in turn to SA (Shah, 2003). The latter pathway was first found in some bacteria and seems to be also active in plants (Figure 1-3) (Wildermuth *et al.*, 2001). Finally, as is the case for jasmonate biosynthesis, the SA pathway also plays a role in volatile production, as methyl salicylate is a compound commonly encountered in volatile blends of diverse plant species. Mutants defective in the SA pathway become more susceptible to pathogen attack. They are impaired in race-specific resistance and are also not able to mount SAR (Halim *et al.*, 2006). Concerning the latter, SA is a well-established player in the onset of SAR, though its definite role is so far unknown.

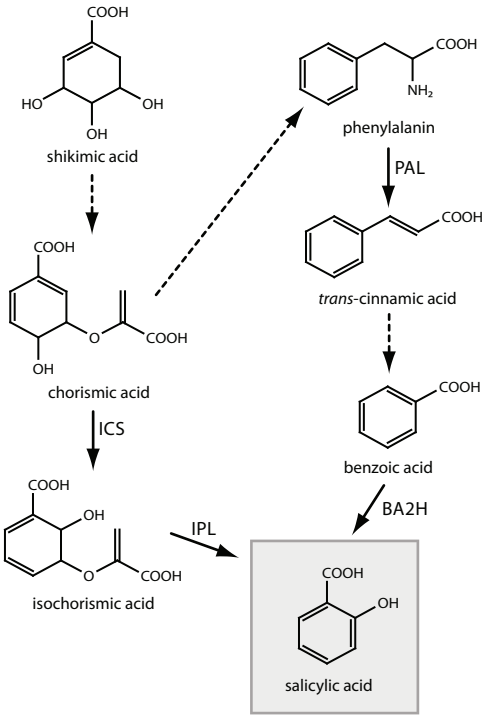


Figure 1-3 Proposed biosynthetic pathways of salicylic acid. Dashed lines indicate multiple biosynthetic steps. Abbreviations: ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; PAL, phenylalanine ammonia lyase; BA2H, benzoic-acid-2-hydroxylase.

Besides its function in defences against pathogens and the onset of SAR in plants, SA has some influence on other physiological properties; the most prominent of them being the calorigen in thermogenesis of floral parts of some plant species (Raskin, 1992). Moreover, SA might act as an allelopathic compound, maybe via interfering with membrane ion transport in roots (Raskin, 1992).

1.5.4 Signal cross-talk at the level of phytohormones

In the literature, predominantly dichotomous approaches are used to explain the interactions between SA-dependent and JA/ethylene-dependent signalling pathway. It is generally assumed that while JA and ethylene act synergistically, they are antagonised by SA. For example, in defence against pathogens, SA and JA have been found to mutually inhibit the expression of many genes; only some genes can be induced both by exogenous SA and JA (Glazebrook, 2005).

As for their biological relevancy, SA seems to mediate the defence against biotrophic pathogens while JA is involved in the response to necrotrophic pathogens, herbivores, and wounding. However, regarding the defence against herbivores, a clear distinction seems to exist between those against chewing herbivores and those against insects with piercing-sucking mode of feeding. That is, the latter shows a clear participation of SA-dependent defences (Kessler & Baldwin, 2002; to be discussed in more detail in Chapter 2). Regarding defences against pathogens, it is easily conceivable that an activation of HR and programmed cell death, as are thought to be

SA-dependent defences, would not be the ideal strategy to hinder infection with necrotrophic pathogens. They would in fact profit from that kind of defence. Thus, a different defensive pathway against those pathogens can be assumed (Glazebrook, 2005). In short, the effectiveness of the respective pathway strongly depends on the attacking organism (Ton *et al.*, 2002). Though most reports are confined to the illustration of antagonistic interaction of the JA and SA pathway, some contradictory studies have been published. Concerning the question whether SAR and JA-related induced systemic resistance (ISR) are mutually exclusive, it has been found recently that ISR does not affect the expression of SAR. On the contrary, SAR and ISR had an additive effect on the protection of *Arabidopsis thaliana* against *Pseudomonas syringae* pv. tomato. However, no significant cross-talk was detected (van Wees *et al.*, 2000). Furthermore, it has been demonstrated that wounding with subsequent accumulation of JA and other oxylipins can increase resistance to following infection with rust fungi, which are biotrophic pathogens (Walters *et al.*, 2006).
Undoubtedly, both the SA and the JA/ethylene pathways contribute to activating induced defence responses. Their actual role in diverse biotic interactions and the crosstalk that may occur between them to fine-tune defence responses are still under investigation. The somewhat contradictory results gathered so far are discussed in more detail in the Chapters 2 and 3.

1.6 Plant-derived volatile organic compounds

Volatile organic compounds emanate predominantly from three biosynthetic pathways: the terpenoid or isoprenoid pathway (Figure 1-4) (Dewick, 2002), the phenylpropanoid/benzenoid pathway, and fatty acid metabolism. To some extent, amino acid derivatives are also represented in volatile blends. The remarkable diversity of plant-derived volatiles, however, is also due to various modifications, such as hydroxylations, acetylations, and methylations, increasing volatility (Dudareva *et al.*, 2006). Some compounds are quite common amongst many plant species, for example, green leaf volatiles, some terpenoids, and indole; others are rather specific (Gatehouse, 2002).
The functions of plant volatiles are quite diverse. Amongst the most prominent are the potential to attract pollinators to scented flowers (Pichersky & Gershenzon, 2002), and the volatile compounds of fruits, giving them their characteristic aroma and smell. Besides visual clues, they contribute to the attraction of animals as seed dispersers. In addition, particularly in valuable reproductive parts, such as flowers and fruits, many volatiles may exert protective effects by their direct antimicrobial properties and thus help to prevent the loss of those costly plant parts (Dudareva *et al.*, 2004). In other biotic interactions, VOCs are also of considerable importance. In plant-herbivore interactions they can act directly on the attacker by having deterrent or toxic properties. Moreover, they serve as semiochemicals, providing natural enemies of the herbivore with information on the location of potential prey. In the defence against pathogens, VOCs might contribute by their antimicrobial properties, thus representing means of direct defence in this instance. Finally, volatiles have been convincingly shown to be perceived in some way by plants neighbouring the respective emitter. It is supposed that this might serve as mode of communication; for example, in the case of herbivore-induced volatiles, VOCs could serve as early warning signs of a nearby infestation. Indeed, volatiles have already been shown to induce resistance in plants. This may take place via direct elicitation or by priming, which leads to accelerated onset of defence responses in the case of subsequent

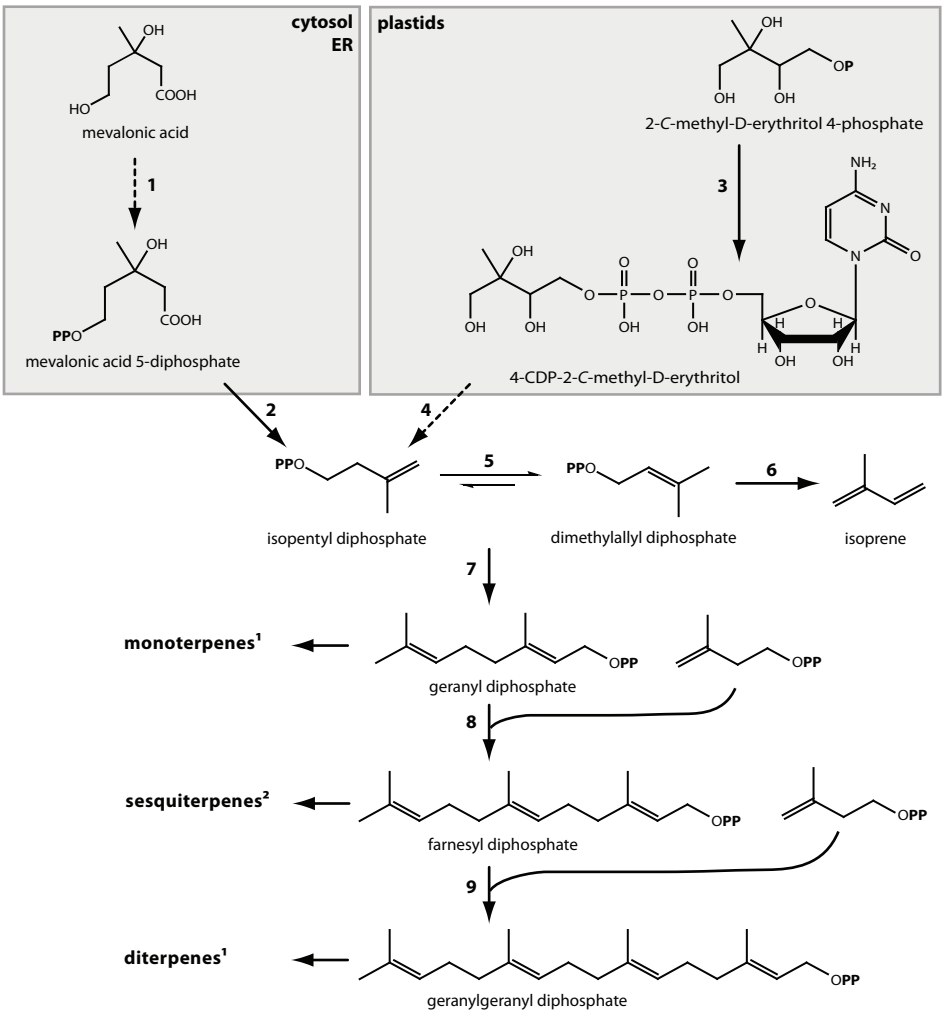


Figure 1-4 Biosynthesis of terpenes. Two pathways are present in plants: the cytoplasmic mevalonic acid (MVA) pathway and the plastidal 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway. Dashed lines indicate multiple biosynthetic steps. 1, two steps of phosphorylation via mevalonate kinase and phosphomevalonate kinase; 2, mevalonate 5-diphosphate decarboxylase; 3, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthase; 4, several, in part indeterminated biosynthetic steps; 5, isopentyl diphosphate isomerase; 6, isoprene synthase; 7, geranyl diphosphate syntase; 8, farnesyl diphosphate synthase; 9, geranylgeranyl diphosphate synthase. Terpenes are finally synthesised via diverse terpene synthases. [1], monoterpenes and diterpenes are mainly synthesised in plastids via the MEP pathway; [2], sesquiterpenes are predominantly synthesised in the cytosol and ER via the MVA pathway. Bold **P** indicate phosphate groups.

infestation (Farmer, 2001; Engelberth *et al.*, 2004; Kessler *et al.*, 2006). This mechanism acts both on a species level and also between different species. It is also possible that VOCs contribute to systemic signalling within one plant, mediating communication between distant plant parts (Bate & Rothstein, 1998; Kishimoto *et al.*, 2005; Heil & Bueno, 2007). Moreover, roots also emit volatiles. In short, the compounds released may serve the same functions belowground as aboveground, but may play an allelopathic role as well.

Finally, not only does the abiotic environment influence VOC patterns as induced in biotic interactions (Takabayashi *et al.*, 1994), but VOCs also are employed to cope with abiotic stresses. Isoprene emission seems to protect plants from heat- and ozone-induced damage (Pichersky & Gershenzon, 2002; Dudareva *et al.*, 2006).

The chemical composition as well as the intensity of volatiles emitted might give a clue about the plant's physiological status and the stresses it has been subjected to.

1.7 *Medicago truncatula* as model plant

Legumes are not only the third largest family of higher plants, they also are the second largest in terms of agricultural importance, topped only by Poaceae (Young *et al.*, 2003). Fabaceae are important forage and pasture crops, and provide sources for vegetable protein in human nutrition. In contrast to other families of higher plants, legumes can be used for extensive studies of symbiosis, as they associate with both AM fungi and rhizobia. Moreover, they may serve as models for the elucidation of some metabolic pathways, like the biosynthesis of isoflavonoids and triterpene saponins, which are not present in model plants such as *Arabidopsis thaliana* (Bell *et al.*, 2001). Also in defensive traits, some differences to this prevailing model plant are emerging (Frugoli & Harris, 2001).

Medicago truncatula Gaertner is an omni-Mediterranean annual herb, also naturalized and cultivated in other regions of Mediterranean climate (Bataillon & Ronfort, 2006). This rather drought tolerant plant is mainly used for dryland grazing and crop rotation. It provides an effective disease break and improves soil fertility. *M. truncatula* is closely related to the world's major forage legume, alfalfa (*Medicago sativa* L.), and to other important European legume crops such as pea (*Pisum sativum* L.), bean (*Vicia faba* L.), chickpea (*Cicer arietinum* L.), and clovers (*Trifolium* spp). But in contrast to most of those, being polyploid and allogamous, it has a small diploid genome (2 x 8 chromosomes, approximately 500 Mbp), a comparatively fast generation time, and self-pollination is possible, all of which makes *M. truncatula* a feasible lab plant (MEDICAGO EU Consortium, 2002). Furthermore, the availability of genetic databases (Cannon *et al.*, 2005) and the ever increasing knowledge about genetic traits make this species a suitable model system for legume genetics (Bell *et al.*, 2001; Frugoli & Harris, 2001). Moreover, *M. truncatula* has some advantages for the study of biotic interactions. Like many legumes, it disposes of an armoury of chemical defences that to date have been poorly characterised. Furthermore, there seems to be considerable ecotypic variation within the species (Bataillon & Ronfort, 2006), and due to its agronomical importance, numerous cultivars are also available that vary with regard to their resistance to various pests and pathogens (Nair & Howie, 2006). In addition to the practical advantages listed above, this provides valuable tools for investigating the influence of slight genetic variation on defensive traits within one model species.

1.8 Aims of the study

We are still far away from detailed knowledge of the mechanisms that lead either to close symbiotic associations or to the activation of defence responses. The elucidation of single interactions is already complicated, but the problem even gets more intricate if considering that single interactions in nature are the exception rather than the rule. Thus, in order to peer into the black box of multiple interactions, it seems to be a reasonable approach to start with the use of one model plant to study those interactions. One prerequisite in this case is that the plant chosen reacts to all the challenges that are to be investigated. Members of the Fabaceae are favourable for such projects in the sense that they readily enter into symbiosis with rhizobia and mycorrhizal fungi. Like any plant, they fall prey to diverse pathogens and herbivores. From this point of view it is favourable to choose a member of this family.

The overall aim of this study was to investigate overlaps and divergences of induced responses of plants in biotic interactions, using *Medicago truncatula* Gaertner as a model plant. The strategy of the present study was to start with the analysis of single defensive traits of this plant. Chapter 2 deals with a part of induced defences in response to herbivore attack. Two different phytophages, a chewing herbivore and a cell content feeder, were chosen to compare the impact of different modes of feeding on inducible defence responses. Furthermore, in view of the wider aim to compare multiple interactions, certain defensive traits that are described as typical for pathogen-induced defences were included in this part of the study in order to assess a first line of potential overlaps between diverse defensive pathways.

The third chapter addresses the impact of different oligosaccharidic elicitors on plant defence components. Paralleling the approach of the previous section, responses thought to be unrelated to pathogen defence or symbiotic interactions were also considered. Different oligosaccharides have long been known to be perceived by plants and to induce specific responses, including compounds derived from detrimental as well as from beneficial microorganisms. Whether structurally related but functionally completely different signalling compounds can trigger the same defence responses in an experimental setup will be addressed in this chapter. The experiments are mere bioassays and offer a mechanistic approach to the question.

The first step further into the investigation of multiple interactions is taken in Chapter 4, where the influence of mycorrhization on defence against herbivores in *M. truncatula* is presented.

Finally, as mentioned earlier, it is crucial to characterise defence responses of a model organism to single stimuli before dealing with mixed effects. As is demonstrated, *M. truncatula* emits a multitude of different VOCs, such that it is of interest to characterise the biochemical potential of the plant more thoroughly, which is done in Chapter 6. In order to do so, different chemical elicitors known to induce VOC emission in other plant species were applied. The spectra recorded in reaction to those stimuli should further contribute to completing the profile of volatiles emitted by *M. truncatula*. Moreover, they offer the possibility to assess the potential specificity of VOC blends emitted in response to different stimuli in more detail.

Broadly speaking, the main question is, to what extent can overlaps in defence responses be found in plants? The ideas that general elicitors of plant defence may parallel PAMPs in animals, and that analogous mechanisms might be activated as in innate immunity in animals are not really new. An extension to that notion would be the assumptions that the innate immunity of plants extends to defence against herbivores and that reactions to abiotic stresses also fall into that category of responses.

As for components of signal transduction, it is commonly assumed that the reactions to different stimuli are mediated via distinct signal transduction pathways. For some well described components, such as SA, JA, ROS, and NO, this is to be verified for *M. truncatula* as a model.

Another question that is investigated throughout this study is to what extent the emission of VOCs can be used to diagnose a plant's physiological or pathological state.

2 Defences induced by piercing-sucking and chewing herbivores

2.1 Introduction

This section contains a comparison of defence reactions to herbivores with different ways of feeding. The evaluation includes traits of direct and indirect defence as well as components of signal transduction. Emitted volatiles, representing a mechanism of indirect defence, were measured and identified by gas chromatography/mass spectrometry (GC-MS). As elements of direct defence, the accumulation of phenolic compounds and of reactive oxygen species (ROS) was assessed using microscopic techniques. Jasmonic acid (JA) and salicylic acid (SA) concentrations were determined as putative components of signal transduction. Furthermore, the behavioural response of predatory mites to the VOCs emitted by *Medicago truncatula* was examined by means of Y-tube olfactometer experiments.

Besides several lines of defence a plant makes use of that act directly against the attacker (cf. 1 General introduction), the emission of certain VOCs, for example in the case of herbivory, provides additional indirect defence involving a third trophic level by attracting natural enemies of the attacker of the plant (Takabayashi & Dicke, 1996; Gatehouse, 2002; Kessler & Baldwin, 2002).

Volatile emission seems to be at least in part mediated by changing concentrations of the phytohormones JA and SA (reviewed by Van Poecke & Dicke, 2004). Previous studies on tomato (*Lycopersicon esculentum* Mill.) suggest that a functional JA biosynthetic pathway is required for the induction of volatile release upon spider mite infestation (Ament *et al.*, 2004). However, it has also been reported that in lima bean (*Phaseolus lunatus* L.) both salicylate- and jasmonate-related signal transduction pathways are essential for mounting an indirect defence against *Tetranychus urticae* Koch, while volatile release in response to caterpillar feeding seems to be mainly controlled by JA concentrations (Ozawa *et al.*, 2000).

Regarding the possible involvement of SA in defence against piercing-sucking insects (Ozawa *et al.*, 2000; Walling, 2000; Arimura *et al.*, 2002) and the relevance of SA in the resistance of plants to microbial pathogens (Delaney *et al.*, 1994; Dempsey *et al.*, 1999), it is tempting to speculate that there might be some other parallels in defence mechanisms against pathogens and herbivores. For example, the oxidative burst (the rapid production of ROS) is a well-described phenomenon occurring in reaction to pathogen attack at the onset of the hypersensitive response (HR) (Lamb & Dixon, 1997). Hydrogen peroxide, the most stable of the radicals produced, has been proposed to fulfil several roles in defence against pathogens. It might act via direct antimicrobial activity, as component of intra- and intercellular signal transduction pathways, or it might contribute to structural defence by oxidative cross-linking of the cell wall (reviewed by Lamb & Dixon, 1997). However, little attention has been paid to the question of whether oxidative responses also play a role in defence against herbivores. There is one report by Bi & Felton (1995) demonstrating significant increases in lipid peroxidation and $OH^{\cdot}$ formation, elevated activity of oxidative enzymes and depletion of cellular antioxidants in soybean (*Glycine max* (L.) Merr.) in reaction to caterpillar feeding. Additionally, the general relevance of ROS as an important defensive factor involved in various forms of stress responses has been suggested recently (Mithöfer *et al.*, 2004).

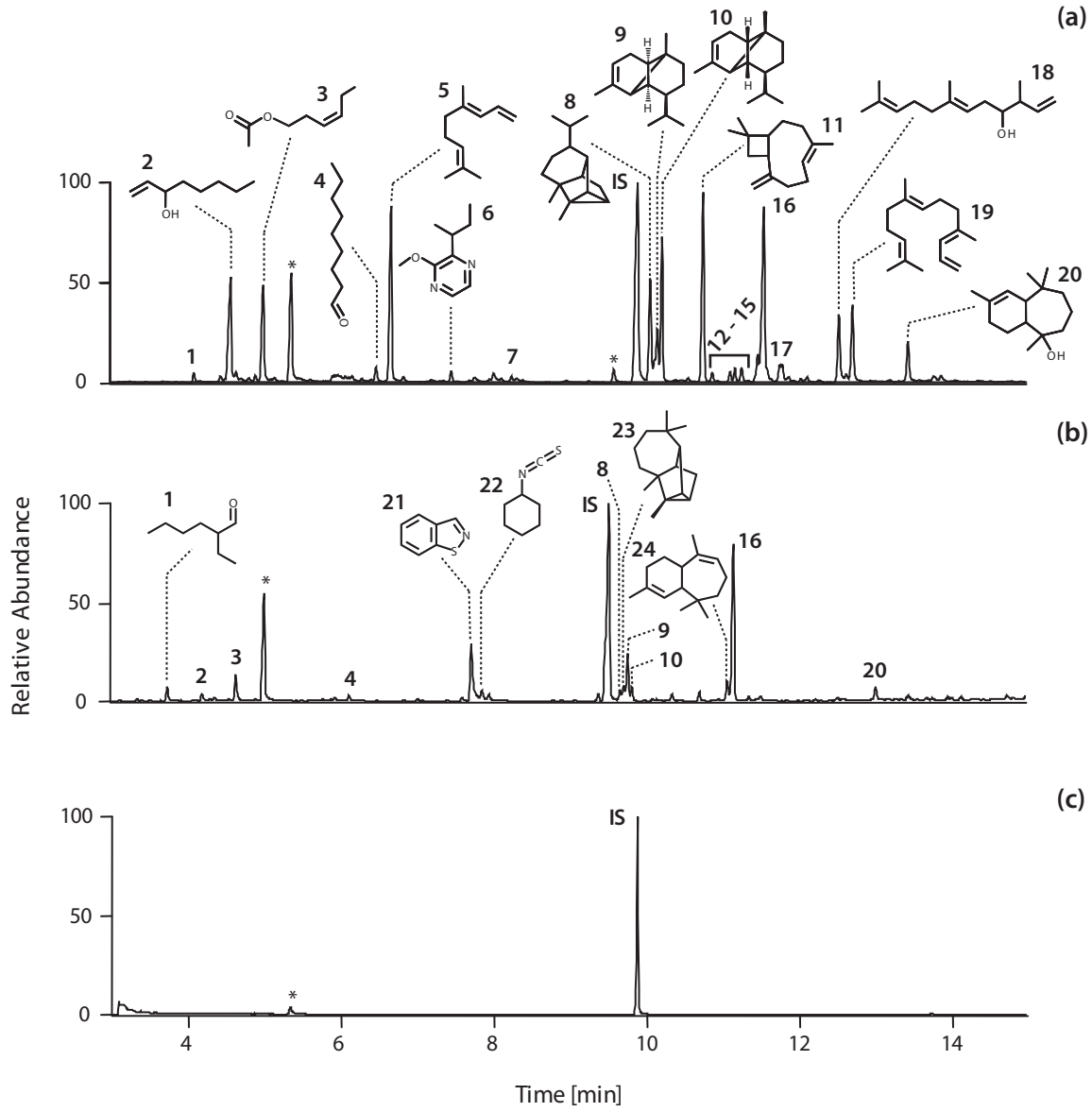


Figure 2-1 Gas chromatograms of volatiles emitted by *Medicago truncatula*. (a) Volatiles induced by *Spodoptera littoralis* feeding. (b) Volatiles induced by *Tetranychus urticae* infestation. (c) Control. For identification of the compounds, see Table 2-1. IS, internal standard (100 µg ml^{-1} n-bromodecane). Asterisks mark contaminations of abiotic origin (plasticiser).

Concomitantly with the HR, the accumulation of phenolic compounds has been described as a mechanism plants use to resist pathogens (Dixon & Paiva, 1995; Kuc, 1995; Dixon *et al.*, 2002) and some animal pests (Ollerstam *et al.*, 2002). Reports on the latter are mainly restricted to more sedentary organisms such as nematodes, mites, galling insects, bark beetles, adelgids, and siricids (Fernandes, 1990; Ollerstam *et al.*, 2002). Although elevated concentrations of phenolic compounds were detected upon feeding by mobile herbivores (Bi *et al.*, 1997a), so far no evidence has been provided for the localised accumulation of these feeding deterrents.

It is known that different herbivores induce different volatile profiles (for an overview, see Van Poecke & Dicke, 2004), but there are few reports on comparative analyses of volatiles induced by herbivores with distinct feeding behaviours (Turlings *et al.*, 1998; Ozawa *et al.*, 2000). This chapter addresses the parallels and differences in the modes of defence a plant uses against different types of herbivores. For the first time, the model legume barrel medic (*Medicago truncatula* Gaertn.) was used to investigate components of signal transduction as well as direct and indirect defences

against either chewing or piercing-sucking herbivores, in this case cotton leafworm (*Spodoptera littoralis* Boisduval) and two-spotted spider mite (*Tetranychus urticae* Koch), respectively.
As limited information is available on the occurrence of localised direct defences, such as the oxidative burst and the deposition of phenylpropanoid metabolites in the context of herbivory, one aim of this study was to assess the accumulation of ROS and phenolic compounds at the wounding sites caused by *S. littoralis* and *T. urticae*. Moreover, in light of the growing importance of *M. truncatula* as a model organism (Cook *et al.*, 1997; Oldroyd & Geurts, 2001) and the increasing insights being gained into the value of indirect defences, another aim was to characterise the volatiles emitted by the vegetative plant parts in reaction to herbivory.
Finally, regarding the specific differences encountered in volatile blends emitted after caterpillar or spider mite infestation, the attractiveness of the respective combinations to predatory mites was evaluated.

2.2 Results

2.2.1 VOC emission

The collection of volatiles upon herbivory in *M. truncatula* revealed a considerable variety of compounds emitted. These included different classes of hydrocarbons such as alkanes, alkenes, aldehydes, alcohols, esters, and aromatics, although the compounds most abundantly present were terpenoids. Figure 2-1 shows examples of gas chromatograms depicting volatile blends after caterpillar and spider mite feeding. These chromatograms illustrate the general picture for most substances emitted, although without the separation of all sesquiterpenoids. Compounds (Table 2-1) were identified according to their fragmentation pattern (MS) and in addition, as far as standard substances were available, by calculation and comparison of retention indices on two different columns with different polarity (see 7.2.2, Identification of VOCs and determination of retention indices, and Appendices I and II for details).
Comparing the blends emitted after feeding by *S. littoralis* and *T. urticae*, some differences become apparent (Figure 2-1, Table 2-1). Overall, fewer substances were released upon spider mite feeding. The total lack of homoterpenes, as well as alkanes and alkenes, in the spectrum is remarkable. Similarly, no emission of methyl salicylate (MeSA) was induced by spider mite infestation. Moreover, the relative amounts of some of the sesquiterpenoids emitted differed, particularly for cyclosativene and α-copaene after spider mite infestation. Although the overall diversity of sesquiterpenoids induced by *T. urticae* was lower than for *S. littoralis*, γ-himachalene and γ-humulene were found exclusively after attack by this herbivore and thus represented the only substances that could be detected after spider mite feeding, but not after caterpillar feeding.

2.2.2 Phytohormone levels

Clear differences were also observed in salicylate and jasmonate concentrations after caterpillar and spider mite infestations. In the comparison drawn here, local and systemic responses, as well as early and late responses, were examined separately. Because attack by different herbivores damages plants at different rates, the comparison presented here is based on measurements at

Table 2-1 List of compounds identified in the volatile blends emitted by *Medicago truncatula* upon *Spodoptera littoralis* or *Tetranychus urticae* infestation.

	Compound[1]	No in Fig. 2-1	*S. littoralis*	*T. urticae*
Alkanes	n-Pentadecane		+	
Alkenes	3-Octanone		+	
	2,6-Dimethyl-1,3,5,7-octatetraene (*E*)[2]		+	
Aldehydes	Benzaldehyde		+	
	n-Decanal		+	+
	2-Ethyl hexanal[3]	1	+	+
	n-Nonanal	4	+	+
Alcohols	2,6-Dimethyl-3,5,7-octatrien-2-ol (*E*)[2]	7	+	
	6-Methyl-1-heptanol		+	+
	1-Octen-3-ol	2	+++	+
Esters	*cis*-3-Hexenylacetate	3	++	+
Aromatics	Cresol			+
	3,5-Dimethoxytoluene		+	
	3,5-Dimethylanisole			+
	Methyl salicylate		+	
	Trimethylbenzene		+	+
Monoterpenes	3-Carene		+	
	Limonene		+	
	α-Pinene		+	+
Sesquiterpenoids	*allo*-Aromadendrene	15	+	
	α-Bisabolol		+	
	Cadalene		+	
	β-Caryophyllene	11	+++	+
	α-Copaene	10	+++	+
	β-Copaene	12	+	
	Cyclosativene	8	+++	+
	β-Farnesene		+	
	α-Himachalene	13	+	+
	β-Himachalene			+
	γ-Himachalene	24	+	+
	β-Himachalol	20	++	+
	α-Humulene	14	+	
	γ-Humulene			+
	β-Ionone		+	
	α-Muurolene	17	+	
	Longicyclene	23	+	+
	E-Nerolidol	18	++	
	α-Ylangene	9	++	++
	unidentified sesquiterpene (RI 1481)	16	+++	+++
Homoterpenes	4,8-Dimethyl-1,3,7-nonatriene (DMNT)	5	+++	
	3*E*,7*E*-4,8,12-Trimethyltrideca-1,3,7,11-tetraene (TMTT)	19	++	
N- or S- containing compounds	1,2-Benzisothiazole	21	+	++
	2-*sec*-Butyl-3-methoxypyrazine	6	+	
	Cyclohexylisothiocyanate	22	+	+

[1] For identification of the compounds (MS and linear retention indices) see Appendices I and II.
[2] Artefacts generated from (*E*)-ocimene during adsorption to the charcoal trap (Kaiser, 1993).
[3] Contamination of abiotic origin.
+, relative abundance below 25%; ++, relative abundance between 25 and 50%; +++, relative abundance above 50%.

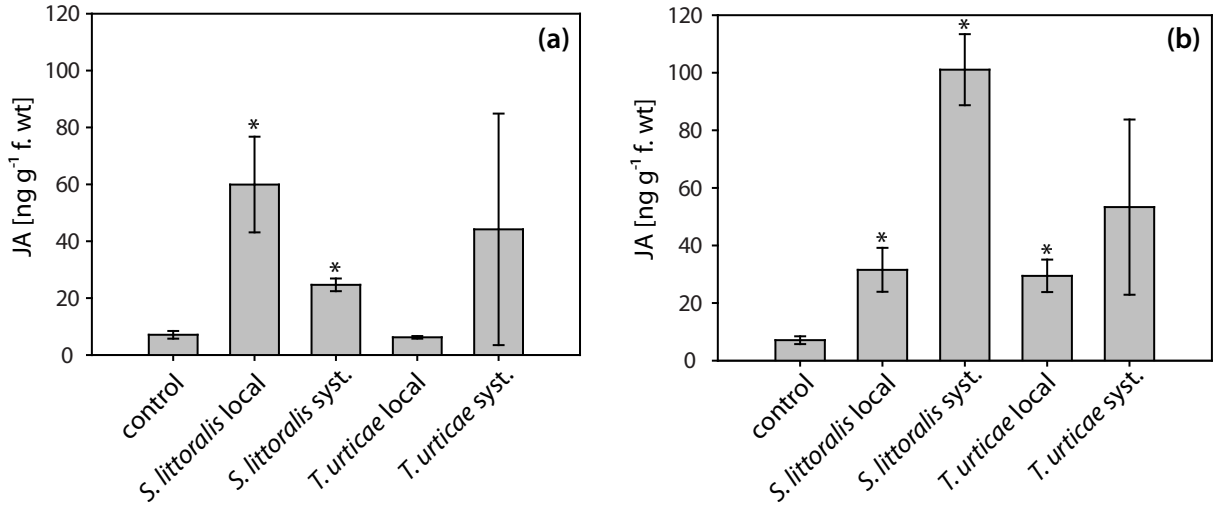


Figure 2-2 Jasmonic acid concentrations in *Medicago truncatula*. (a) JA concentrations at early stages of infestation (for *Spodoptera littoralis*, 6 h after the onset of feeding; for *Tetranychus urticae*, after the appearance of yellowish spots). (b) JA concentrations at late stages of infestation (for *S. littoralis*, 48 h after the onset of feeding; for *T. urticae*, after yellowing of the initially infested leaves of *M. truncatula*). Data are the mean ± standard deviation for three independent experiments. Asterisks indicate a statistically significant increase of JA values for the particular treatment group ($p < 0.05$; Student's *t*-test) compared with the control. syst., systemic.

a given time for caterpillar feeding and at a certain stage of symptom development for spider mite infestation. Thus, the data sets were divided into early and late stages of infestation. In the case of caterpillar feeding, the early and late stages of infestation were taken to be 6 and 48 h after the onset of feeding, respectively (larvae were allowed to feed on the plants for 4 h). After spider mite infestation, samples representing the early stages of damage were collected following the appearance of yellowish spots, while samples representing the late stages of damage were collected when initially infested leaves yellowed. This classification of samples ensured that the degree of damage was very similar for caterpillar feeding and spider mite infestation (in terms of leaves affected). However, secondary infections by opportunistic pathogens could not be fully excluded, particularly in light of the long incubation time. Thus, leaves were screened for infections diagnosable by microscopic means, such as fungal infections, which could then be excluded. A virus infection of the leaves via a herbivore vector, as described for whitefly *Bemisia argentifolii* (Bellows & Perring) (Mayer *et al.*, 2002), is very unlikely as Ortlob (1968) showed that *T. urticae* is unable to transmit viruses. Samples for determining local SA and JA concentrations were taken from damaged leaves; systemic concentrations were measured using the uppermost undamaged leaves of the infested plants. Control samples were taken from undamaged, healthy plants.

At early stages of infestation, feeding by *S. littoralis* caused a marked increase of local JA concentrations, up to 8.4-fold of the control values, while JA concentrations after *T. urticae* infestation did not exceed baseline values. Conversely, systemic concentrations of JA were only slightly increased early after caterpillar feeding (3.5 times), whereas the response to *T. urticae* attack was relatively strong, with an average rise up to 6.2-fold of the control values, although the result in the latter case was not significant (Figure 2-2 a). At later stages, the situation changed; JA concentrations increased (4.0 – 4.5-fold) after both types of herbivory in local tissue, indicating a transient increase after caterpillar feeding and a late increase after spider mite infestation.

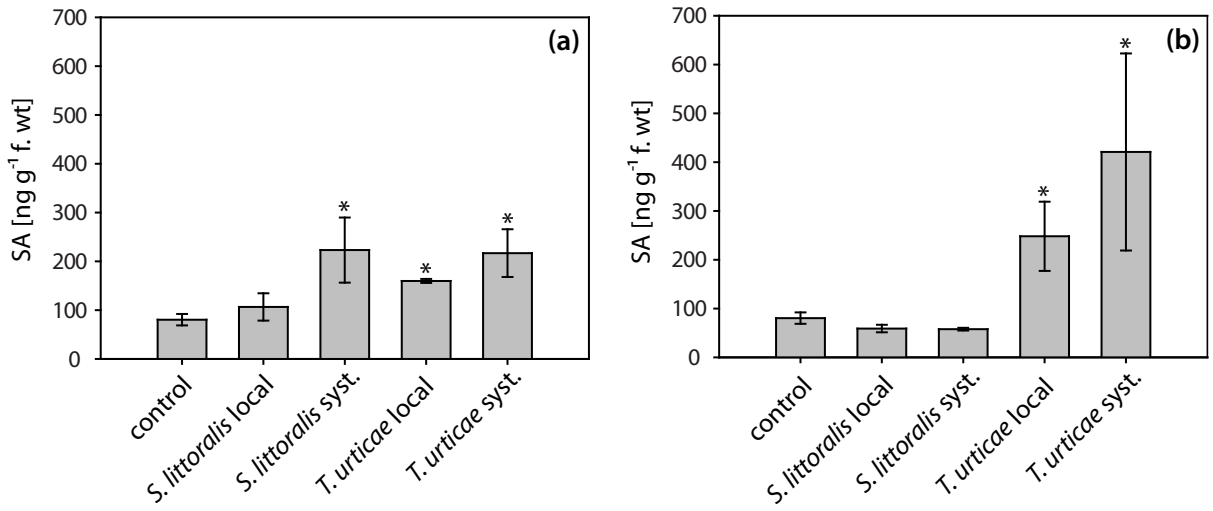


Figure 2-3 Salicylic acid concentrations in *Medicago truncatula*. (a) SA concentrations at early stages of infestation (for *Spodoptera littoralis*, 6 h after the onset of feeding; for *Tetranychus urticae*, after the appearance of yellowish spots). (b) SA concentrations at late stages of infestation (for *S. littoralis*, 48 h after the onset of feeding; for *T. urticae*, after yellowing of the initially infested leaves of *M. truncatula*). Data are the mean ± standard deviation for three independent experiments. Asterisks indicate a statistically significant increase of SA values for the particular treatment group ($p < 0.05$; Student's *t*-test) compared with the control. syst., systemic.

Regarding systemic tissue, concentrations rose strongly in plants damaged by caterpillar feeding (14.2-fold), but no changes could be observed in spider-mite-infested plants with respect to the concentrations measured at early stages (Figure 2-2 b).

For SA concentrations, the alterations with time were notably different. In tissue local to the wounding site there was no accumulation of SA after *S. littoralis* feeding, neither at early nor at late stages. Conversely, concentrations were elevated in systemic tissue after *S. littoralis* and *T. urticae* infestation, as well as locally after *T. urticae* attack at early stages (2.0 – 2.8-fold; Figure 2-3 a). After 48 h, SA concentrations were very similar in caterpillar-damaged and control plants, while both local and systemic tissues of spider-mite-infested plants at late stages showed a large accumulation of SA (3.1- and 5.2-fold increases, respectively; Figure 2-3 b).

Thus, it appears that feeding by *S. littoralis* produces a transient increase of JA locally in wounded tissue, whereas systemic concentrations are already elevated 6 h after the onset of feeding and continue to rise up to 48 h. In wounded tissue, no increase of SA concentrations could be detected, whereas a moderate increase took place in systemic tissue 6 h after wounding.

After spider mite infestation, the local response with regard to JA concentrations appeared late and was only moderate. In systemic tissue, there was a clear increase in JA concentration; the onset of this rise is likely to occur at the stage at which the leaves begin to yellow, thus the high standard deviation might indicate that samples were taken during the main period of JA accumulation. Regarding SA determination, a persistent increase in local as well as systemic tissues was found, although the response was not as strong in local tissue as in systemic tissue.

2.2.3 Direct defences

2.2.3.1 Deposition of phenolic compounds

The detection of phenolic compounds after caterpillar feeding (Figure 2-4, a – c) revealed a two-phasic, time-dependent deposition of these autofluorescent compounds around the bite zone. Immediately after wounding, a bright yellow fluorescent edge at the bite zone could be seen.

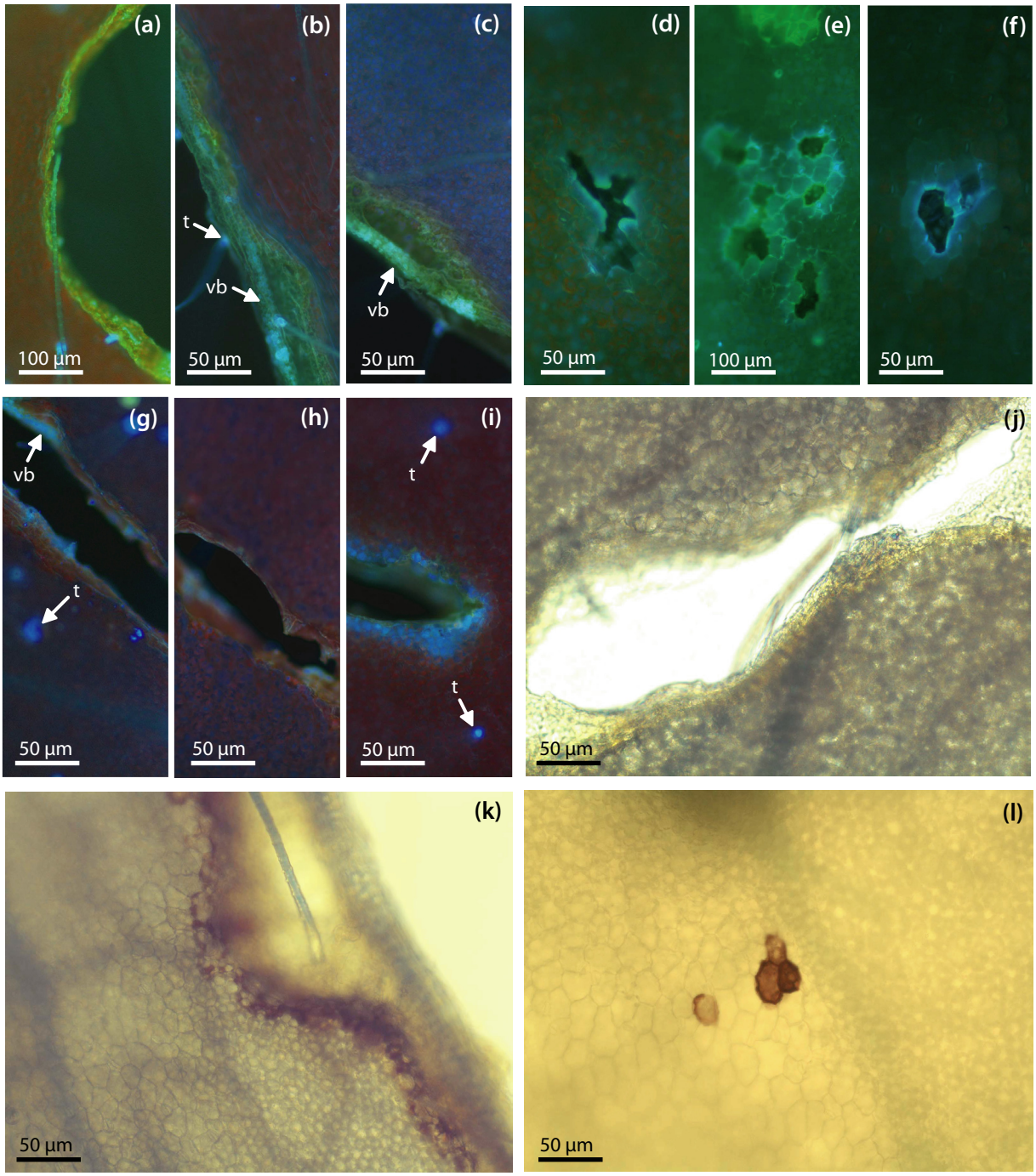


Figure 2-4 Detection of phenolic compounds (a - i) and reactive oxygen species (ROS) (j - l) at wounding sites in *Medicago truncatula*. (a - c) Autofluorescence at the bite zone after feeding by *Spodoptera littoralis*: (a) immediately after wounding; (b) 6 h after wounding; (c) 48 h after wounding. (d - f) Autofluorescent phenolics around cells damaged by *Tetranychus urticae*: (d) at the stage of limited lesions; (e) at the onset of yellowing of the leaf; (f) after almost the entire leaf has yellowed. (g - i) Deposition of phenolic compounds after mechanical wounding (control): (g) immediately after wounding; (h) 4 h after wounding; (i) 48 h after wounding. (j - l) Detection of ROS using an iodine-starch stain: (j) 48 h after mechanical damage; (k) 48 h after feeding by *S. littoralis*; (l) after feeding by *T. urticae* (at the onset of yellowing of the leaf). vb, vascular bundle; t, basal cell of trichome (filled with autofluorescent compounds).

Six hours after wounding, the area adjacent to the wounding zone became blue fluorescent with excitation at 365 nm. This localised fluorescence subsequently spread around the wounding site. The widespread faint blue fluorescence after 48 h might also in part be attributable to the reduced autofluorescence of chlorophyll as a consequence of yellowing around the wounding

site (Figure 2-4 c). Similarly, a weak yellow fluorescence was present after spider mite infestation at the stage of limited necrotic lesions (Figure 2-4 d). At the time of expansion of the lesions, yellow to blue fluorescence spread from the wounding site, which was mainly localised to the cell wall (Figure 2-4 e). At the final stage, in almost entirely yellowed leaves, a strictly localised blue fluorescence around the wounding site remained (Figure 2-4 f). As a control, leaves were mechanically damaged with a pin (Figure 2-4, g – i). Immediately after wounding and up to 24 h afterwards, no increased autofluorescence could be seen around the wounding site. Starting at 24 h, and increasing up to 48 h, blue autofluorescence was seen locally around the wounding sites (Figure 2-4 i) that resembled the reactions seen after feeding by *S. littoralis* after 6 h.

Because the reactions described above were originally described as defence mechanisms against pathogen invasion (Dixon & Paiva, 1995; Kuc, 1995; Dixon *et al.*, 2002), a pathogen-derived elicitor (branched β-1,3-β-1,6-glucans from the cell wall of *Phytophthora sojae*, cf. 3 Oligosaccharidic elicitors) was applied to wounds on the leaf to serve as positive control. As can be seen in Figure 2-5 (a – d), the pattern of autofluorescence around the elicited wounds was very similar to that observed after herbivory, but the accumulation of phenolics was clearly stronger after elicitor application. Again, a rather strong yellow fluorescence could be observed one hour after application of the elicitor (Figure 2-5 a). Later, this fluorescence spread along the cell walls, presumably due to fortification of cell walls and impregnation with defensive compounds (Figure 2-5 b). From 48 h after elicitation onwards, two cases could be observed. In some instances, necrotic lesions spread around the wounding site that were surrounded by cells filled with autofluorescent blue compounds. In other cases, the elicited wound was surrounded by cells with strongly blue fluorescent cell walls.

Thus, it may be stated that the accumulations of phenolic compounds in reaction to herbivory and pathogen-derived elicitors are indeed rather similar in the qualitative aspect, although they clearly differ in the quantitative dimension.

2.2.3.2 Accumulation of reactive oxygen species

The principle of the iodine-starch stain used to detect ROS is the oxidation of iodide in the presence of ROS to iodine, which forms a coloured complex with starch. As can be seen in Figure 2-4 (j – l), mechanical wounding did not cause the accumulation of ROS, whereas both damage caused by *S. littoralis* and that caused by *T. urticae* induced the production of ROS around the wounding site. Interestingly, staining appeared only in the late stages of damage from herbivore feeding, with the first positive results not appearing before 24 h after the start of caterpillar feeding (weakly), or at the beginning of the yellowing of the leaves during spider mite infestation. In the case of caterpillar feeding, clear staining did not appear until 48 h.

Again, elicitation with β-glucans (cf. 2.2.3.1 Deposition of phenolic compounds) served as a positive control. For this comparison, however, an alternative staining method using 3,3'-diaminobenzidine (DAB) was applied. Experiments with wounding and caterpillar feeding were also repeated using this method in order to ensure comparable results. As can be seen in Figure 2-5 (e – g), the patterns of ROS accumulation observed after wounding and herbivore feeding were the same as those seen with the iodine-starch stain. 48 h after β-glucan elicitation, a ring of cells producing ROS could be detected (Figure 2-5 g). The area of cells affected coincided with expanding necrotic lesions around the wounds (cf. Figure 2-5 c). For experiments involving β-glucan elicitation, staining with DAB turned out to be advantageous, as due to the rather widespread but weak staining, the

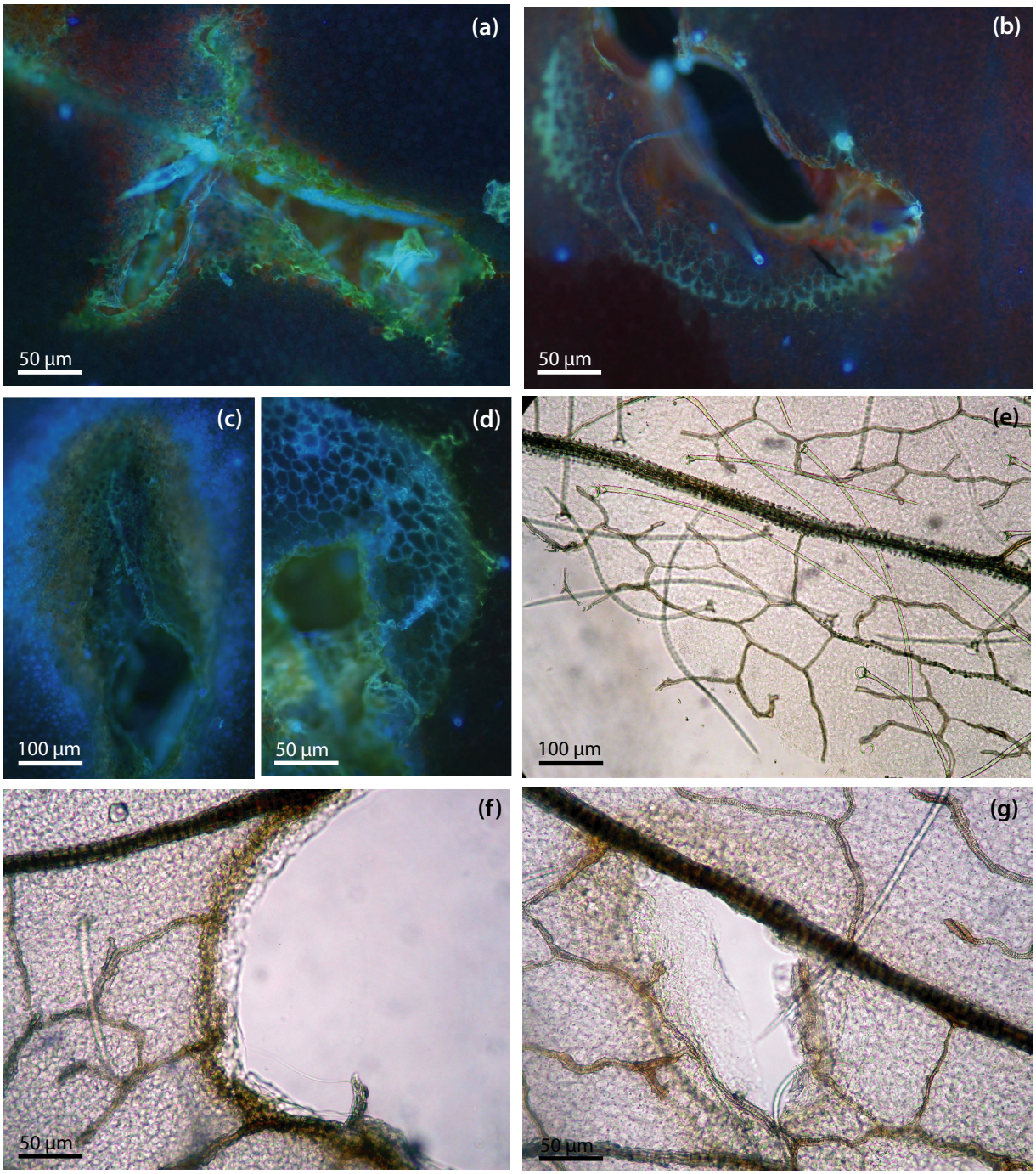


Figure 2-5 Detection of phenolic compounds (a - d) and reactive oxygen species (ROS) (e - g) at wounding sites in *Medicago truncatula*. (a - d) Autofluorescence at wounding sites treated with a pathogen-derived β-glucan elicitor: (a) 1 h after the application of the elicitor; (b) 4 h after elicitation; (c) 72 h after elicitation; (d) 48 h after elicitation. (e - g) Detection of ROS using DAB-staining: (e) 30 min after mechanical damage; (f) 48 h after caterpilar feeding; (g) 48 h after elicitation with β-glucan elicitor.

bleaching applied after the iodine-starch stain was not strong enough to yield a sufficient contrast for photography. However, results also were the same using this method. In summary it may be said that regarding the production of ROS, a quite similar pattern was observed after herbivory and elicitation with a pathogen-derived elicitor.

2.2.4 Attraction of predatory mites to different VOC blends

In the Y-tube olfactometer experiments, four different combinations were tested: uninfested plants were offered combined with empty vials, spider-mite-infested, and caterpillar-damaged plants. Finally, the attractiveness of caterpillar- and spider-mite-infested plants to predatory mites (*Phytoseiulus persimilis* Athias-Henriot) was compared.

As shown in Figure 2-6, undamaged plants were more attractive to predatory mites than an empty vial, with 39 individuals out of 60 choosing the former odour source ($p < 0.05$, binomial test). Both, the volatiles emitted by spider-mite-infested plants as well as those from caterpillar-damaged plants were able to attract predatory mites. With a total of 80 individuals tested, 61 were attracted by the volatiles emitted by spider-mite-infested plants ($p < 0.001$, binomial test). In the case of caterpillar-damaged plants, 53 out of 80 predatory mites preferred plants damaged by *S. littoralis* over control plants ($p < 0.01$, binomial test). However, comparing the behavioural responses of predatory mites in those two experiments, no significant differences were found depending on whether the plants were infested with host or non-host organisms ($p > 0.05$, contingency table analysis, Fisher's exact test). At last, when offered the choice between caterpillar- and spider-mite-infested plants, predatory mites were not able to locate the plant bearing its host ($p > 0.05$, binomial test).

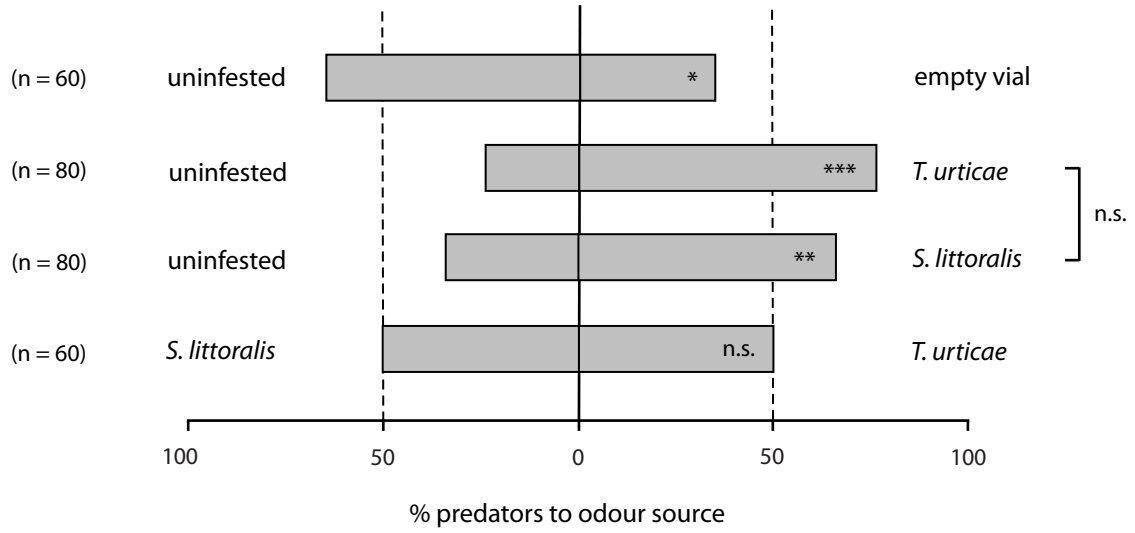


Figure 2-6 Choices of the predatory mite *Phytoseiulus persimilis* between the volatiles emitted by *Tetranychus urticae* infested and *Spodoptera littoralis* damaged *Medicago truncatula* plants. Statistical analysis was done using a binomial test for single choice tests and Fisher's exact test for the comparison between different experiments. n.s., $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

2.3 Discussion

Amongst the group of piercing-sucking arthropods there is a clear distinction, in terms of the responses of plants, between phloem-feeding herbivores and herbivores that feed on cellular contents; spider mites belong to the latter group (Walling, 2000). Phloem-feeding herbivores, such as aphids, leafhoppers, and whiteflies, cause only minor tissue damage and seem to induce defence signalling pathways largely resembling those activated against pathogens. However, these herbivores can induce unique volatile blends (Du *et al.*, 1998; Birkett *et al.*, 2000). Mites that

lacerate cells cause more extensive tissue damage, and thus the response of plants to these mites is more similar to their response to chewing herbivores (Walling, 2000). Nevertheless, there are some substantial differences in plant reactions to chewing and cell-content-feeding herbivores. Regarding volatile release in *M. truncatula*, the quantitative composition of the blends clearly differed depending on the type of herbivory (Figure 2-1; Table 2-1). This is in accordance with previous findings that the blend emitted specifically attracts predators or parasitoids of the attacker (Takabayashi & Dicke, 1996; De Moraes *et al.*, 1998; Du *et al.*, 1998; Birkett *et al.*, 2000; Shimoda *et al.*, 2002; Horiuchi *et al.*, 2003). There were relatively small qualitative differences, but it is remarkable that MeSA, TMTT (3*E*,7*E*-4,8,12-trimethyltrideca-1,3,7,11-tetraene) and *E*-nerolidol were not found in the chromatograms recorded after spider mite infestation, as MeSA and TMTT have been suggested to be spider-mite-inducible volatiles in lima bean (Dicke *et al.*, 1999) as well as in tomato (Kant *et al.*, 2004). Moreover, all three compounds have been described as typical constituents of volatile blends produced by tomato in reaction to spider mites, being absent only in plants with impaired JA accumulation (Ament *et al.*, 2004). This is clearly not the situation in barrel medic, as elevated concentrations of JA were detected in this study, although the measured concentrations fluctuated greatly. This might have been a result of the sampling mode, in which symptoms were used as a measure. JA concentrations seem to rise during the expansion of localised necrotic lesions. Nevertheless, it is useful to establish comparisons in this way, because the time courses of damage development are very distinct after caterpillar and spider mite infestation. Furthermore, the time that elapsed before the first symptoms of disease became visible after the onset of spider mite feeding differed considerably in various infestations, and it therefore seemed preferable to use damage symptoms as criteria for sampling (in accordance with Kant *et al.*, 2004). Finally, the sampling time might matter more than first assumed. An ongoing re-evaluation of volatiles emitted after spider mite infestation, this time with sampling dependent on the time after the onset of feeding, indicates drastic changes of the volatile pattern emitted with the time after infestation.

The fact that previously described attractants of predatory mites (De Boer *et al.*, 2004; Kappers *et al.*, 2005) were not emitted by spider-mite-infested plants was a sound reason to test the effectiveness of VOCs emitted by *M. truncatula* in tritrophic interactions. The results of the olfactometer experiments provided evidence that these compounds are able to attract predatory mites to the wounded plants. However, the behavioural response was not specific to a certain volatile blend. This is unsurprising when some ecological details as well as the experimental setup are taken into account.

As spider mites are rather polyphagous herbivores and each plant species has its own repertoire of volatiles, predatory mites would profit from being able to detect some of the more common volatile compounds or a multitude of different compounds. It is thus rather unlikely that only the homoterpenes DMNT and TMTT, *E*-nerolidol, and MeSA are powerful attractants. Still, those compounds are quite commonly encountered substances in volatile blends emitted by damaged plants. *M. truncatula* emits a considerable number of different compounds, yet there are still a lot of other candidates that may be responsible for attracting predatory mites to damaged *M. truncatula* plants.

Regarding the experimental setup, it has to be stressed that only inexperienced predatory mites (reared on lima bean infested with spider mites) were used in all experiments. As control

experiments, predatory mites were given the choice between undamaged and spider-mite-infested lima bean plants (data not shown). They were strongly attracted to the infested plants, and a comparison of the attraction to damaged *M. truncatula* plants revealed a significantly lower attraction to the latter (contingency table analysis, Fisher's exact test), which could be due to a learning effect resulting from the predatory mites' rearing history (De Boer *et al.*, 2005).
Finally, it has to be considered that this study was working with a generalist system. Both *S. littoralis* and *T. urticae* are polyphagous herbivores and thus a generalised defence reaction on the side of the plant seems plausible. Furthermore, *M. truncatula* reacts with volatile emission to a wide range of stimuli (cf. Chapter 5 Volatile profiling). Thus, the response by itself cannot be considered specific. However, from the point of view of a predatory mite feeding on a generalist herbivore, any stressed plant merits recognition to increase the probability of encountering prey. The fact that predatory mites also were able to detect undamaged plants when offered with empty glasses can be explained by the emission of green leaf volatiles from undamaged plants as well.

Clear differences were found in phytohormone concentrations depending on the type of herbivory (Figures 2-2 and 2-3). In contrast to results obtained for *Helicoverpa zea* Boddie larvae feeding on cotton (Bi *et al.*, 1997b), concentrations of SA were largely unaffected by caterpillar feeding on *M. truncatula*; JA concentrations rose markedly. For spider mite infestation, enhanced production of both JA and SA was detected. Similar effects have recently been demonstrated for lima bean (Arimura *et al.*, 2002), although no assessment of differences between local and systemic reactions was carried out. It is noteworthy that in *M. truncatula* a generally greater accumulation in systemic tissue than in local tissue at the feeding sites was observed. Furthermore, up-regulation of genes activated via the JA and SA pathways has been shown after spider mite infestation of tomato (Kant *et al.*, 2004). Thus, it can be concluded that caterpillar feeding mainly activates JA-related signalling pathways, whereas spider mites induce reactions involving JA as well as SA. These differences might be connected to the specific volatiles released, as JA treatment mimics the effect of caterpillar feeding with respect to the attraction of specific predators (Van Poecke & Dicke, 2002), whereas a combination of JA and MeSA is able to attract natural enemies of herbivorous mites (Shimoda *et al.*, 2002).
The clear involvement of SA in the defence against spider mite infestation calls into question the hypothesis that SA signalling contributes to herbivore-induced pathways only if there is very limited tissue damage, as in the case of phloem feeders (Walling, 2000). These doubts are supported by another recent report showing that the puncture-feeding *Tupiocoris notatus* Distant not only induced defences similar to those induced by SA, but also repressed the expression of JA-induced genes in *Nicotiana attenuata* Torrey ex Watson (Heidel & Baldwin, 2004).

In this context, it is intriguing to ask whether other parallels can be found in reaction known to be involved in defence against pathogens and protection against herbivory. Localised deposition of phenolic compounds has been described after pathogen attack (Bennett *et al.*, 1996; Silva *et al.*, 2002) and after the feeding of some sedentary herbivores (reviewed in Fernandes, 1990; Ollerstam *et al.*, 2002) in species such as *Lactuca* spp. (in reaction to *Bremia lactucae* Regal, downy mildew fungus), *Coffea* spp. (in reaction to *Hemileia vastatrix* Ber. & Br., orange rust), *Solanum dulcamara* L. (in reaction to *Eriophyes cladophthirus* Nal., a gall mite), and *Salix viminalis* L. (in reaction to *Dasineura marginemtorquens* Bremi, the gall midge). Also, increased production of defensive

compounds was demonstrated in reaction to caterpillar feeding (Bi *et al.*, 1997a). In the course of this study it was possible to show a localised accumulation of phenolic compounds in *M. truncatula* surrounding the wounding site resulting from both types of herbivory that was distinct from the reaction seen after mechanical wounding (Figure 2-4, a – i). This is consistent with findings that transcripts of an enzyme involved in phenylpropanoid phytoalexin biosynthesis, isoflavon-3'-hydroxylase, accumulate after *Spodoptera exigua* Hübner feeding on *M. truncatula* leaves (Liu *et al.*, 2003). The progression of deposition of phenolic compounds appears to be two-phasic, similar to that reported for pathogen infection (Bennett *et al.*, 1996) and treatment with pathogen-derived elicitors (Figure 2-5, a – d). In contrast, mechanical damage induces only the accumulation of blue fluorescent compounds, which occurs later than that induced by herbivores. Thus, the data presented here accord with a report on potato (*Solanum tuberosum* L.) demonstrating more rapid accumulation of the mRNAs of defence-related genes induced by herbivory compared with simple wounding (Korth & Dixon, 1997). It may be concluded that the second phase is a general reaction to wounding, perhaps with the purpose of preventing opportunistic microbes using wounds as penetration sites, while the first phase seems to be specific for biotic interactions. Further confirming of this assumption will certainly require identifying the respective compounds.
As for the similarities of some components of plant defences against diverse attackers, it was intriguing to learn that production of ROS, as is typical after pathogen attack, can also be found after herbivory (Figure 2-4, j – l). It has already been reported that wounding induces ROS production, at least in some species. For example, in *Zinnia elegans* L. (Olson & Varner, 1993), ROS production upon wounding has been shown using the same method as in this study. Yet another interesting report by Orozco-Cardenas & Ryan (1999) demonstrated that the production of ROS upon mechanical damage does not occur in all species. For example, the species tested belonging to the families Solanaceae, Cucurbitaceae, and Poaceae showed wound-inducible H_2O_2 accumulation, whereas only one of the five legume species tested, *Pisum sativum* L., tested positive for this trait. This finding is consistent with the results presented above, which showed that mere mechanical damage did not induce the accumulation of ROS in *M. truncatula*, but that herbivore feeding did. However, the late occurrence of ROS after arthropod feeding is an argument against their involvement in signal transduction. Instead they may play a role in propagating cell death or acting against other potential invaders at the wounding site. Nevertheless, it cannot be ruled out that the method applied was not sensitive enough to detect an early, transient increase in ROS concentrations.

To summarise the results presented in this section, it can be stated that *M. truncatula* emitted a large variety of volatile substances in reaction to herbivory, differing in their quantitative and qualitative composition depending on the attacking organism. However, the behavioural response of predatory mites to these different volatile blends was not specific, as they were attracted to all the damaged *M. truncatula* plants tested. Furthermore, the response in terms of the phytohormones JA and SA clearly differed with the type of herbivory, with a greater involvement of SA in the reaction to spider mite feeding and a different time course for the accumulation or JA. Both spider mite and caterpillar infestation induced the deposition of phenolic compounds around the wounding site in a seemingly two-phase manner, in contrast to mechanical wounding, which caused only the respective second phase. ROS production occurred in the late stages of infestation, whereas wounding did not affect this defensive trait. Defences against herbivores are distinct from

reactions to mechanical wounding, and different types of herbivores are also recognised. While some responses seem to be more general, even being produced in defence against pathogens, others are clearly specific to the reaction to a particular attacker. The components of signal transduction, which shape the recognition and differentiation of the attacking organism, remain to be determined. Furthermore, a thorough investigation of VOCs able to attract predatory mites to damaged *M. truncatula* plants would certainly be of interest.

3 The impact of microbial oligosaccharide signals on defensive traits

3.1 Introduction

As outlined in the general introduction, plants are able to recognise a multitude of chemical signals from their environment and to react appropriately to diverse challenges. Some of these signalling compounds act as general elicitors of defence reactions (Ebel & Cosio, 1994; Boller, 1995). They are perceived at low concentrations and comprise diverse structures, including carbohydrates, (glyco-) proteins, lipids, and sterols (Ebel & Cosio, 1994; Boller, 1995; Nürnberger *et al.*, 2004). The relevant compounds are in general evolutionarily conserved amongst microorganisms (not only pathogens), not present in the potential host plant, and important for the fitness of the microbe; thus, they parallel pathogen-associated molecular patterns (PAMPs) important for non-self recognition in the animal immune system (Gomez-Gomez, 2004; Nürnberger *et al.*, 2004; Zipfel & Felix, 2005). Elicitor-induced defence responses include the oxidative burst, the strengthening of cell walls, the hypersensitive reaction, and the activation of genes encoding for pathogenesis-related (PR) proteins and enzymes of phytoalexin synthesis (Ebel & Mithöfer, 1998). Interestingly, beneficial microorganisms like rhizobia that might primarily be perceived as intruders, take advantage of compounds structurally related to certain elicitors, i.e. nodulation factors (Nod-factors), to communicate their presence to the plant. In fact, while Nod-factors induce root hair deformations, cortical cell divisions, and in some cases even complete nodule-like structures in their host plants, they are able to cause reactions that occur in the context of pathogen defence in non-host plants (Staehelin *et al.*, 1994; Baier *et al.*, 1999; Bueno *et al.*, 2001) and in host plant cell cultures (Savouré *et al.*, 1997).

Although plants' reactions to pathogen attack are mostly seen as distinct from plant-insect-interactions, the induced defence reactions against both arthropods and pathogens intersect considerably. For instance, plants' defences against certain sedentary herbivores (e.g. galling insects or mites) include reactions that are typical for the response to pathogen attack, such as the hypersensitive response and the accumulation of phenolic compounds (Fernandes, 1990; Fernandes & Negreiros, 2001; Ollerstam *et al.*, 2002). These similarities might be due to the minor tissue damage those pests inflict on the plant (Walling, 2000). But even lepidopteran larvae induce defence reactions that so far have only been reported to occur after pathogen attack or the application of pathogen-derived elicitors, such as the local accumulation of defensive compounds at the wounding site or hydrogen peroxide production (cf. Chapter 2, Defences induced by piercing-sucking and chewing herbivores; Maffei *et al.*, 2006).

In this context it was intriguing to search for further potential parallels in the defences against pathogens and herbivores. Based on the observations presented in Chapter 2, the emission of VOCs as well as changes in the levels of the phytohormones jasmonic acid (JA) and salicylic acid (SA) were obvious candidates for further comparison.

The release of VOCs is a trait typically associated with defence against herbivores. However, in some studies volatile emission has also been reported after infection by different strains of the bacterial pathogen *Pseudomonas syringae* in tobacco (*Nicotiana tabacum* L.) or bean (*Phaseolus*

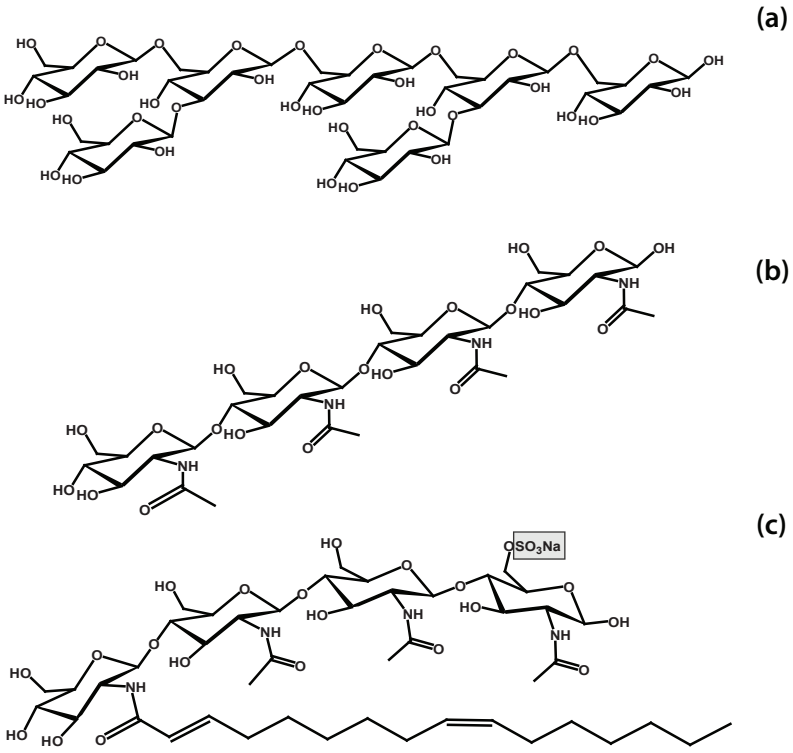


Figure 3-1 Chemical structures of the signalling compounds used in this study. (a) β-(1,3)-β-(1,6)-glucan from the oomycete *Phytophthora sojae* cell wall as an example for an elicitor structure that is active in legumes; (b) *N*,*N*',*N*'',*N*'''-tetraacetylchitotetraose (CH4); (c) LCO-IV (C16:2, S) & LCO-IV (C16:2); Nod-factors. Both molecules differ only in the presence or absence of the sulphate group (marked grey) that provides for host specificity in LCO-IV (C16:2, S).

vulgaris L.) (Croft *et al.*, 1993; Huang *et al.*, 2003). In peanut plants (*Arachis hypogaea* L.), the white mold (*Sclerotium rolfsii* Sacc.) has been reported to induce VOC release upon infection (Cardoza *et al.*, 2002). These results give rise to the question, what kinds of elicitors confer this property.

The accumulation of phytohormones is generally separated into pathogen- and herbivore-induced responses. The JA pathway is thought to be involved in the activation of defences against herbivores and necrotrophic pathogens, whereas the SA-mediated pathway contributes to the defence against biotrophic pathogens (Gatehouse, 2002; Kessler & Baldwin, 2002; Glazebrook, 2005). If rhizobia are regarded simplistically as intruders of the plant, the question arises how stress-associated phytohormone levels change during the establishment of symbiosis. Indeed, previous studies showed that for successful nodulation to occur, it is crucial to suppress the activation of signalling cascades involving the SA and the JA pathways. In contrast to interaction with wild type rhizobia, SA accumulates in the roots of *Medicago sativa* L. inoculated with mutants of *Sinorhizobium meliloti* that are incapable of synthesising Nod-factors, while at the same time nodulation is clearly reduced (Martínez-Abarca *et al.*, 1998). Furthermore, the exogenous application of SA or JA inhibits nodulation (Martínez-Abarca *et al.*, 1998; Sun *et al.*, 2006).

Thus, in order to gain deeper insight into general and specific traits of reactions to biotic stressors of a plant, several microbial oligosaccharidic signalling compounds (Figure 3-1) were used in this part of the study and their impacts on defensive traits of the plant were compared. Besides pathogen-derived elicitors (β-glucans and *N*,*N*',*N*'',*N*'''-tetraacetylchitotetraose), *Medicago truncatula* Gaertn. cv. Jemalong A17 was challenged with symbiotic signalling substances (Nod-factors), and the responses in terms of VOC emission and phytohormone levels were evaluated. For better comparability, the effect of damage caused by *Spodoptera* spp. was also included in the determination of phytohormone levels. In addition, some results on the involvement of reactive oxygen species (ROS) and nitric oxide (NO) in signal transduction are presented, in an attempt to link the induced stress responses observed to level of ROS-mediated reactions.

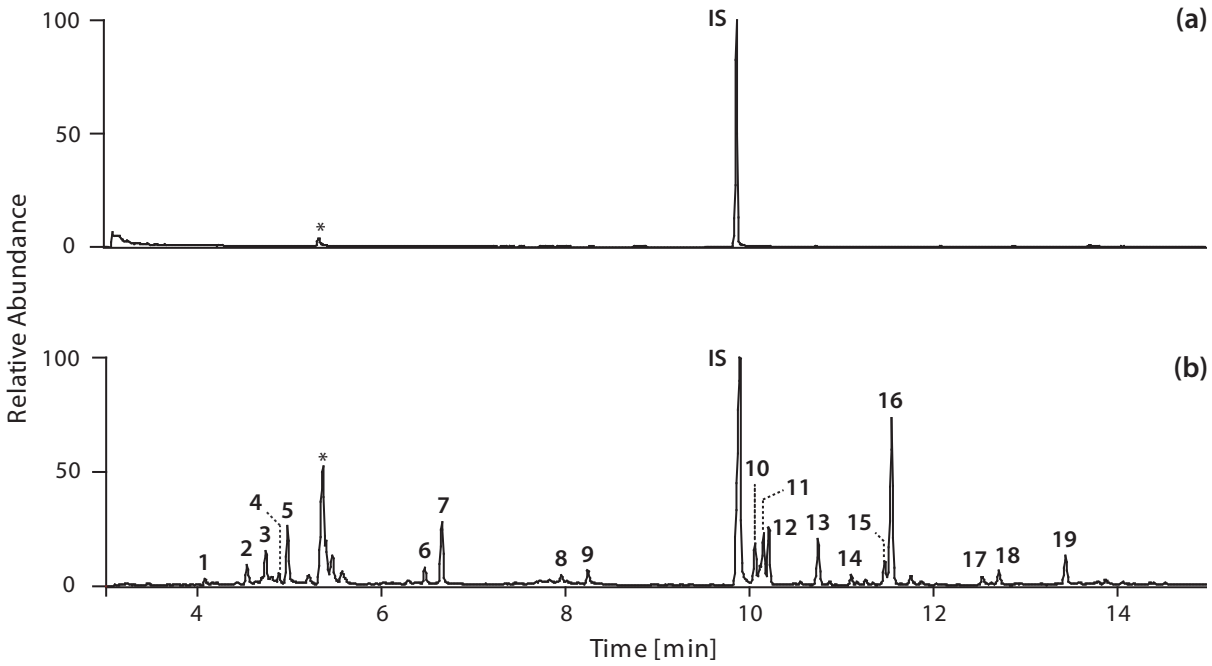


Figure 3-2 Gas chromatograms of volatiles released by *Medicago truncatula* in reaction to β-glucans. (a) control (detached plant placed in tap water); (b) volatiles induced in plants treated with 200 µg ml^{-1} β-glucans. For identification of the compounds, see Table 3-1.

3.2 Results

3.2.1 VOC emission

Certain oligosaccharides are well-known signalling compounds of microbial origin, which are often involved in plant-microbe interactions. Out of these structurally diverse elicitors, branched β-(1,3)-β-(1,6)-glucans from the phytopathogenic oomycete *Phytophthora sojae* as well as *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose (CH4) were used in this study to simulate pathogen attack (Figure 3-1 a, b).

Treating *M. truncatula* plants with the β-glucan elicitor strongly induced the emission of VOCs compared to control plants that were cut and placed in tap water (Figures 3-2 & 3-3, a, c). Thirteen sesquiterpenoids, and the homoterpenes 3*E*,7*E*-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) and 4,8-dimethyl-1,3,7-nonatriene (DMNT) were found to be induced, but no monoterpenoids could be detected. The VOC blend emitted was similar to that found after herbivore feeding in *M. truncatula* (cf. Figures 2-1 and 3-2). The qualitative composition of the blends was almost identical, except for some minor differences concerning compounds found in trace amounts. Regarding the quantitative aspect, there was a slight trend towards higher emission rates after herbivore attack. In contrast to the substantial induction of volatiles by β-glucans, CH4 was inactive in this respect. No significant differences were found between volatile blends of control plants and those treated with CH4, except for an elevated emission of n-tetradecane (Figure 3-3 b).

Furthermore, two Nod-factors that are involved in *Sinorhizobium meliloti – M. truncatula* symbiosis were tested for their ability to induce the emission of VOCs. These molecules share the chitotetraose backbone with a fatty acid chain (C16:2) attached to it by an amide bond. But

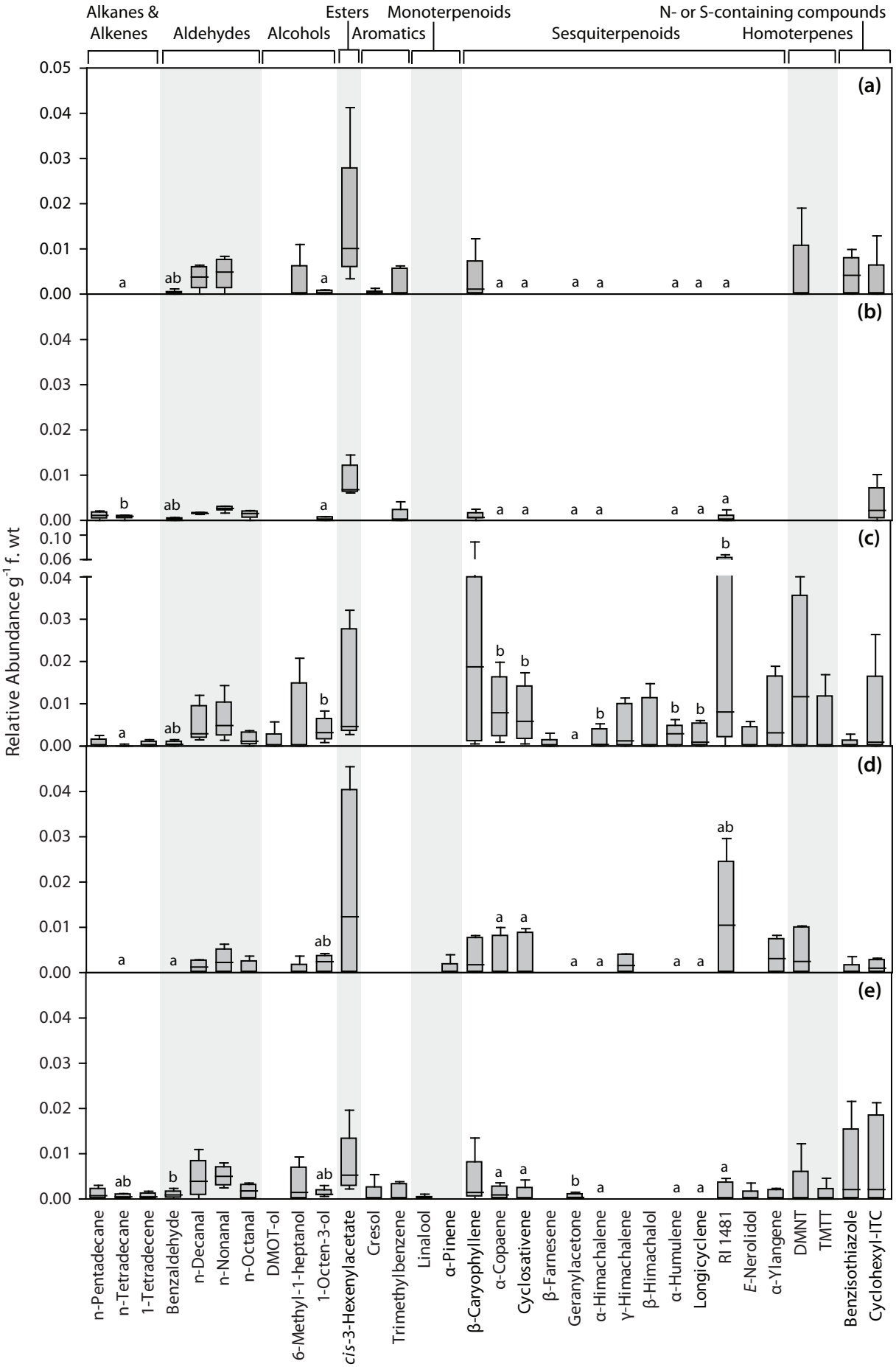

Alkanes & Alkenes
Aldehydes
Alcohols
Esters
Aromatics
Monoterpenoids
Sesquiterpenoids
N- or S-containing compounds
Homoterpenes
Relative Abundance g-1 f. wt
(a)
(b)
(c)
(d)
(e)
n-Pentadecane
n-Tetradecane
1-Tetradecene
Benzaldehyde
n-Decanal
n-Nonanal
n-Octanal
DMOT-ol
6-Methyl-1-heptanol
1-Octen-3-ol
cis-3-Hexenylacetate
Cresol
Trimethylbenzene
Linalool
α-Pinene
β-Caryophyllene
α-Copaene
Cyclosativene
β-Farnesene
Geranylacetone
α-Himachalene
γ-Himachalene
β-Himachalol
α-Humulene
Longicyclene
RI 1481
E-Nerolidol
α-Ylangene
DMNT
TMTT
Benzisothiazole
Cyclohexyl-ITC

Figure 3-3 (previous page) Box-plots representing the relative quantification of volatiles induced by treatment with microbial oligosaccharides. (a) control (detached plant placed in tap water); (b) 100 µM *N,N',N'',N'''*-tetraacetylchitotetraose; (c) 200 µg ml^{-1} β-glucan elicitor; (d) 10 µM LCO-IV (C16:2); (e) 10 µM LCO-IV (C16:2, S). n = 6 for control and Nod-factors; n = 5 for β-glucan and CH4. Small letters indicate significant differences as determined by the Newman-Keuls *post hoc* test (for results of the one-way ANOVA, see Table 3-1). Abbreviations: DMOT-ol, 2,6-dimethyl-3,5,7-octatrien-2-ol; RI 1481, unidentified sesquiterpene with RI 1481; Cylcohexyl-ITC, cylcohexylisothiocyanate.

while the lipochitooligosaccharide LCO-IV (C16:2, S) carries a sulphate group, providing for activity and host specificity, LCO-IV (C16:2), which lacks this substituent, is unable to induce nodulation in *M. truncatula* (Figure 3-1 c) (D'Haeze & Holsters, 2002). Strikingly, both compounds induced volatile release by *M. truncatula* (Figure 3-3 d, e). Though the quantity of VOCs emitted varied highly within each treatment group, the blends were qualitatively clearly distinct from the control.

Table 3-1 Summary of compounds identified in the volatile blends emitted by *Medicago truncatula* in response to elicitation with microbial oligosaccharides, numbered according to Figure 3-2, and results of one-way ANOVA analysis (overall difference of means; for pairwise comparison see Figure 3-3).

	Compound[1]	No in Fig. 3-2	p (ANOVA)
Alkanes/Alkenes	n-Pentadecane		*
	n-Tetradecane		**
	1-Tetradecene		*
Aldehydes	Benzaldehyde		*
	2-Ethyl hexanal[2]	1	ns
	n-Decanal	8	ns
	n-Nonanal	6	ns
	n-Octanal	4	ns
Alcohols	2,6-Dimethyl-3,5,7-octatrien-2-ol (*E*)[3]		ns
	6-Methyl-1-heptanol	3	ns
	1-Octen-3-ol	2	**
Esters	*cis*-3-Hexenylacetate	5	ns
Aromatics	Cresol		ns
	Trimethylbenzene		ns
Monoterpenoids	Linalool		ns
	α-Pinene		ns
Sesquiterpenoids	β-Caryophyllene	13	*
	α-Copaene	12	**
	Cyclosativene	10	**
	β-Farnesene		ns
	Geranylacetone		**
	α-Himachalene	14	**
	γ-Himachalene	15	*
	β-Himachalol	19	*
	α-Humulene		**
	Longicyclene		**
	unidentified sesquiterpene (RI 1481)	16	**
	E-Nerolidol	17	ns
	α-Ylangene	11	*
Homoterpenes	4,8-Dimethyl-1,3,7-nonatriene (DMNT)	7	ns
	3*E*,7*E*-4,8,12-Trimethyltrideca-1,3,7,11-tetraene (TMTT)	18	ns
N- or S-containing compounds	Benzo(iso)thiazole		ns
	Cyclohexylisothiocyanate	9	ns

[1] for identification of the compounds (MS and linear retention indices) see Appendices I and II.
[2] contamination of abiotic origin.
[3] artefact generated from *E*-ocimene during adsorption to the charcoal trap (Kaiser, 1993).
ns, not significant; *, $p < 0.05$; **, $p < 0.01$.

However, due to the above-mentioned variability of VOC emission, hardly any of those differences were statistically significant using analysis by ANOVA and Newman-Keuls post hoc test. Still, there was a trend towards the emission of certain sesquiterpenoids in higher abundance, namely α-copaene, cyclosativene, α-ylangene, and the unidentified sesquiterpene (RI 1481) for both Nod-factors, γ-himachalene after treatment with LCO-IV (C16:2), and *E*-nerolidol after treatment with LCO-IV (C16:2, S). Furthermore, some of the changes in the emission pattern seemed to be rather specific, as for example the emission of geranylacetone that could be found only after treatment with LCO-IV (C16:2, S) and the higher level of emission of benzaldehyde compared to the treatment with LCO-IV (C16:2). In contrast, the emission patterns observed after treatment with LCO-IV (C16:2) seemed to be concentrated between control and β-glucan treatment without any specific differences. Additionally, in contrast to induction with pathogen-derived elicitors, small amounts of monoterpenoids were detected: α-pinene in the case of LCO-IV (C16:2) treatment and linalool upon LCO-IV (C16:2, S) treatment. Again, however, these differences were not statistically significant using ANOVA analysis. In conclusion, it can be noted that *M. truncatula* emits numerous compounds in response to certain oligosaccharides. All substances detected in these volatile blends are listed in Table 3-1, which also includes the *p*-value summary of the one-way ANOVA analysis (overall difference of means) and the numbering according to Figure 3-2.

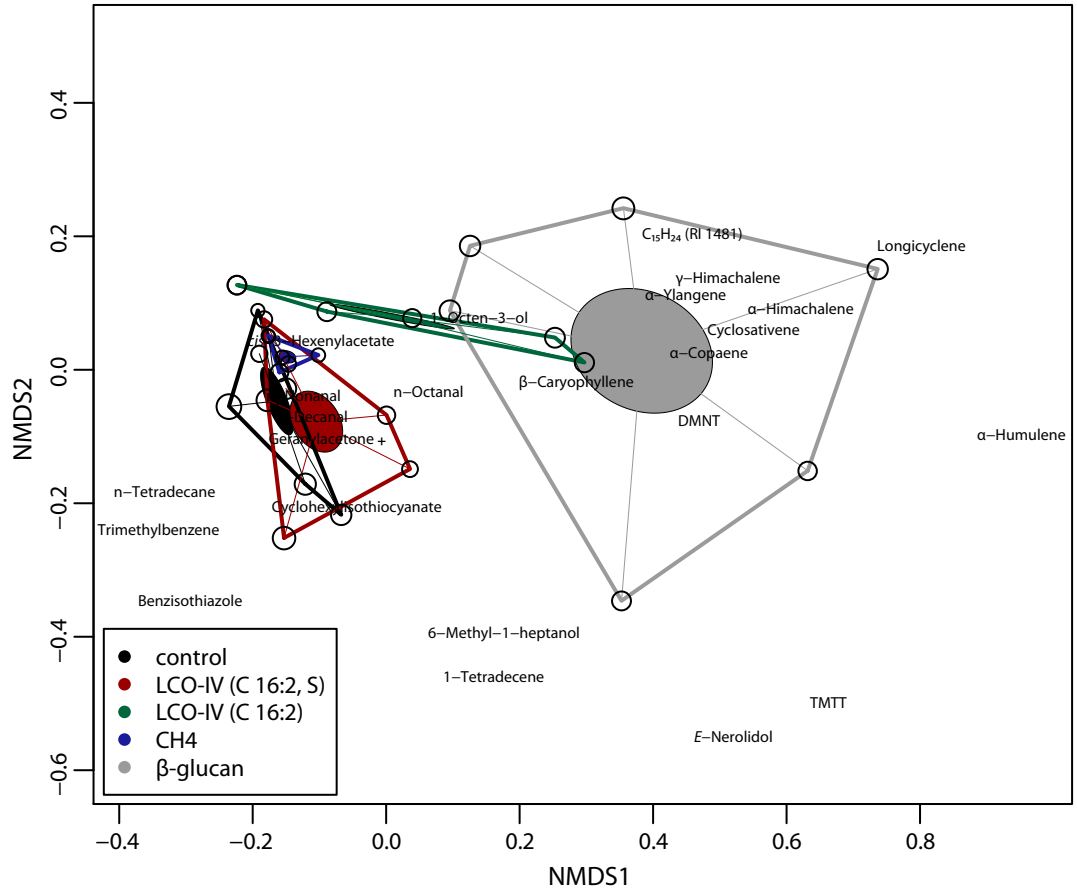


Figure 3-4 Non-metric multidimensional scaling (NMDS) plots of VOC patterns in response to microbial oligosaccharides using square root transformed data and **Euclidean distance** as a dissimilarity measure. Stress, 9.04. Open circles indicate the relative location of single volatile samples in ordination space. The size of the circles represents the goodness of the fit into the model for each particular sample. The centroids are given by the intersection of the spiderweb-like lines within each treatment group; the groups' standard errors are given by filled ellipses. The size of the ellipse can be interpreted as a measure of consistency for the respective group. The relative distances between any sample or substance shown represent similarities, and positive (low distances) or negative (high distances) correlations, respectively. Variables are plotted according to their weighted averages.

However, for the description of complex volatile patterns, multivariate statistical methods might be superior to univariate ones. Furthermore, considering the low number of samples, methods depicting single samples might be advantageous. Hence, non-metric multidimensional scaling (NMDS) was used to visualise multivariate patterns. In the first line of analysis, square root transformed data and Euclidean distance as a dissimilarity measure were used (Figure 3-4). With a stress of 9.04 the fit of the model was fairly good. The results were largely in line with those of ANOVA and *post hoc* test. Treatment with β-glucans resulted in the emission of clearly distinct VOCs, such that this group could easily be distinguished from the others. However, there was a large overlap between the effects of the active Nod-factor, LCO-IV (C16:2, S), and the control, though a part of the analysed samples displayed fairly distinct patterns. The inactivity of CH4 with respect to its impact on VOC emission is depicted very clearly; in comparison to all other treatments, including the control, the variation between single samples was the smallest by far. Finally, treatment with the inactive Nod-factor, LCO-IV (C16:2), resulted in strongly varying emission patterns, but roughly spanning the area between control and β-glucan treatment. It was striking, however, to find that in terms of the qualitative composition of the VOC blends detected, the overlap between effects produced by β-glucans and the active Nod-factor was bigger than between β-glucans and the inactive Nod-factor (Table 3-2). As a consequence, a binomial variant of NMDS was calculated as completion, based on a probabilistic distance measure, hence disregarding relations between concentrations of certain compounds, but emphasising qualitative traits of volatile blends. The results of this analysis were clearly distinct from those gained using Euclidean distance, and showed increased grouping performance in contrast to originally scaled measurements (Figure 3-5), as evaluated by a slightly lower stress of 8.26. To give but one example, the formerly rather broad group of β-glucan treatments got more pronounced and well shaped. The remaining groups were configured more distinct, with resolved overlaps. This could be interpreted that only regarding qualitative traits of volatile patterns results in good separability of the different treatments. It also showed that treatment with both Nod-factors produced rather variable effects: about half of the plants tested responded to the treatment, whereas the other half remained largely unaffected. In conclusion, qualitative and quantitative aspects of the volatile blends emitted gave different pictures and it remains to be answered whether quantity or quality matters more in the biological context.

Table 3-2 Comparison of qualitative differences in volatile blends induced by microbial oligosaccharides. Any substance that was found in controls was excluded from the list.

	β-glucan	LCO IV (C16:2, S)	LCO IV (C16:2)	CH4
Longicyclene	x			
α-Himachalene	x			
α-Humulene	x			
1-Tetradecene	x	x		
Geranylacetone	x	x		
E-Nerolidol	x	x		
TMTT	x	x		
γ-Himachalene	x		x	
α-Ylangene	x	x	x	
Cyclosativene	x	x	x	
α-Copaene	x	x	x	
$C_{15}H_{24}$ (RI 1481)	x	x	x	x
n-Octanal	x	x	x	x
n-Tetradecane	x	x	x	x

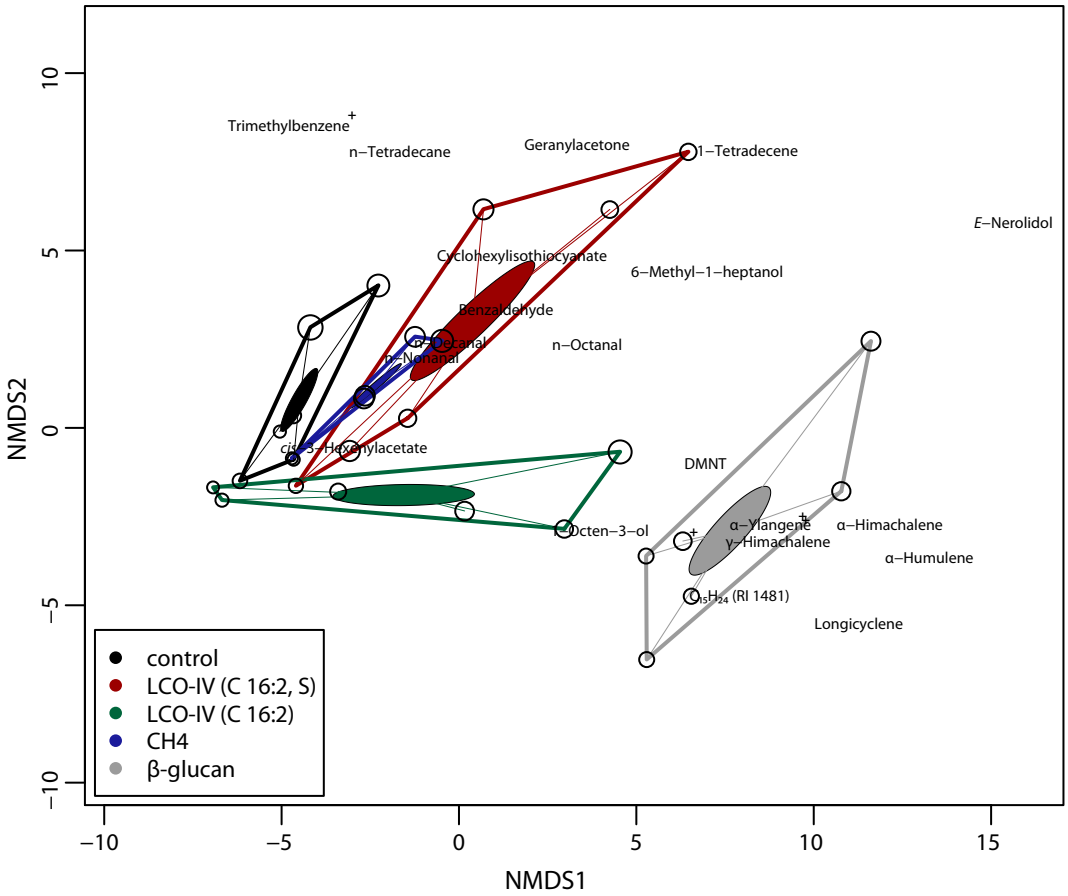


Figure 3-5 Non-metric multidimensional scaling (NMDS) plots of VOC patterns in response to microbial oligosaccharides using square root transformed data and **binomial distance** as a dissimilarity measure. Stress, 8.26. Open circles indicate the relative location of single volatile samples in ordination space. The size of the circles represents the goodness of the fit into the model for each particular sample. The centroids are given by the intersection of the spiderweb-like lines within each treatment group; the groups' standard errors are given by filled ellipses. The size of the ellipse can be interpreted as a measure of consistency for the respective group. The relative distances between any sample or substance shown represent similarities, and positive (low distances) or negative (high distances) correlations, respectively. Variables are plotted according to their weighted averages.

3.2.2 Phytohormone levels

Two classical phytohormones involved in plants' defence responses are SA and JA: Conventionally, SA is seen to be mainly linked to defence reactions upon pathogen attack, whereas JA is usually implicated in defence against herbivores (Bostock *et al.*, 2001). Here, the levels of both hormones were determined in response to elicitation with microbial oligosaccharides. Hardly any significant differences in SA levels between any of the treatments and the control were detected within the first 24 h after elicitation (Figure 3-6 a, b). Only two samples deviated from control values. These were taken 8 h after CH4 treatment and 2 h after treatment with LCO-IV (C16:2). These variations might be attributed to a Type I error due to the small sample number, as the rest of the data fit perfectly in the control curve. When parallel measurements on the effect of caterpillar feeding were included, it became clear that the rise of SA concentrations was due only to the detachment of the plant. Even substantial tissue damage as induced by feeding *Spodoptera* spp. did not cause SA levels to rise, as long as the stem was not cut. (Figure 3-6 b). It is noteworthy that a transient increase of SA levels by caterpillar feeding was detected in systemic tissue, as demonstrated in Chapter 2 (Figure 2-3). This slight augmentation might be leveled by measuring the SA content

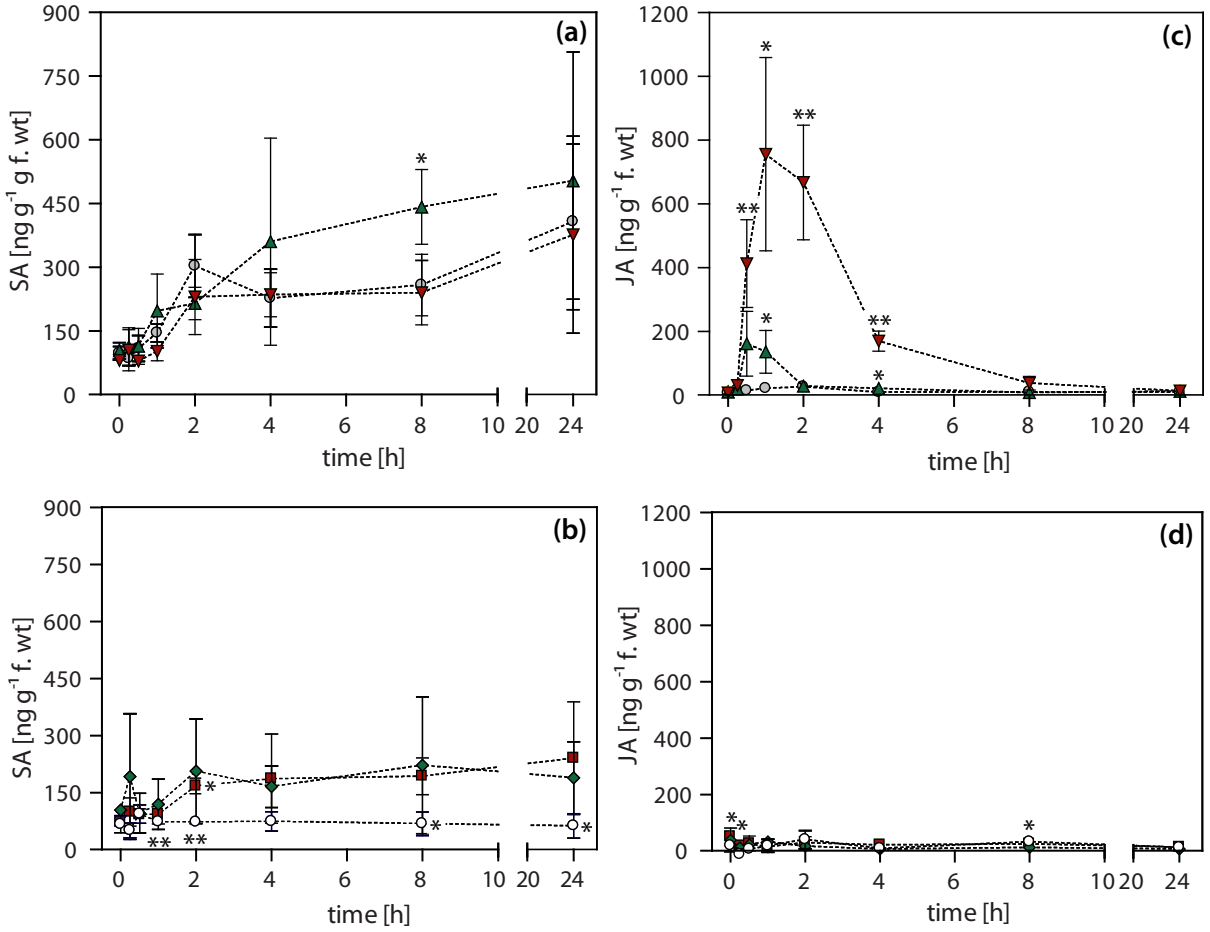


Figure 3-6 Salicylic acid (SA) levels (a, b) and jasmonic acid (JA) levels (c, d) after treatment with microbial oligosaccharides.
(a) SA levels in control plants (cut and placed into tap water, ○), plants treated with 200 µg ml^{-1} β-glucan (▼), and plants treated with 100 µM *N,N′,N″,N‴*-tetraacetylchitotetraose (▲). (b) SA levels of plants treated with 10 µM LCO-IV (C16:2) (inactive Nod-factor, ■), 10 µM LCO-IV (C16:2, S) (active Nod-factor, ◆), and after feeding by *Spodoptera* sp. (○).
(c) JA levels in control plants (cut and placed into tap water, ○), plants treated with 200 µg ml^{-1} β-glucan (▼), and plants treated with 100 µM *N,N′,N″,N‴*-tetraacetylchitotetraose (▲). (d) JA levels of plants treated with 10 µM LCO-IV (C16:2) (inactive Nod-factor, ■), 10 µM LCO-IV (C16:2, S) (active Nod-factor, ◆), and after feeding by *Spodoptera* sp. (○).
The results shown are the mean ± standard deviation of three independent experiments. Asterisks indicate statistically significant differences as determined by two-tailed *t*-test. *, $p < 0.05$; **, $p < 0.01$.

of entire plant. Furthermore, it cannot be excluded that feeding lepidopteran larvae influence phytohormone levels by mechanisms that differ from the impact of simple wounding.

In contrast, JA levels rose markedly though transiently after treatment with both β-glucans and CH4, showing the highest concentration within the first 60 minutes, whereas neither of the Nod-factors caused the JA levels to rise above those found in the control (Figure 3-6 c, d). Astoundingly, also JA levels after caterpillar feeding did not differ from those in the control. Again, these results differ from those shown in Chapter 2, demonstrating that feeding *Spodoptera* spp. do indeed induce the accumulation of JA. As mentioned for SA levels, instead of picking just two sampling times, a complete time course was assessed in this row of experiments; furthermore, local and systemic tissues were not separated, but the phytohormone levels of the entire damaged plant were determined. These methodological differences might explain the results to some extent. But the accumulation of JA was still drastically higher (up to about 8-fold) after treatment with pathogen-derived elicitors, even if taking the maximal values of the determinations presented in Chapter 2 (cf. Figure 2-2) for comparison.

Figure 3-7 (right) Cross-section of a stem of *Medicago truncatula* with astra blue and safranin staining (cellulose stained blue, lignified cell walls stained red). p, parenchyma; sc, sclerenchyma; ph, phloem; c, cambium; x, xylem; prx, protoxylem.

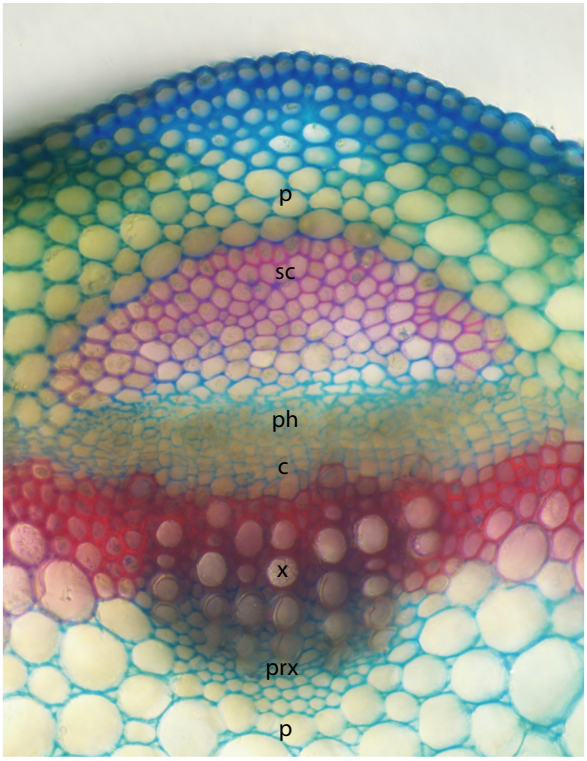
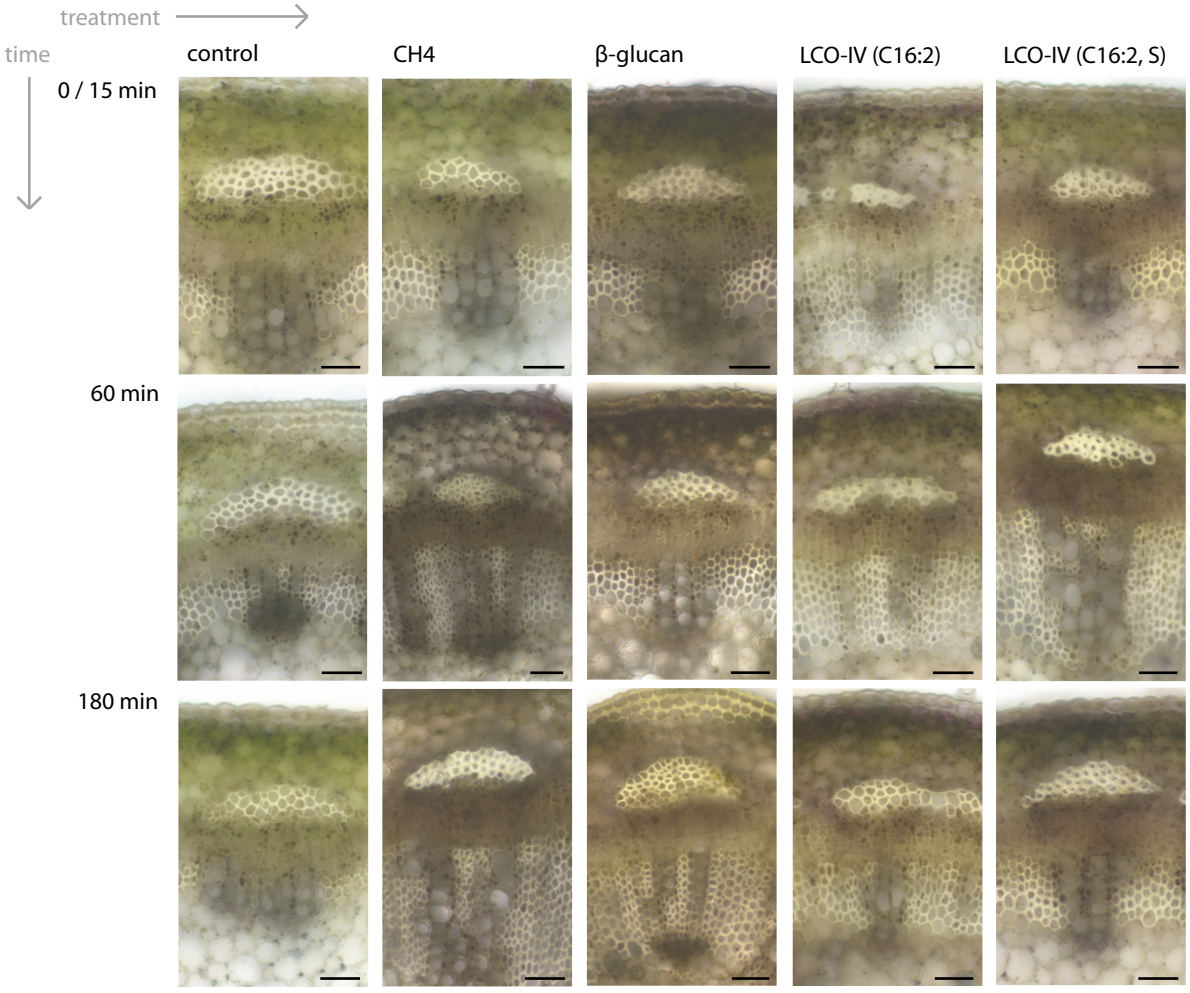


Figure 3-8 (below) Detection of ROS in cross-sections of *Medicago truncatula* in reaction to microbial oligosaccharides using Nitroblue tetrazolium (NBT, purple-blue staining).
Each column shows the reaction to a certain elicitor; each row depicts the temporal development of ROS production.
control, plants cut and placed in tap water; CH4, treatment with 100 µM *N,N',N'',N'''*-tetraacetylchitotetraose; β-glucan, elicitation with 200 µg ml^{-1} β-glucans; LCO-IV (C16:2), treatment with 10 µM inactive Nod-factor; LCO-IV (C16:2, S), treatment with 10 µM active Nod-factor. Scaling bars represent 50 µM.
Only samples of the control were taken immediately after wounding; the first samples of any other treatment were analysed 15 minutes after elicitation.



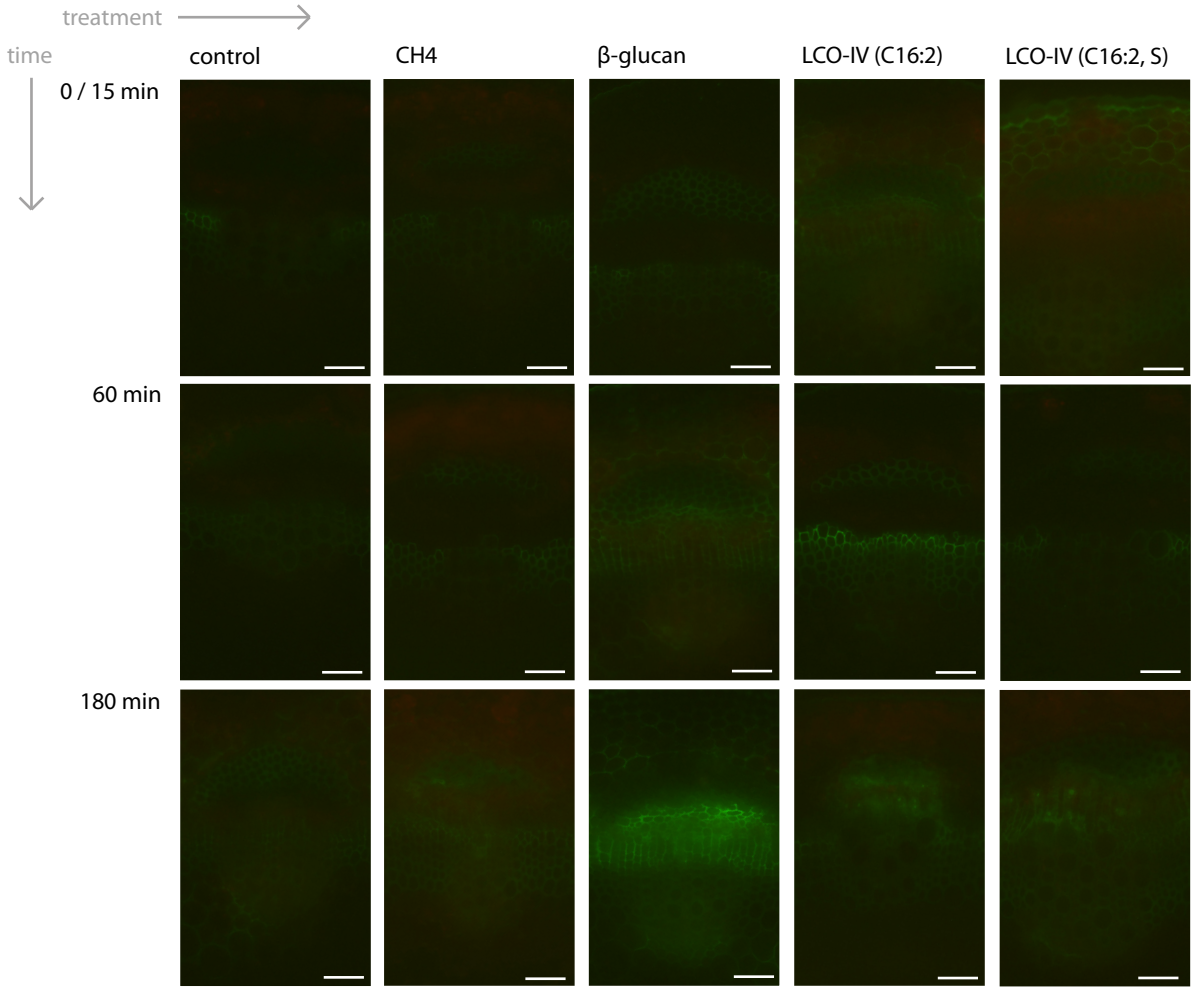


Figure 3-9 Detection of nitric oxide (NO) in cross-sections of *Medicago truncatula* stems in reaction to microbial oligosaccharides using 4,5-diaminofluorescein diacetate (DAF 2-DA, green fluorescence). Pictures are overlays of the fluorescence signals produced by DAF 2-DA and chlorophyll autofluorescence.
Each column shows the reaction to a certain elicitor; each row depicts the temporal development of NO production.
control, plants cut and placed in tap water; CH4, treatment with 100 µM *N,N',N'',N'''*-tetraacetylchitotetraose; β-glucan, elicitation with 200 µg ml^{-1} β-glucans; LCO-IV (C16:2), treatment with 10 µM inactive Nod-factor; LCO-IV (C16:2, S), treatment with 10 µM active Nod-factor. Scaling bars represent 50 µM.
Only samples of the control were taken immediately after wounding; the first samples of any other treatment were analysed 15 minutes after elicitation.

3.2.3 Accumulation of reactive oxygen species and nitric oxide

Besides the fact that the results with herbivory were somewhat contradictory to the data gathered earlier, the fact that VOC emission and phytohormone levels could not be correlated was fairly puzzling. In order to find a link between the primary stimulus and VOC emission, the accumulation of ROS and NO were assessed using microscopic techniques.

The excessive production of ROS and NO is a trait that has been described in the plant's reaction to pathogen attack and elicitation with pathogen-derived elicitors (Lamb & Dixon, 1997; Durner & Klessig, 1999). In preliminary studies for NO detection using elicited leaves, hardly any reaction could be observed. Only in a few cases, where vascular bundles were uncovered by smashing the leaf, could the staining of this tissue after the treatment with β-glucans be seen (data not shown). This led to the assumption that there might be strong tissue specificity of NO production. Thus, in all following experiments, cross-sections of the stem were used for microscopic analysis. On the one hand, effects concerning vascular bundles could easily be visualised using this experimental

setup, and on the other hand this procedure better fit the results presented above, as the site of action of the elicitors was kept comparatively constant. However, morphological studies showed that the tissue treated with elicitors in the experiments on VOC emission and phytohormone levels still belonged to the root (stems were cut directly at the soil surface), whereas the plant parts used to assess ROS accumulation were actually stems (Figure 3-7).

For all experiments only examples of vascular bundles are presented, as all observable effects were restricted to this tissue and its near surroundings. The accumulation of ROS was assessed with nitroblue tetrazolium (NBT), producing purple to blue precipitate in the presence of ROS (Figure 3-8). Regarding controls, some precipitates could be observed in all samples. Slight staining of the xylem in all samples can, however, be regarded as "background" due to lignification in that region; but the phloem also exhibited faint staining. The responses observed to the treatments with all oligosaccharides tested were considerably stronger. All substances applied induced the pronounced accumulation of ROS. Phloem and cortical parenchyma were particularly affected; in some cases the staining also extended to the inner parenchyma. However, no pronounced or consistent differences between the different elicitors could be observed.

The detection of NO gave a much more distinct picture of effects induced by the different oligosaccharides (Figure 3-9). Wounding and treatment with CH4 did not bring about any pronounced production of NO, though in some cases, slight staining of the phloem and parenchyma tissue could be observed. Considerably stronger effects could be detected after elicitation with β-glucans. After 60 minutes, distinct staining became visible in phloem and meristematic tissue; the signal increased with time, yielding strong staining after 180 minutes, again concerning phloem and cambium and in some cases also parts of the cortical parenchyma.

Also, both Nod-factors induced NO accumulation. Though the temporal development of the reaction in response to the inactive Nod-factor, LCO-IV (C16:2), was highly variable, NO accumulation could consistently be shown after 180 minutes, affecting the phloem and cambium, comparable to β-glucan treatment, though the staining was of somewhat lower intensity. An entirely different pattern was observed for the active Nod-factor. In this case, staining was observed as early as 15 minutes after application, mostly in the cortical parenchyma, and thereafter it was drastically decreased. However, again after 180 minutes, pronounced staining was visible in the phloem, cambium, and cortical parenchyma, in several cases as pronounced as the staining shown for β-glucans. Altogether, the intensity of the effects observed varied greatly after all treatments. This was particularly prominent for the two Nod-factors. However, this variation is consistent with the high variability of the effects observed on induced VOC emission.

Sections for the detection of ROS and NO were done not only directly at the contact site between the wounded stem and the elicitor solution, but also 1 cm above. Results were basically the same within this distance. That is, the observed effects can be considered not to be strictly localised to the wounding site and independent of the immediate contact with the elicitor. In summary, the combined results on ROS and NO accumulation in reaction to different oligosaccharides gave three distinct patterns: for the control, no increased production, of ROS or of NO, could be detected. Although ROS accumulated in response to CH4 treatment, this elicitor failed to induce excessive NO production. Finally, β-glucans as well as both Nod-factors induced overproduction of ROS and NO, though the latter accumulated in differing temporal and spatial patterns.

3.3 Discussion

Signal perception systems for pathogen-derived β-glucan elicitors, chitin fragments, and Nod-factors have been convincingly shown to be present in *Medicago* species (Côté *et al.*, 2000; Felle *et al.*, 2000; Cullimore *et al.*, 2001). However, investigation of subsequent reactions predominantly concentrated on traits that were in close connection either with the activation of defence responses or with the establishment of a functional symbiosis.
So far, the possibility that plants may respond to those stimuli with the synthesis and emission of VOCs has largely escaped notice. That *M. truncatula* is in principle able to produce and emit these compounds has been shown in several studies. Gomez and co-workers (Gomez *et al.*, 2005) described the transcript induction of two putative mono- or diterpene and two putative sesquiterpene synthases after jasmonate application and herbivory. Moreover, about 30 triterpene saponins have been identified in *M. truncatula* cell cultures, some of which are induced by JA (Huhman & Sumner, 2002; Suzuki *et al.*, 2005). The emission of a variety of VOCs in reaction to herbivory was demonstrated in Chapter 2. Finally, with the results presented above, VOC emission could be shown after elicitation with microbial oligosaccharidic signals.
NMDS represents a straightforward and robust exploratory tool for visualising volatile blends in response to diverse induction treatments in a reduced multidimensional space suitable for direct interpretation (cf. Chapters 4 & 5); both qualitative and quantitative patterns can readily be evaluated using different levels of abstraction of data, i.e. originally scaled, or only with regard to the presence or absence of certain compounds. As each sample is depicted individually, the method is also applicable to small sample sizes. Treatments can be studied in direct connection to each other; mapped substances can also be interpreted in conjunction with the treatments they are most likely to correlate with, as any compound is directly projected onto the ordination. Thus, patterns could be found, which univariate methods would have been unable to capture. Furthermore, as the volatile patterns detected were rather distinctive in this set of experiments, it might be expected that they can also be classified or used for prediction. This question, however, will be considered in the next Chapter.

The emission of volatiles induced by herbivory has often been causally linked with elevated levels of JA (Walling, 2000; Gatehouse, 2002; Ament *et al.*, 2004). In the present study, no conclusive correlation between JA levels and VOC release could be found, as treatment with CH4 resulted not in volatile emission but in the accumulation of JA, whereas both Nod-factors induced VOC emission but not increased JA levels. Only elicitation with β-glucans gave rise to enormous accumulation of JA and accordingly to the highest level of volatile emission. Also, increases in SA levels cannot be interpreted as responsible for volatile release, as the pattern of accumulation was the same in treated and in control plants. As these results make clear, working with detached plants is not favourable in this context. Cutting the plant led to an increase in SA concentration that was not further enhanced by the application of any of the elicitors tested. In contrast, even the substantial tissue damage a caterpillar inflicts on a plant was not sufficient to enhance SA accumulation, as long as the stem remained intact.
These results are in line with previous reports indicating that neither of the two phytohormones is responsible of VOC emission in reaction to bacterial pathogen attack in tobacco (Huang *et al.*, 2003). In a succeeding study, the impact of ethylene on volatile emission was assessed (Huang

et al., 2005). But again, no sound evidence was found that changing levels of any of the studied phytohormones were somehow linked to alterations in volatile emission.
In the studies of Huang and co-workers (Huang *et al.*, 2003; Huang *et al.*, 2005) different strains of *Pseudomonas syringae* were used to induce VOC release. These pathogens produce several toxins, amongst them coronatine, which is known to mimic JA functionally and to induce secondary metabolism (Weiler *et al.*, 1994); the presence of coronatine may explain volatile release independent of phytohormonal changes in this case. Still, further studies with other bacterial (Buonaurio & Servili, 1999) and fungal (Cardoza *et al.*, 2002) pathogens strengthened the idea that volatile release may be a general reaction of plants to pathogen attack. Yet, no conclusive results on causal relationships concerning the mode of induction were found, though the study conducted by Buonaurio & Servili (1999) suggests an involvement of the lipoxygenase pathway.
However, recent studies questioned the essential role of JA in the induction of secondary metabolism (Zhao *et al.*, 2005). For example, treating *Petroselinum crispum* cell cultures with inhibitors of JA accumulation did not influence phytoalexin production or PR gene expression in response to elicitation (Hahlbrock *et al.*, 2003); in soybean cell cultures, elicitation with β-glucans induced phytoalexin accumulation, while endogenous levels of JA, OPDA, and SA remained at the resting level (Fliegmann *et al.*, 2003); treating *Hyoscyamus muticus* root cultures with either methyl jasmonate or a fungal elicitor resulted in the induction of sesquiterpenes in quantitatively and qualitatively different patterns (Singh *et al.*, 1998).
Even though CH4 induced higher JA levels compared to herbivory, it failed to induce the release of VOCs. Conversely, both Nod-factors, the biologically active as well as the inactive, did not influence JA or SA levels but led to slightly elevated sesquiterpene emissions. Still, in the case of β-glucan elicitation, elevated JA levels and increased VOC emission coincided, which might be regarded as incidental given the other results. Finally, the divergence of the results concerning herbivory in Chapters 2 and 3 might be attributed to the different modes of sampling.

In view of the inconsistencies outlined above, an alternative hypothesis needs to be stated.
First, a multitude of other oxylipins exists that is only poorly described regarding their biological activities and physiological roles (Blee, 2002; Schulze *et al.*, 2006). Furthermore, the involvement of the lipoxygenase pathway is not inevitably required for the induction of secondary metabolism. Yet another way to link the interactions with pathogens and symbionts to volatile emission is via the production of ROS. The oxidative burst is a well-described phenomenon in the context of the hypersensitive response (Lamb & Dixon, 1997). Also, β-glucans (Mithöfer *et al.*, 1997) and chitin fragments (Yamaguchi *et al.*, 2005) have been shown to induce ROS production. Still, elevated levels of ROS have not only been detected after pathogen attack or elicitor challenge, but they have proved to be a rather common trait of plants' responses to other biotic threats such as herbivory or abiotic stresses (Mithöfer *et al.*, 2004). Finally, ROS can even be detected in roots after the application of Nod-factors (Ramu *et al.*, 2002) and in root nodules (Santos *et al.*, 2001). As shown in Figures 3-8 and 3-9, ROS and NO can also be detected in response to elicitation with certain microbial oligosaccharides, displaying distinct patterns of accumulation depending on the respective induction treatment.
Amongst the variety of effects produced by ROS in a cell, radical-mediated lipid peroxidation can lead to the formation of cyclic oxylipins independent of enzymatic participation. These linolenic acid-derived compounds, namely phytoprostanes, can be induced by wounding, heavy metals,

and pathogen attack via the production of ROS (Imbusch & Mueller, 2000; Thoma *et al.*, 2003). Moreover, the physiological role of NO in plants has gained increasing attention in the last few years. However, its mode of action is still largely unknown. Only recently have *S*-nitrosylated proteins been identified in *Arabidopsis thaliana*, paralleling the well-described posttranslational modifications in animals (Lindermayr *et al.*, 2005; Lindermayr *et al.*, 2006). Furthermore, it has been demonstrated lately that in animal systems nitrated fatty acids might be important signalling compounds (Baker *et al.*, 2005; Schopfer *et al.*, 2005). Analogous reactions are clearly possible in plant cells and would presume the existence of another class of signal components in plants that has so far escaped notice. As the occurrence of nitrated compounds, particularly fatty acids, in plants has not yet been investigated, the analysis of such putative signalling compounds is an interesting target for further investigation. Finally, the hypothesis stated by Mithöfer *et al.* (2004), regarding ROS as link in mediating diverse stress responses, may also be extended by the impact of NO and to some cases of beneficial biotic interactions too.

Besides open questions regarding signalling cascades leading to the emission of VOCs, physiological and ecological functions remain to be discussed. In the case of pathogen attack or induction with pathogen-derived elicitors, the problem has already been addressed, and the antimicrobial properties of certain emitted compounds have been confirmed. These compounds belong to the class of lipid-derived volatiles from the lipoxygenase pathway such as (*Z*)-3-hexenol, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate; the monoterpenoid linalool and methyl salicylate have also been shown to inhibit pathogen growth (Croft *et al.*, 1993; Wright *et al.*, 2000; Cardoza *et al.*, 2002; Kishimoto *et al.*, 2006). Consequently, the emission of VOCs might represent means of direct defence in this respect. On the other hand, volatile release could contribute to the engagement of host resistance mechanisms, both systemically or in plant-to-plant communication (Farmer, 2001; Holopainen, 2004). The ability of certain VOCs (terpenoids and C6 components) to trigger the onset of resistance has already been shown in *Arabidopsis thaliana* (Bate & Rothstein, 1998; Kishimoto *et al.*, 2005), lima bean (Arimura *et al.*, 2000; Arimura *et al.*, 2001), and tomato (Farag & Pare, 2002; He *et al.*, 2006). The situation is somewhat more complicated in the case of Nod-factors. On the one hand, it has been proposed that the establishment of a functional symbiosis could rely on the suppression of the plant's defence response (e.g. Mithöfer, 2002). This could imply the initiation of certain defence reactions before the onset of an effective suppression or an incomplete suppression of these defences. On the other hand, emitted volatiles could be employed as signals mediating the interaction between the host plant and the microbial symbiont. In a recent study, Horiuchi and co-workers (Horiuchi *et al.*, 2005) showed that in response to plant-derived volatiles the soil nematode *Caenorhabditis elegans* transfers the nodulating bacterium *Sinorhizobium meliloti* to the rhizosphere of *M. truncatula*. Finally, elevated emission of VOCs could be a symptom of induced resistance as has been proposed to be an effect of interaction with beneficial microorganisms.

In summary, it was possible to demonstrate that *M. truncatula* emits a variety of VOCs in reaction to pathogenic and symbiotic oligosaccharidic signals. From a mechanistic point of view it is intriguing that diverse, though structurally related molecules induce similar responses albeit seemingly via different signal pathways. Those signalling nets, however, remain elusive. Furthermore, the biological relevance of VOCs in different biotic interactions still needs to be thoroughly investigated and defined.

4 The influence of mycorrhization on herbivore-induced volatile emission

4.1 Introduction

Colonisation of a plant with arbuscular mycorrhizal (AM) fungi is associated with drastic changes of the physiology and ecology of the plant. Out of the multitude of effects produced by AM fungi, two are the major objects of this part of the study: Plants associated with AM fungi undergo radical changes in secondary metabolism, and become more resistant to all kinds of pests, pathogens as well as phytophages. Both phenomena are well documented, though the reports on the latter are in part rather inconsistent.

Several effects on secondary metabolism have been documented so far. In *Medicago truncatula* Gaertn. and *Medicago sativa* L., patterns of flavonoid accumulation and correlating biosynthetic enzymes change during the establishment of mycorrhizal symbiosis. While the phytoalexin medicarpin is transiently increased in early phases of symbiosis and thereafter decreases below control levels, accumulation of other flavonoids clearly increases in AM roots (Harrison & Dixon, 1993). In *Medicago sativa*, those changes have been shown to depend not only on the temporal progression of the interaction but also on the fungal species involved (Larose *et al.*, 2002). Cell-wall-bound phenolics increase with the duration of symbiosis in onion (*Allim cepa* L.) (Grandmaison *et al.*, 1993); in cucumber (*Cucumis sativus* L.), three triterpenoids were found to be induced by mycorrhizal colonisation (Akiyama & Hayashi, 2002). In barley (*Hordeum vulgare* L.), levels of hydroxycinnamic acid amides transiently increase in mycorrhizal roots (Peipp *et al.*, 1997), and the continuous accumulation of apocarotenoids in AM roots seems to be a widespread phenomenon (Fester *et al.*, 2002). Furthermore, in most cases studied the transcript levels of phenylalanine ammonia lyase and chalcone synthase increase in AM roots, as do the transcripts of the key enzymes of the methylerythritol phosphate (MEP) pathway, 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR); the latter correlates with the accumulation of apocarotenoids in AM roots (Walter *et al.*, 2000). Finally, jasmonates, which are often associated with changes in secondary metabolism, and related biosynthetic enzymes occur at elevated levels in arbuscule-harbouring cells (Hause *et al.*, 2002).

Though most of these reports concentrated on changes that occur in the roots, changes in secondary metabolites in aboveground plant parts have also been described. For example, changes in the concentration of essential oils have been reported in three genotypes of oregano (*Origanum vulgare* L.) and in *Ocimum basilicum* L. var. *Genovese*; in the latter case these changes correlated with an increased number of peltate glandular trichomes, though the qualitative composition of essential oils was not altered (Copetta *et al.*, 2006; Khaosaad *et al.*, 2006). Furthermore, mycorrhization of *Citrus jambhiri* Lush leads to an induced accumulation of leaf sesquiterpenoid volatiles (cited in Strack *et al.*, 2003).

Mycorrhization also seems to entail a certain degree of bioprotection against different biotic and abiotic stresses. Protective capacities have been shown against root-feeding nematodes (Castillo *et al.*, 2006; de la Pena *et al.*, 2006) and certain pathogens (Cordier *et al.*, 1996; Bodker *et al.*, 1998; Fritz *et al.*, 2006), presumably via enhanced tolerance (Kjoller & Rosendahl, 1996). The interaction

of mycorrhization with herbivores has been studied in depth, but the results so far are still quite inconsistent. As summarised by Gehring & Whitham (2002), some chief motifs can be stated: In the majority of the cases studied, aboveground herbivores reduce mycorrhizal colonisation and alter the mycorrhizal fungi community composition, which could be due to the reduced ability of the attacked plant to supply the fungus with nutrients. However, belowground herbivores have been reported to facilitate fungal colonisation for *Agrostis capillaris* (Currie *et al.*, 2006). Conversely, influences of mycorrhizal fungi on the performance of aboveground herbivores have been observed. The quality of this impact, however, ranges from positive over neutral to negative. This substantial variation can to some extent be attributed to the species involved in the interaction, including fungal and herbivore species. For example, the performance of chewing and leaf-mining insects is predominantly negatively affected by AM symbiosis of the host plant, whereas sucking insects seem to profit from this interaction (Gange & West, 1994); counter-examples, however, also exist (Goverde *et al.*, 2000). Another tendency indicates that AM plants are favourable for specialist herbivores and detrimental for generalists. But although this effect was observed for chewing herbivores, it was not detected for sucking herbivores (Gange *et al.*, 2002). The effects of mycorrhization on parasitoids of herbivores have been assessed as well. Interestingly, in field experiments parasitism of herbivores was reduced on mycorrhized plants (Gange *et al.*, 2003). In the lab, this effect turned out to be strongly dependent on the fungal species associated with the plant. Both herbivore damage to the plant and parasitism on the herbivore were either reduced or remained unchanged by AM fungi, depending on the fungal species involved in the interaction (Gange *et al.*, 2003). But consistently, AM symbiosis did not improve the searching efficiency of the parasitoid. It has been reasoned that this could be due to increased plant size, which may impede the search of the parasitoid for its host (Gange *et al.*, 2003). But changes in induced volatile patterns as an effect of mycorrhization have not been considered as explanation.

Other mutualists have also been shown to be affected by the symbiotic state of the plant, as pollinators were more strongly attracted to flowers of mycorrhized fireweed (*Chamerion angustifolium* L.) than to those of non-mycorrhized fireweed (Wolfe *et al.*, 2005). In this study it has been argued that the observed effect could correlate with the development of larger inflorescences by mycorrhized plants, because other floral traits, such as nectar production or composition, were not influenced in this species. Again, the putative impact of changes in volatile emission has not been examined.

In regard to these heterogeneous reports on the impact of AM symbiosis on plant-herbivore and tritrophic interactions, this study aimed to further investigate what components could contribute to the effects observed. In order to do so, VOC release was assessed in non-mycorrhized and mycorrhized *Medicago truncatula* Gaertn. plants. Both the emission by intact plants and the emission by plants damaged by generalist lepidopteran larvae (*Spodoptera* spp.) were monitored. Whether mycorrhization alone could change volatile profiles of undamaged plants or whether the VOC pattern was altered when induction by herbivore attack occurred was the focus of the investigation. Furthermore, two different cultivars of *Medicago truncatula* were used in this study to evaluate the influence of subtle changes in the host, yet another possible reason for the inconsistencies of previous reports. Finally, measured VOC blends were used to assess whether classification and prediction of volatile patterns are possible, and thus may serve as indicators of the physiological state of the plant.

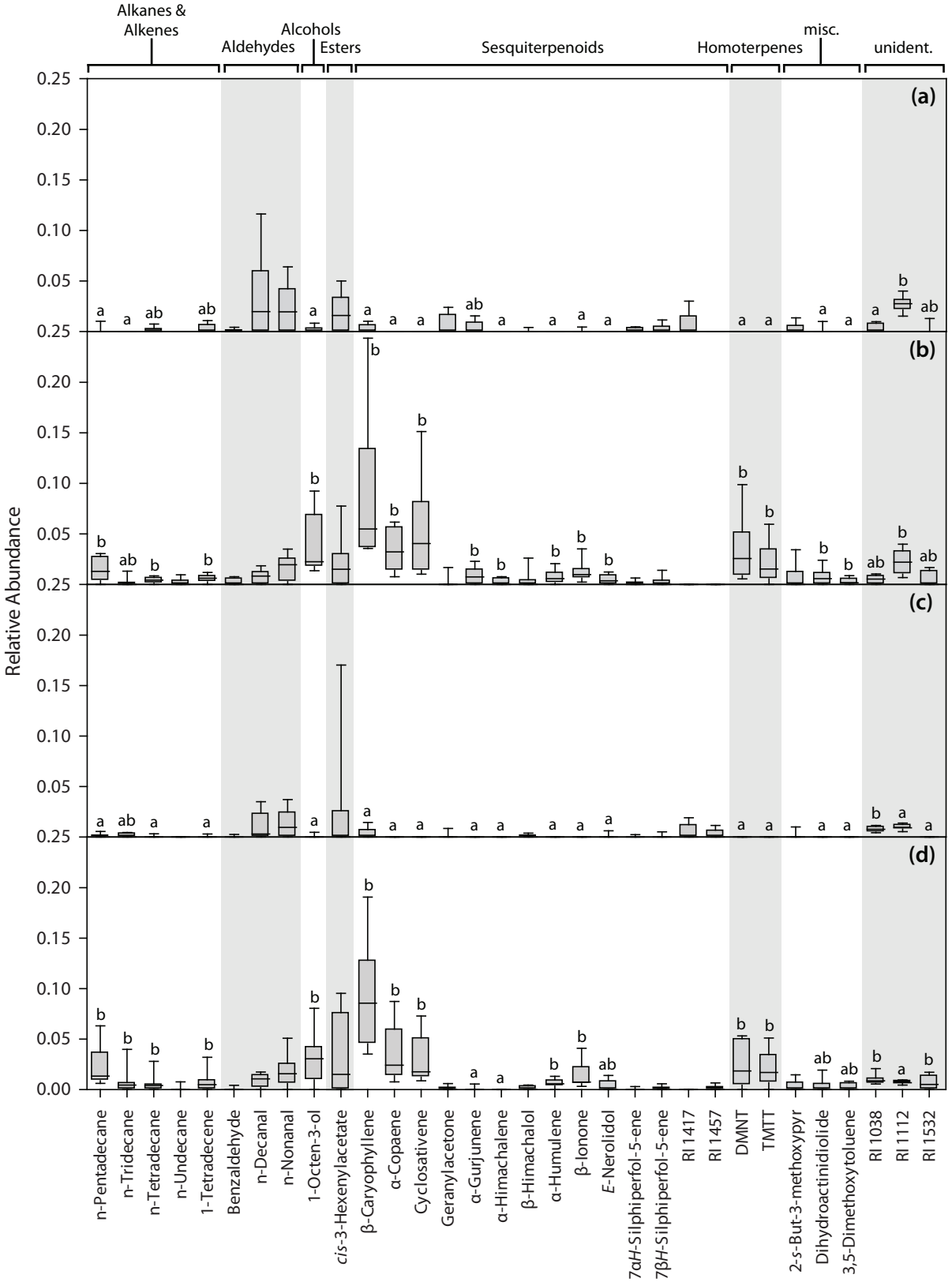


Figure 4-1 Box plots representing relative quantification of volatiles emitted by non-mycorrhized and mycorrhized *Medicago truncatula* cv. Jemalong A17 plants in reaction to herbivory. (a) control (non-mycorrhized), unwounded; (b) control, damaged by *Spodoptera* sp.; (c) mycorrhized, unwounded; (d) mycorrhized, wounded by *Spodoptera* sp. n = 9 - 10. Small letters indicate significant differences between the different treatments as determined by ANOVA and Newman-Keuls *post hoc* test. Abbreviations: unidentified compounds are given by their respective retention index; DMNT, 4,8-dimethylnona-1,3,7-triene; TMTT, (3*E*,7*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene; 2-*s*-But-3-methoxypyr, 2-*sec*-butyl-3-methoxypyrazine.

4.2 Results

4.2.1 VOC emission by *Medicago truncatula* cv. Jemalong A17

As demonstrated in Chapter 2, *M. truncatula* reacts to herbivory by emitting a great variety of volatile compounds. These experiments were repeated with non-mycorrhized plants that were grown under the same culture conditions as the mycorrhized ones (cf. Material and Methods, 7.1.5) as well as with plants inoculated with *Glomus intraradices* Schenck & Smith. Volatiles were collected from nine to ten plants for each treatment group. These groups were non-mycorrhized control plants, non-mycorrhized plants damaged by *Spodoptera* spp. larvae, undamaged mycorrhized plants, and mycorrhized, herbivore-wounded plants.

As can be seen in Figure 4-1, the results presented in Chapter 2 could approximately be reproduced. Herbivore damage induced the emission of several VOCs, in which sesquiterpenoids were the group of compounds most abundantly present. The overall pattern of VOC emission was quite similar in mycorrhized and non-mycorrhized plants for both damaged and undamaged plants. Most changes in the volatile pattern could be attributed to herbivore attack. However, some subtle differences between mycorrhized and non-mycorrhized plants could also be observed. These variations mainly concerned substances found in rather low abundance or even only trace amounts, with exception of two compounds that have not yet been identified. These substances exhibited an emission pattern that specifically changed with mycorrhization. One of them, RI 1038[1], was emitted at significantly higher levels in mycorrhized plants, be they wounded or not, whereas the emission RI 1112 was clearly reduced in mycorrhized plants (Figure 4-2 a, b). For all other compounds, whose release seemed to be somehow influenced by mycorrhization, the overall tendency was a reduced emission of the respective compounds in reaction to herbivory by mycorrhized plants. The compounds affected were α-gurjunene, α-himachalene, *E*-nerolidol, dihydroactinidiolide, and 3,5-dimethoxytoluene (representing this pattern, the relative abundance of α-gurjunene emitted is shown in Figure 4-2 c). Only n-tridecane showed a slight trend to elevated emission after herbivore attack in mycorrhized plants.

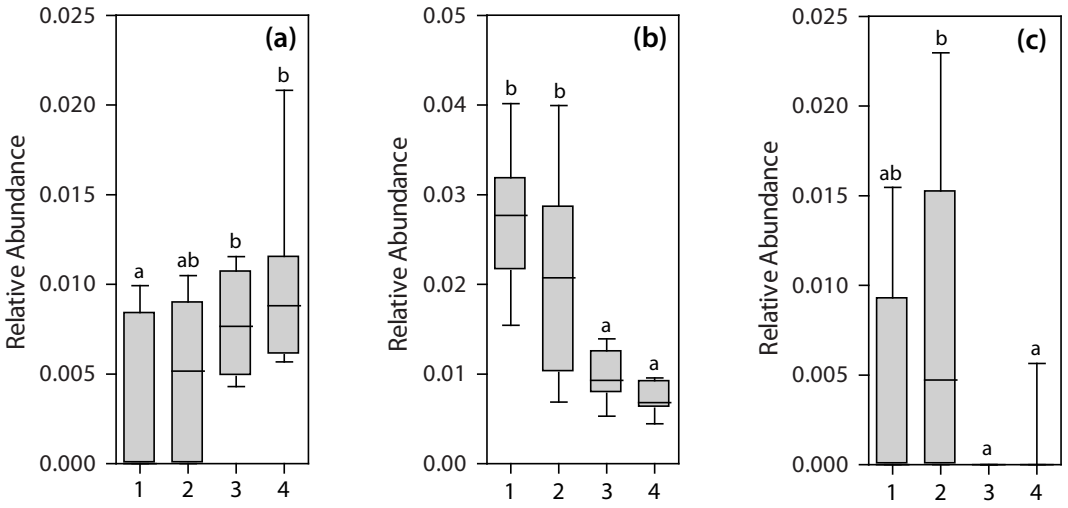


Figure 4-2 Examples of volatile compounds emitted by *Medicago truncatula* cv. Jemalong A17, whose release seems to be influenced by mycorrhization. (a) unidentified compound, RI 1038; (b) unidentified compound, RI 1112; (c) α-gurjunene. 1, control, unwounded; 2, control, wounded by *Spodoptera* sp.; 3, mycorrhized, unwounded; 4, mycorrhized, wounded by *Spodoptera* sp. n = 9 - 10. Small letters indicate significant differences as determined by ANOVA and Newman-Keuls *post hoc* test.

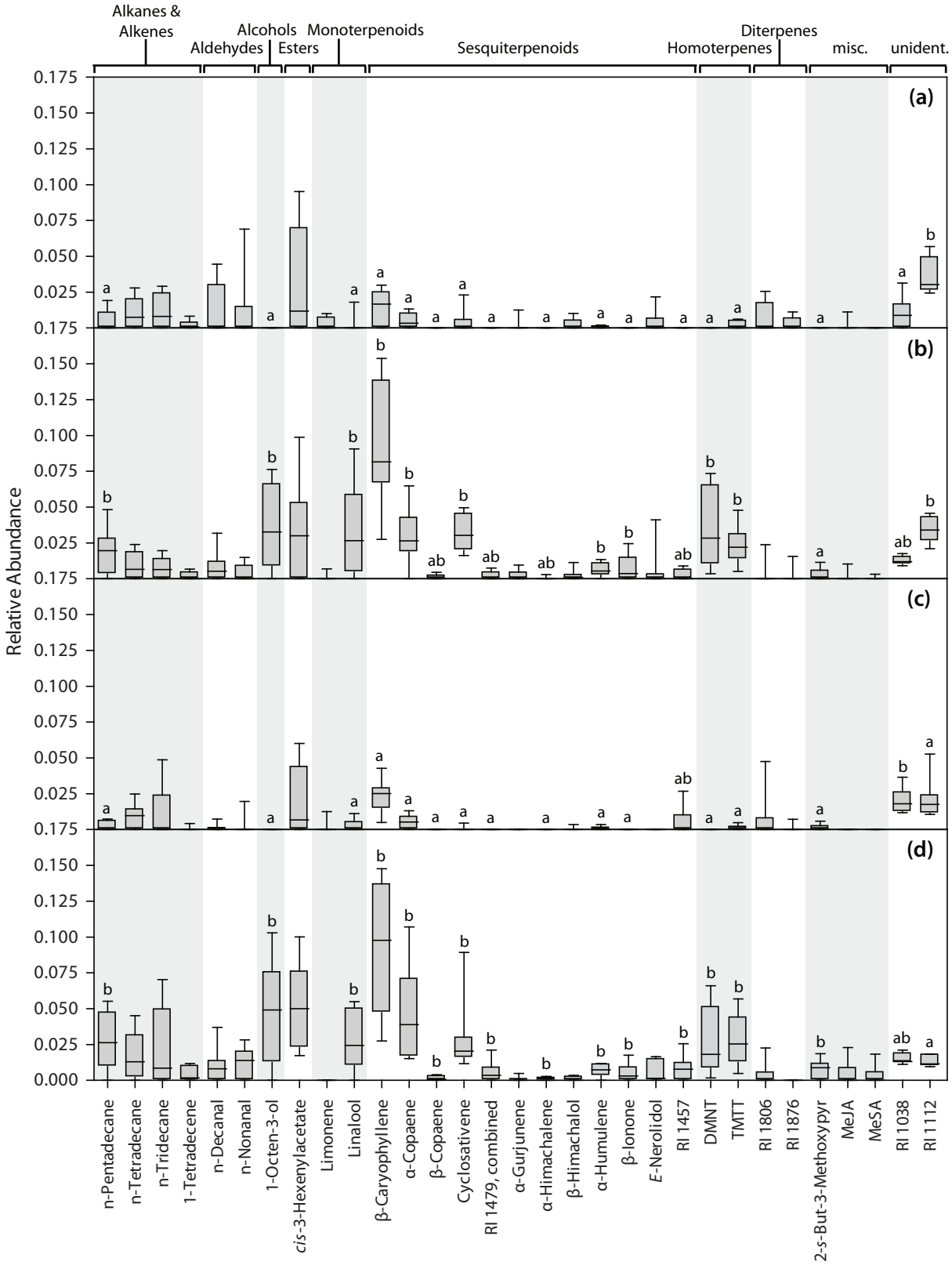


Figure 4-3 Box plots representing relative quantification of volatiles emitted by non-mycorrhized and mycorrhized *Medicago truncatula* plants (mixed cultivars) in reaction to herbivory. (a) control (non-mycorrhized), unwounded; (b) control, damaged by *Spodoptera* sp.; (c) mycorrhized, unwounded; (d) mycorrhized, wounded by *Spodoptera* sp. n = 7 - 10. Small letters indicate significant differences between the different treatments as determined by ANOVA and Newman-Keuls *post hoc* test. Abbreviations: unidentified compounds are given by their respective retention index; RI 1479 combined, combined quantification of germacrene D and γ-muurolene; DMNT, 4,8-dimethylnona-1,3,7-triene; TMTT, (3*E*,7*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene; 2-*s*-But-3-methoxypyr, 2-*sec*-butyl-3-methoxypyrazine; MeJA, methyl jasmonate; MeSA, methyl salicylate.

Surprisingly, some substances detected in these experiments have not been found before after any induction treatment in this cultivar, including caterpillar feeding. For example, 7α*H*-silphiperfol-5-ene and 7β*H*-silphiperfol-5-ene have not been found in any other set of experiments. The same holds true for both the unidentified compounds, RI 1038 and RI 1112. The latter case is particularly puzzling as this substance was found in rather high abundance in undamaged as well as damaged non-mycorrhized plants.

4.2.2 VOC emission in a cultivar mixture of *Medicago truncatula*

Besides *M. truncatula* cv. Jemalong A17 a mixture of cultivars (including cv. Jemalong but without definition of the line, and cv. Parragio), was used for experiments paralleling those described above (Figure 4-3).

First of all it was striking that though the differences between those cultivars cannot be assumed to be very high in terms of VOC emission patterns, some clear discrepancies could be observed regardless of the symbiotic state of the plants (cf. Figures 4-1 and 4-3). These variations, however, did not affect the main components that were found to be induced by herbivory. But while no monoterpenoids were found to be induced in the cultivar Jemalong A17 at all, a considerable amount of linalool and low levels of limonene were emitted by the other cultivar. Furthermore, the blend of sesquiterpenoids detected differed to a certain extent. Moreover, in Jemalong A17 all compounds that seemed to be somehow affected by mycorrhization were released to a lower extent after caterpillar feeding, whereas it was the other way round in this case. The emission of several sesquiterpenoids, such as β-copaene, α-himachalene, RI 1457, germacrene D and γ-muurolene (not thoroughly separable under the GC-conditions used, thus quantified together as RI 1479), and 2-*sec*-butyl-3-methoxypyrazine (Figure 4-4 c) turned out to be induced more strongly in mycorrhized caterpillar-damaged plants than in non-mycorrhized ones. Still, one clear parallel regarding the effect of mycorrhization was found in the emission patterns of RI 1038 and RI 1112 (Figure 4-4 a, b). Those substances showed more or less the same increase or reduction, respectively, in reaction to mycorrhization as observed with the cultivar Jemalong A17.

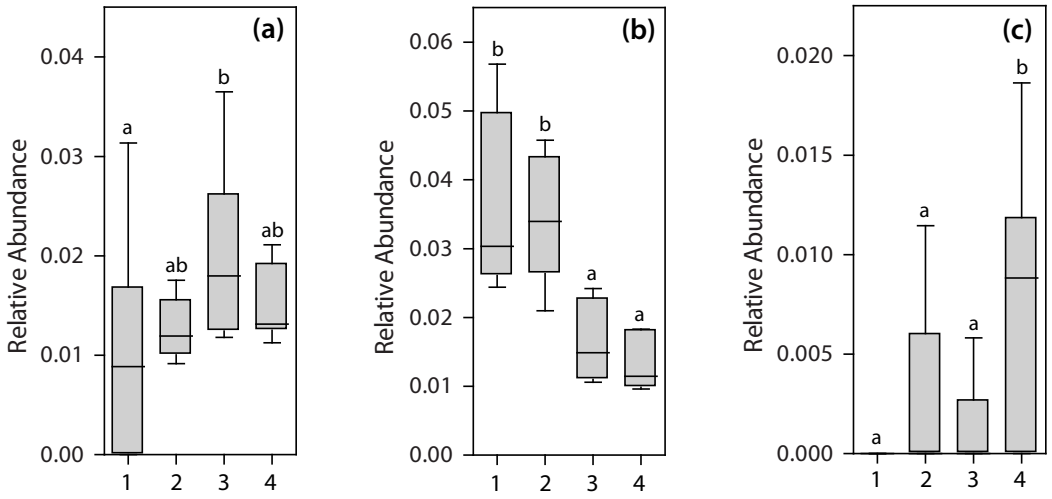


Figure 4-4 Examples of volatile compounds emitted by *Medicago truncatula* (mixed cultivars), whose release seems to be influenced by mycorrhization. (a) unidentified compound, RI 1038; (b) unidentified compound, RI 1112; (c) 2-*sec*-butyl-3-methoxypyrazine 1, control, unwounded; 2, control, wounded by *Spodoptera* sp.; 3, mycorrhized, unwounded; 4, mycorrhized, wounded by *Spodoptera* sp. n = 7 - 10. Small letters indicate significant differences as determined by ANOVA and Newman-Keuls *post hoc* test.

The characteristics were, however, not very pronounced, as drastic deviations occurred in some samples (excluded from statistical analysis).

4.2.3 Visualisation and classification of volatile patterns

As introduced in Chapter 3, multivariate patterns of VOC emission were again visualised by means of Non-metric Multidimensional Scaling (NMDS). In the first place, emission patterns were compared within each batch of plants used (Figures 4-5 & 4-6). Basically, using this approach it was shown that herbivore-induced volatile blends are clearly distinct from those measured from undamaged plants. The changes due to colonisation by AM fungi, however, are too subtle to be perceivable in this kind of data representation. Though there seems to be some variation, particularly in the mixed cultivars (Figure 4-6), the overall pattern is essentially the same. In view of the obvious differences between the VOC blends emitted by the different cultivars, it was interesting to compare the influence of the plants' genetic background (Figures 4-7 & 4-8). Regarding undamaged plants of both plant batches, including mycorrhized and non-mycorrhized plants, no compelling differences in the basal level of VOC emission could be observed (Figure 4-7).

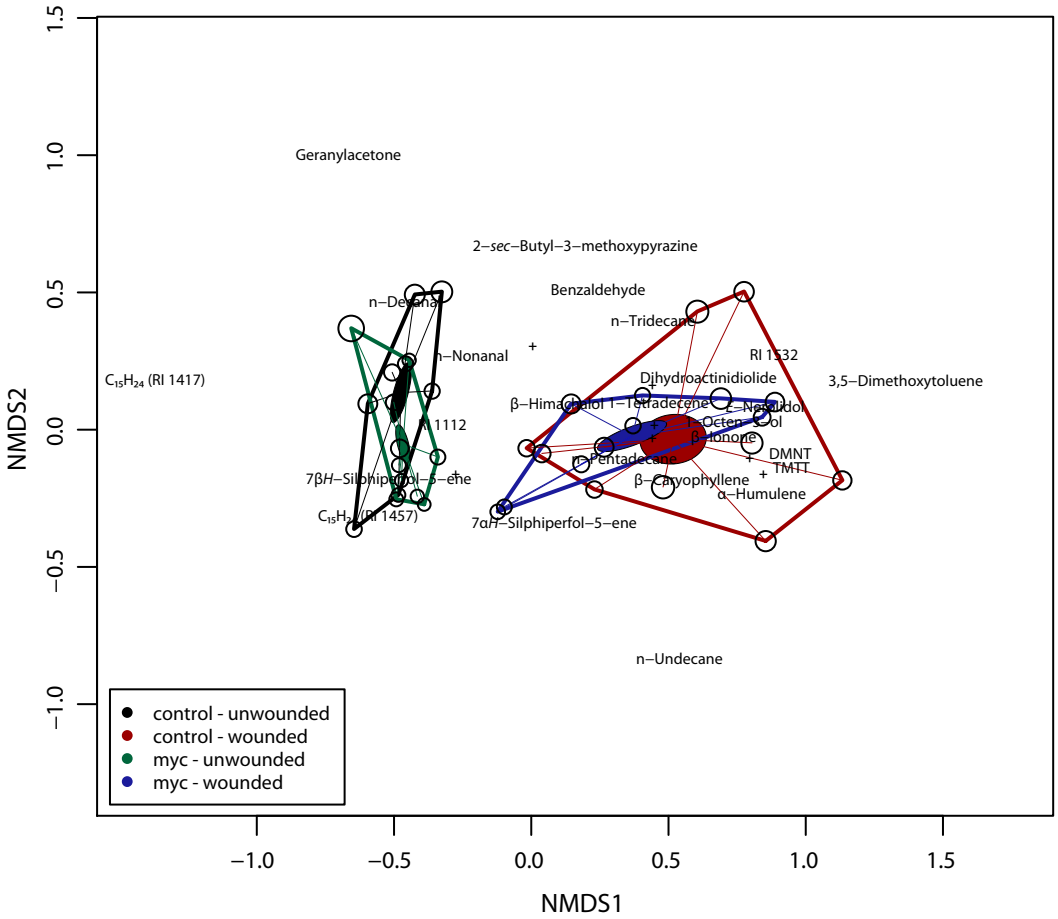


Figure 4-5 Non-metric multidimensional scaling (NMDS) plots of VOC blends emitted by *Medicago truncatula* cv. Jemalong A17 in response to mycorrhization and feeding by *Spodoptera* sp. using square root transformed data and Euclidean distance as a dissimilarity measure. Stress, 9.12. Open circles indicate the relative location of single volatile samples in ordination space. The size of the circles represents the goodness of the fit into the model for each particular sample. The centroids are given by the intersection of the spiderweb-like lines within each treatment group; the groups' standard errors are given by filled ellipses. The size of the ellipse can be interpreted as a measure of consistency for the respective group. The relative distances between any sample or substance shown represent similarities, and positive (low distances) or negative (high distances) correlations, respectively. Variables are plotted according to their weighted averages.

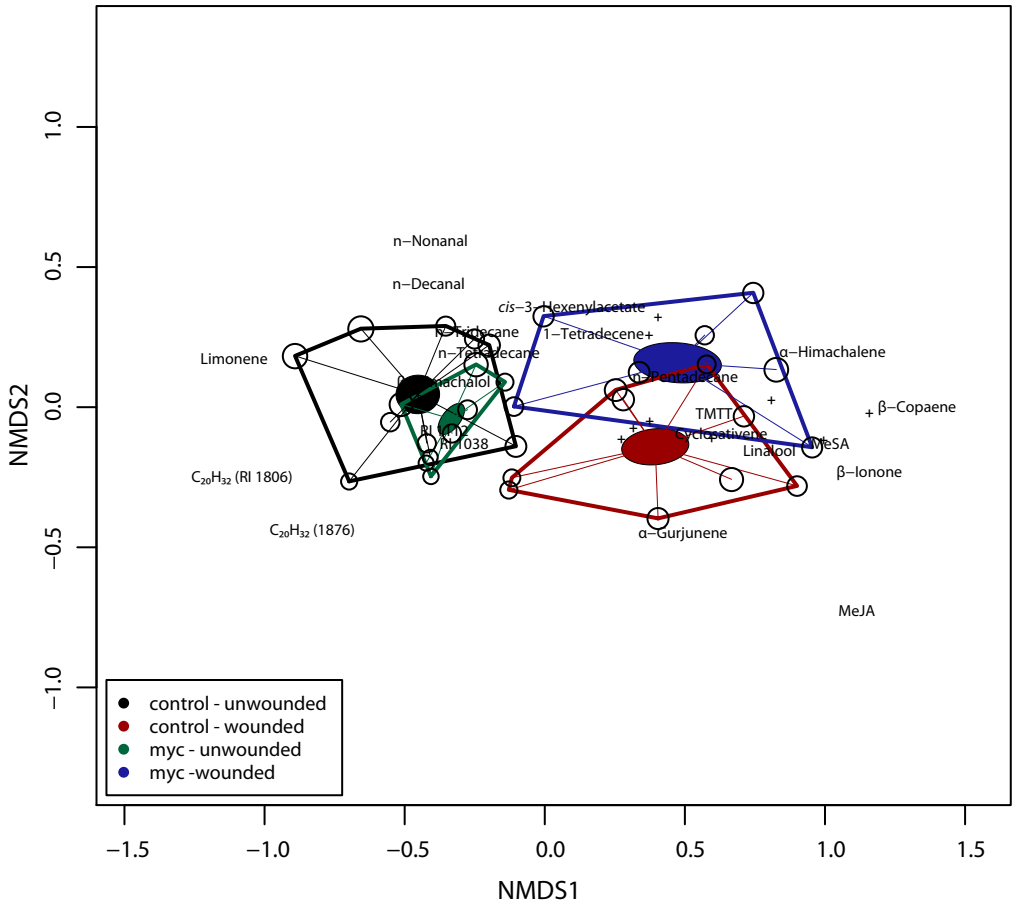


Figure 4-6 Non-metric multidimensional scaling (NMDS) plots of VOC blends emitted by *Medicago truncatula* (mixed cultivars) in response to mycorrhization and feeding by *Spodoptera* sp. using square root transformed data and Euclidean distance as a dissimilarity measure. Stress, 10.94. Open circles indicate the relative location of single volatile samples in ordination space. The size of the circles represents the goodness of the fit into the model for each particular sample. The centroids are given by the intersection of the spiderweb-like lines within each treatment group; the groups' standard errors are given by filled ellipses. The size of the ellipse can be interpreted as a measure of consistency for the respective group. The relative distances between any sample or substance shown represent similarities, and positive (low distances) or negative (high distances) correlations, respectively. Variables are plotted according to their weighted averages.

The non-mycorrhized control group of the mixed cultivars spans the entire range of variation. This is not very surprising considering that the cultivar Jemalong was also present in the mixture; the high variability may thus be due to the heterogenous genetic background of the plants tested. But it is remarkable that mycorrhized plants of the cultivar mixture somehow differed from cv. Jemalong. This could have been due to some peculiar effect of mycorrhization in this instance or by chance only plants of cv. Parragio were picked for those experiments. But when plants were damaged by lepidopteran larvae, the induced VOC blends differed drastically (Figure 4-8). The differences between mycorrhized and non-mycorrhized plants proved to be minor, with some slight changes in cv. Jemalong A17; the different cultivars, however, could be easily separated by means of the induced VOC blends. Still, no distinction between mycorrhized and non-mycorrhized plants could be depicted. In the results presented above, NMDS was calculated using Euclidean distance as a dissimilarity measure. Binomial NMDS led to the same results, as there were no qualitative differences in the VOC blends of mycorrhized and non-mycorrhized plants (data not shown; cf. Chapter 3). Nevertheless, from the basic data it was obvious that at least a slight influence is perceivable in the quantitative aspect. That these discrepancies could not be depicted using NMDS is not surprising. This is an exploratory method that aims only to depict

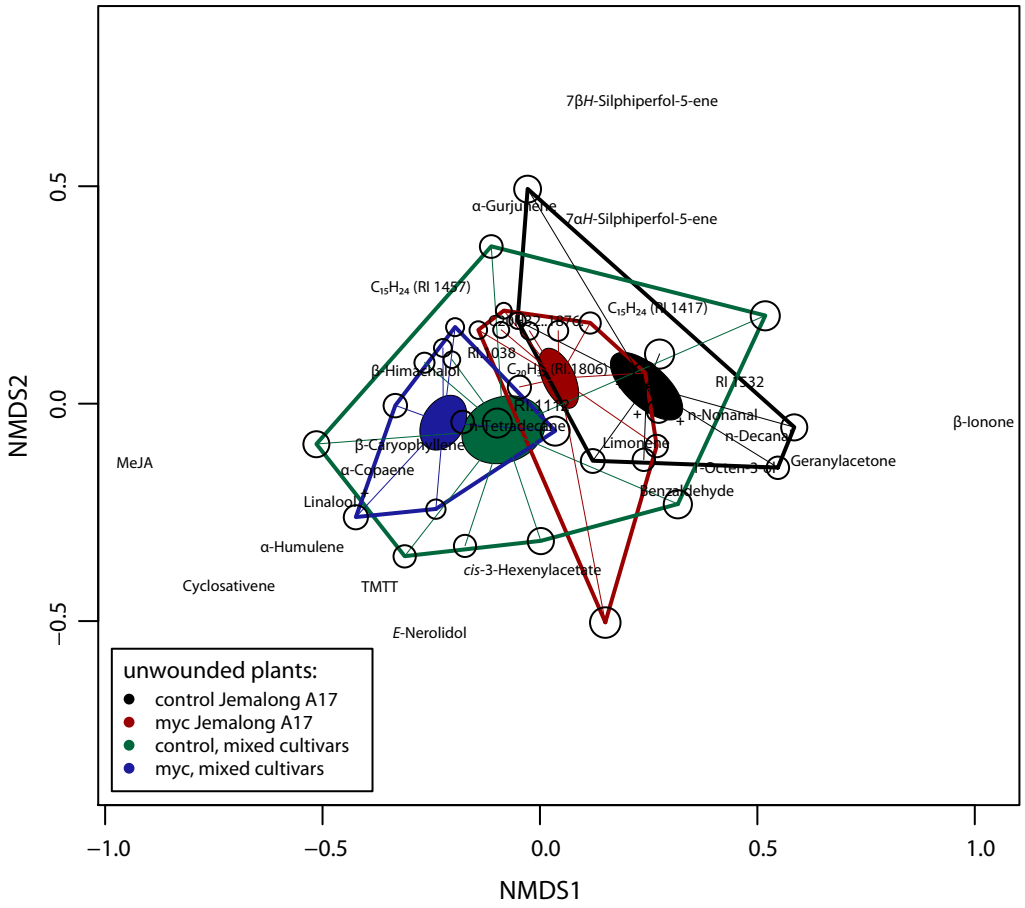


Figure 4-7 Non-metric multidimensional scaling (NMDS) plots of VOC blends emitted by different *Medicago truncatula* cultivars in response to mycorrhization using square root transformed data and Euclidean distance as a dissimilarity measure. Stress, 15.54. Open circles indicate the relative location of single volatile samples in ordination space. The size of the circles represents the goodness of the fit into the model for each particular sample. The centroids are given by the intersection of the spiderweb-like lines within each treatment group; the groups' standard errors are given by filled ellipses. The size of the ellipse can be interpreted as a measure of consistency for the respective group. The relative distances between any sample or substance shown represent similarities, and positive (low distances) or negative (high distances) correlations, respectively. Variables are plotted according to their weighted averages.

complex patterns in a low-dimensional space, so that the *interitem* distances between any points represent the original similarities (or dissimilarities) observed as well as possible. NMDS is mainly a way to visualise multivariate patterns and does not search for any disparities. Thus, in order pinpoint those dissimilarities, linear discriminant analysis (LDA) was tested for its applicability to this problem. In contrast to NMDS, the underlying algorithm of LDA tries to produce a low-dimensional representation of the data that yields maximal separation of the given groups; thus, it serves to describe differences between certain groups (McLachlan, 1992; Venables & Ripley, 2002). The first attempts to use this method, however, were not very successful due to the high multicollinearity of the data sets (for a glossary of statistical terms, see Appendix III). This refers to the fact that a range of variables, i.e. compounds detected, carry the same information, as for example several sesquiterpenes show highly similar emission patterns in response to herbivory. This problem can be overcome by eliminating collinear variables, for instance using stepwise classification. It is important to note that with this approach, the biological relevance of complete VOC blends remains in the background; the resulting rules of classification are quite artificial and to some extent replaceable, as only parts of the data are considered. In fact, several models with approximately the same level of significance have been calculated; in the following only two

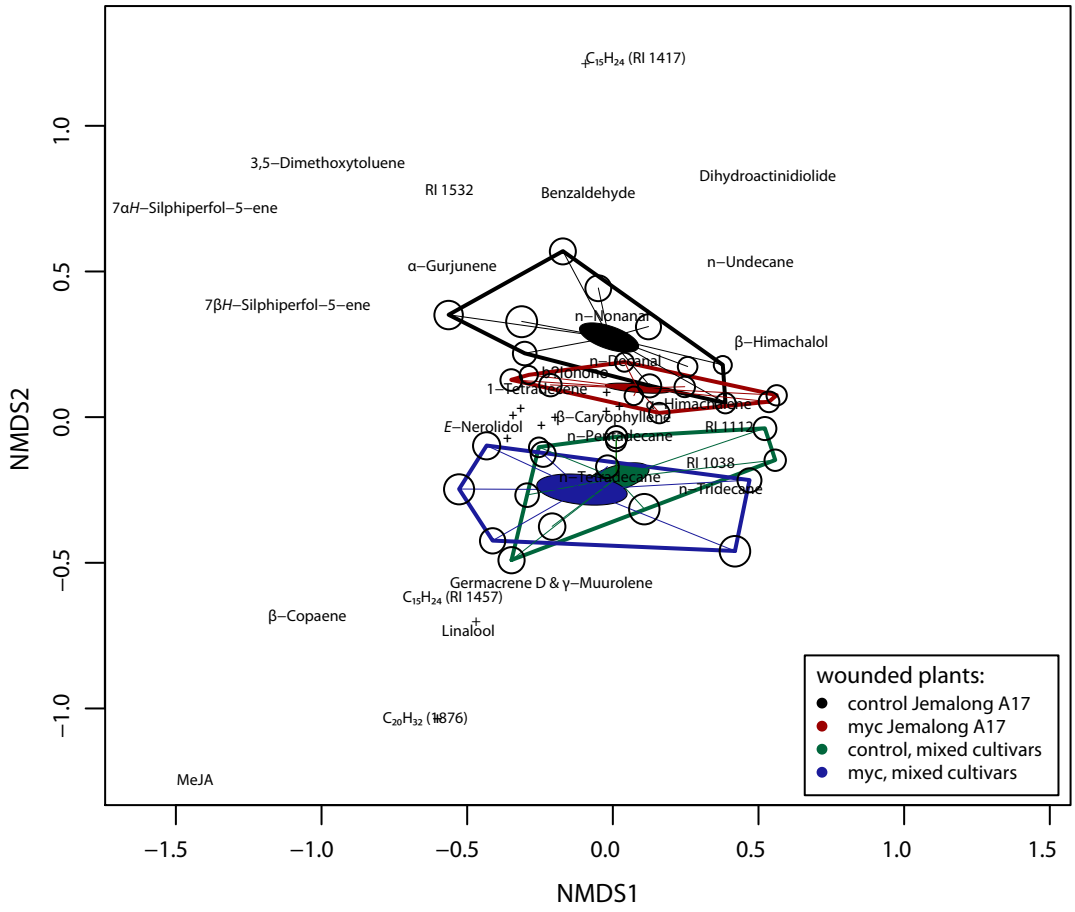


Figure 4-8 Non-metric multidimensional scaling (NMDS) plots of VOC blends emitted by different *Medicago truncatula* cultivars in response to feeding by *Spodoptera* sp. and mycorrhization using square root transfomed data and Euclidean distance as a dissimilarity measure. Stress, 16.20. Open circles indicate the relative location of single volatile samples in ordination space. The size of the circles represents the goodness of the fit into the model for each particular sample. The centroids are given by the intersection of the spiderweb-like lines within each treatment group; the groups' standard errors are given by filled ellipses. The size of the ellipse can be interpreted as a measure of consistency for the respective group. The relative distances between any sample or substance shown represent similarities, and positive (low distances) or negative (high distances) correlations, respectively. Variables are plotted according to their weighted averages.

examples, one for each batch of plants, will be presented; in both cases, selection of variables was performed using the Wilk's lambda as criterion.

For the cultivar Jemalong, 10 out of 32 compounds were selected; namely, α-copaene, RI 1112, α-gurjunene, RI 1457, 7β*H*-silphiperfol-5-ene, geranylacetone, 7α*H*-silphiperfol-5-ene, RI 1038, n-tridecane, and n-pentadecane. Using those variables as predictors, quite distinct grouping of all treatment classes can be achieved (Figure 4-9). The resulting model was statistically significant with a Wilk's lambda of 0.011, and an overall *p*-value < 0.0001 (8.42^{-14}). Evaluation of the model using cross-validation revealed an error rate of 11.41 % (Figure 4-9). The proportion of classification, i.e. which samples were allocated to which group during cross-validation, can be read from a confusion matrix (Figure 4-9). All groups could be predicted with reasonable success; errors occurred predominantly in the classification of undamaged, non-mycorrhized plants.

In the case of the mixed cultivar, classification could be achieved with only 6 out of 31 compounds (Figure 4-10). The VOCs selected were 2-*sec*-butyl-3-methoxypyrazine, β-caryophyllene, RI 1112, RI 1038, MeSA, and 1-tetradecene. To test the level of significance of the model, the same analyses as those used for cv. Jemalong A17 were performed. With a value for Wilk's lambda of 0.023 and an overall *p*-value < 0.0001 (2.19^{-13}) this model also prove to be significant. The error rate, as determined

true \ predicted	control - unwounded	control - wounded	myc - unwounded	myc - wounded
control - unwounded	44.2	0.9	34.3	0.5
control - wounded	0.3	98.1	0.0	1.6
myc - unwounded	0.1	0.0	99.9	0.0
myc - wounded	0.0	2.7	1.7	85.6

error rate = 11.41 %

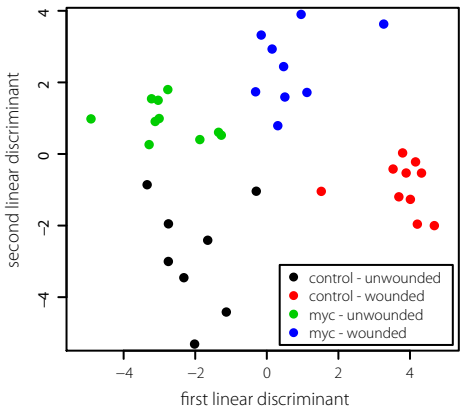


Figure 4-9 Results of Linear Discriminant Analysis (LDA) for volatile blends emitted by *Medicago truncatula* cv. Jemalong A17 in response to mycorrhization and feeding by *Spodoptera* sp. The table represents the proportions of classification as determined by cross-validation (left). For visual inspection of the grouping, the first two linear discriminants were plotted (right).

by cross-validation, was 11.36 % (Figure 4-10). Misclassification occurred mainly between non-mycorrhized unwounded and wounded plants, and between unwounded mycorrhized and non-mycorrhized plants (Figure 4-10).

Taken together, these results indicate that mycorrhizal fungi do influence herbivore-induced VOC emission, though not very conspicuously. Still, these slight differences are sufficient to create classification rules, and to distinguish plants with distinct physiological status. Thus, in this instance, VOC blends can indeed be used as diagnostic criteria. Strikingly, only a small proportion of the compounds detected was sufficient to build up rules of classification.

4.3 Discussion

The results presented in this section again showed that herbivory induces the emission of VOCs in *Medicago truncatula*. It is noteworthy, however, that the volatile blends in this second row of experiments were not identical to those formerly detected (cf. Chapter 2). This could, for example, be due to minor variations in the growth conditions of the plants (cf. Material and Methods). Still, the high abundance of some of these compounds was rather surprising; in the case of trace

true \ predicted	control - unwounded	control - wounded	myc - unwounded	myc - wounded
control - unwounded	80.0	1.2	18.8	0.0
control - wounded	15.8	74.0	0.2	0.0
myc - unwounded	0.5	0.0	68.9	0.6
myc - wounded	0.0	0.1	0.3	69.6

error rate = 11.36 %

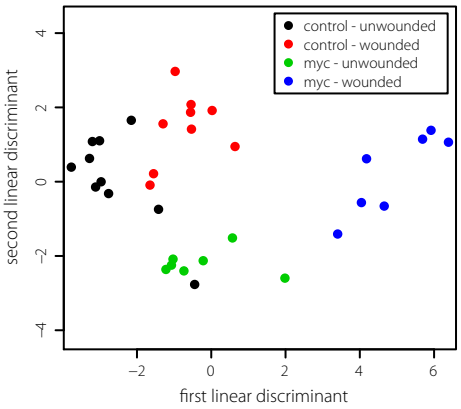


Figure 4-10 Results of Linear Discriminant Analysis (LDA) for volatile blends emitted by *Medicago truncatula* (mixed cultivars) in response to mycorrhization and feeding by *Spodoptera* sp. The table represents the proportions of classification as determined by cross-validation (left). For visual inspection of the grouping, the first two linear discriminants were plotted (right).

components, it could easily be argued that those were overlooked in previous experiments. Furthermore, some striking differences between the two cultivars used were detected. The VOCs released constitutively by healthy plants were more or less the same, but the blends emitted in response to herbivory were clearly distinct in the different batches of plants used. Although the main herbivore-inducible sesquiterpenoids were emitted in a similar manner, several compounds could be detected that were present only in one of the cultivars. It was particularly striking that no compounds derived from the MEP pathway could be found in cv. Jemalong A17, whereas both monoterpenoids and diterpenoids were detected in VOC blends emitted by the mixed cultivars.

Overall, the effects of mycorrhization on VOC emission were not very prominent. No qualitative changes could be detected, though a certain divergence in the quantitative aspect was recorded. Although in the cultivar Jemalong A17 some of the herbivore-induced VOCs were reduced in mycorrhized plants, plants of the mixed cultivar tended to emit higher amounts of certain VOCs when mycorrhized. In this context it is remarkable to find in cultivar descriptions that cv. Paraggio is generally more resistant to all kinds of stresses than is cv. Jemalong (Nair & Howie, 2006). For example, cv. Jemalong is susceptible to different species of aphids and boron, whereas Paraggio resists or at least moderately resists all those stresses. One can assume that in nature plants are predominantly mycorrhized; thus, that one genotype reduces its indirect defence under these circumstances whereas the other one exhibits increased VOC emission may be important. Whether this trend extends to other induced defences as well would be interesting to know. In addition, the qualitative differences in the composition of defensive compounds might also influence the resistance to diverse stressors.

However, the results are in line with previous findings, namely that mycorrhization influences the content of secondary metabolites in aboveground plant parts, and that these changes are somehow dependent on the genetic background of the plant (Khaosaad *et al.*, 2006). The latter furthermore gives a possible reason for the fact that the observed effects on herbivores vary so much. The fungal and herbivore species involved in the interaction have been shown to greatly influence the outcome (Goverde *et al.*, 2000; Gange *et al.*, 2002; Gange *et al.*, 2003). To what degree the plant species control these interplays is, however, only poorly understood.

In the quest for possible reasons for the observed effects, two modes of explanation might be taken into consideration: AM fungi basically have to evade the plant's defence responses in order to successfully colonise the roots. Thus, as suggested for the interaction with rhizobia, it is often assumed that these symbionts are able to suppress the plant's defence mechanisms, which could in part explain why induced defences are reduced in cv. Jemalong A17. This is, however, not very conclusive, as on the other hand it is considered as a basic fact that mycorrhization increases the plant's resistance to diverse stressors. A systemic suppression of induced defences would therefore be quite counterproductive. However, in the case of the mixed cultivars, the slightly enhanced emission of certain VOCs may be a symptom of increased resistance or priming by AM fungi.

Anyway, changes in volatile patterns, be they increased or decreased emissions, are plausible causes for changed behaviour of plant mutualists. Most of the changes detected were, however, so small that their influence on such interactions seems improbable. Only the amounts of the two unidentified compounds (RI 1112 and RI 1038) varied to a large extent, and they were present in an abundance that may lend support to their putative physiological and ecological roles. In order to carry out any investigation on that topic, the identification of those compounds is mandatory.

But our knowledge about the importance of trace compounds is limited. Chemical detection limits do not necessarily transport crucial information about biological importance, as they do not take into account the sensitivity of biological perception systems.

Although the differences observed in volatile emission are only marginal, classification is clearly possible. This theoretical consideration is certainly of some interest, demonstrating that the VOCs emitted by *Medicago truncatula* are specific enough to allow discrimination of different stimuli, even if the influences seem to be only minor. With regard to practical applicability, the problem gets more complicated. All experiments were conducted under highly controlled conditions. As only VOCs of one plant at a time were analysed, no problems with normalisation were encountered. Because the culture conditions were kept as constant as possible, variation due to the abiotic environment can assumed to be negligible. Also, care was taken to prevent any infection or infestation prior to the experiments, so that life history traits would not influence emission patterns in any way. Finally, differences in the genetic background were comparatively low. In short, any influence that could alter the plants' emission patterns was reduced as much as possible. It is easily imaginable that the parameters listed above, and probably many more, severely impede successful discrimination of VOC blends in the natural environment. Regarding the statistical methods used, there is a clear need for further tests. Though the results gained with LDA are quite satisfactory, other methods that are more robust with regard to multicollinearity, and do not rely on linear correlations (e.g. neural networks, learning vector machines, etc.) could be expected to perform better. Indeed, several methods have been compared for other problems, such as classification of aromas, that bear some similarity from the statistical point of view (Baroni *et al.*, 2006; González-Arjona *et al.*, 2006).

Finally, the commonplace notion that insight into multiple interactions is substantially hindered by the fact that every organism involved has a drastic influence on the outcome seems to be the conclusion. Thus, general effects are very likely to be difficult to find, as the data available to date indicate that even the slightest variation in one of the partners of any interaction considerably changes the overall consequences.

Footnotes

[1] Unidentified compounds were all classified by their linear retention index on an EC-5 column under temperature programmed conditions.

5 Profiling volatile emission by *Medicago truncatula*

5.1 Introduction

In previous sections of this thesis, emission of volatile organic compounds (VOCs) has received a sizeable share of attention. It has already been demonstrated that *Medicago truncatula* Gaertn. releases VOCs in response to diverse stimuli, and that the blends detected in part exhibit considerable specificity. Hence, adding to the profile of volatiles emitted by this species by testing the release of VOCs in response to varied modes of chemical elicitation seemed appropriate. Therefore, different substances that had tested positively for their ability to induce volatile emission in other species were examined for their capacity to do so in *M. truncatula*. The compounds used as elicitors of VOC emission were alamethicin (ALA), copper sulphate, coronalon, jasmonic acid (JA), and acetylsalicylic acid (acetyl-SA).

Alamethicin is a mixture of antibiotic peptides originally isolated from cultures of the fungus *Trichoderma viride*. It exerts its effects via voltage-dependent insertion into membranes and formation of oligomeric pores of varying sizes (Duclohier & Wróblewski, 2001). Regarding its antibiotic activity, like other antimicrobial peptides with high α-aminoisobutyric acid content, it is assumed to lead to membrane permeabilisation and consequently to cell lysis (Duclohier & Wróblewski, 2001). ALA has also been shown to act on plants; it induces tendril coiling in several plant species and leads to induced volatile emission (Engelberth *et al.*, 2001). The latter has been studied in more detail in lima bean (*Phaseolus lunatus* L.); there, it was striking that the diversity of VOCs emitted in response to ALA treatment was reduced compared to the blends detected in reaction to herbivory or JA treatment. Only the two homoterpenes 4,8-dimethylnona-1,3,7-triene (DMNT) and 4,8,11-trimethyltrideca-1,3,7,11-tetraene (TMTT), and methyl salicylate (MeSA) were emitted. A finely tuned interplay of the octadecanoid pathway and SA seemed to mediate the highly specific response in terms of VOC emission. While JA was only transiently induced, SA accumulation correlated with enhanced MeSA emission. Also, pre-treatment with acetyl-SA abolished JA accumulation but did not influence the increase of OPDA levels (Engelberth, 2000; Engelberth *et al.*, 2001).

Heavy metal ions represent one form of abiotic stress plants may encounter in nature. Induction of certain secondary metabolites in reaction to heavy metal stress has been observed in several plant species (summarised by Mithöfer *et al.*, 2004). Moreover, though emission of VOCs is usually associated with defence against herbivores or at most with defences against biotic stresses, it has been demonstrated that certain heavy metal ions also induce volatile release in lima bean (Schulze, 2005). Interestingly, the VOC blends induced by all of the active metals closely resembled those detected after ALA treatment. Again, accumulation of endogenous SA was detected in connection with increased volatile emission. In this case, however, JA remained at the resting level and only OPDA increased. This would be consistent with the notion that SA inhibits the JA pathway downstream of OPDA biosynthesis (Schulze, 2005) and could contribute to the explanation of similarities reported in VOC emission in response to ALA and heavy metals. The most potent elicitor for VOC emission in lima bean, copper sulphate, was chosen to serve as an elicitor in this study.

Coronalon is a structural mimic of coronatine (Figure 5-1, a, b). This phytotoxin is produced by several pathovars of the bacterial phytopathogen *Pseudomonas syringae* van Hall and has been shown to mimic the effect of octadecanoid signal compounds in plants to some extent, without inducing endogenous accumulation of JA (Weiler *et al.*, 1994). Although coronatine is a profitable tool for investigating plants' stress responses, its synthesis is quite complicated due to its complex stereochemistry (Schüler *et al.*, 2004). In comparison, indanoyl isoleucine conjugates are synthesised much more easily and share some of the eliciting properties of coronatine. One of the most potent analogues found is the 6-ethyl indanoyl isoleucine conjugate coronalon (2-[(6-ethyl-1-oxo-indane-4-carbonyl)-amino]-3-methyl-pentanoic acid methyl ester). It induces VOC release and tendril coiling, promotes fruit and leaf drop, and stimulates the synthesis and accumulation of secondary metabolites in several plant species (Schüler *et al.*, 2001; Schüler *et al.*, 2004; Mithöfer *et al.*, 2005), and thus can be used to mimic effects of coronatine in bioassays.

Although SA (Figure 5-1, d) is generally thought to be uninvolved in the induction of secondary metabolism *in planta*, it has been demonstrated in several experimental systems that exogenous application of this phytohormone (or derivatives such as acetyl-SA or MeSA) can lead to the induced accumulation of certain defensive compounds (Zhao *et al.*, 2005). Often acetyl-SA has been applied, which seems to rapidly decompose to SA in aqueous solution and basically induces the same effects (Raskin, 1992). As VOC emission is viewed mainly as a response to herbivory, it is interesting to note that herbivore feeding, particularly feeding by sucking insects or cell content feeders, also increases endogenous SA levels (cf. Chapter 2; Van Poecke & Dicke, 2004); Moreover, the combined application of JA and MeSA yields the emission of different volatile blends compared to the application of JA alone (Van Poecke & Dicke, 2004). Finally, SA seems to play a role in the regulation of VOC emission in response to ALA and heavy metal ions (see above).

In contrast to SA, JA (Figure 5-1, c) ranges amongst the usual suspects when it comes to naming the compound responsible for mediating VOC emission. It has been shown in numerous studies that exogenous application of JA up-regulates secondary metabolism (including VOC emission), and that VOC emission correlates with an increase of endogenous JA levels (Van Poecke & Dicke, 2004; Zhao *et al.*, 2005). Furthermore, the VOC blends emitted in reaction to JA largely resemble those induced by herbivory (Boland *et al.*, 1995) and are able to attract carnivores to the damaged plant, though not as efficiently as herbivore-induced volatiles (Dicke *et al.*, 1999).

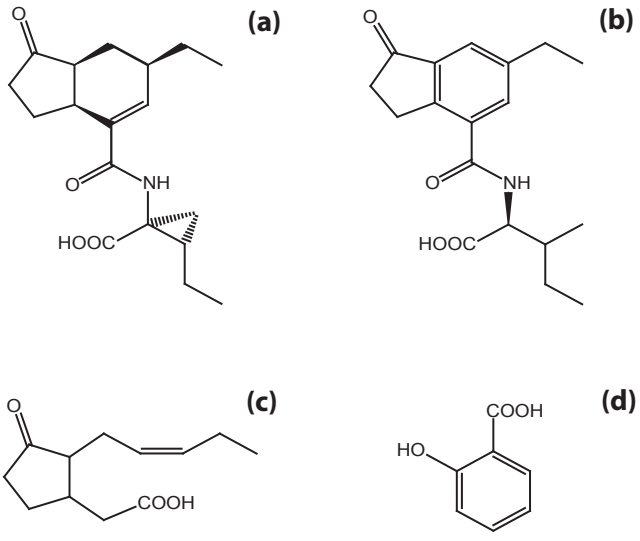
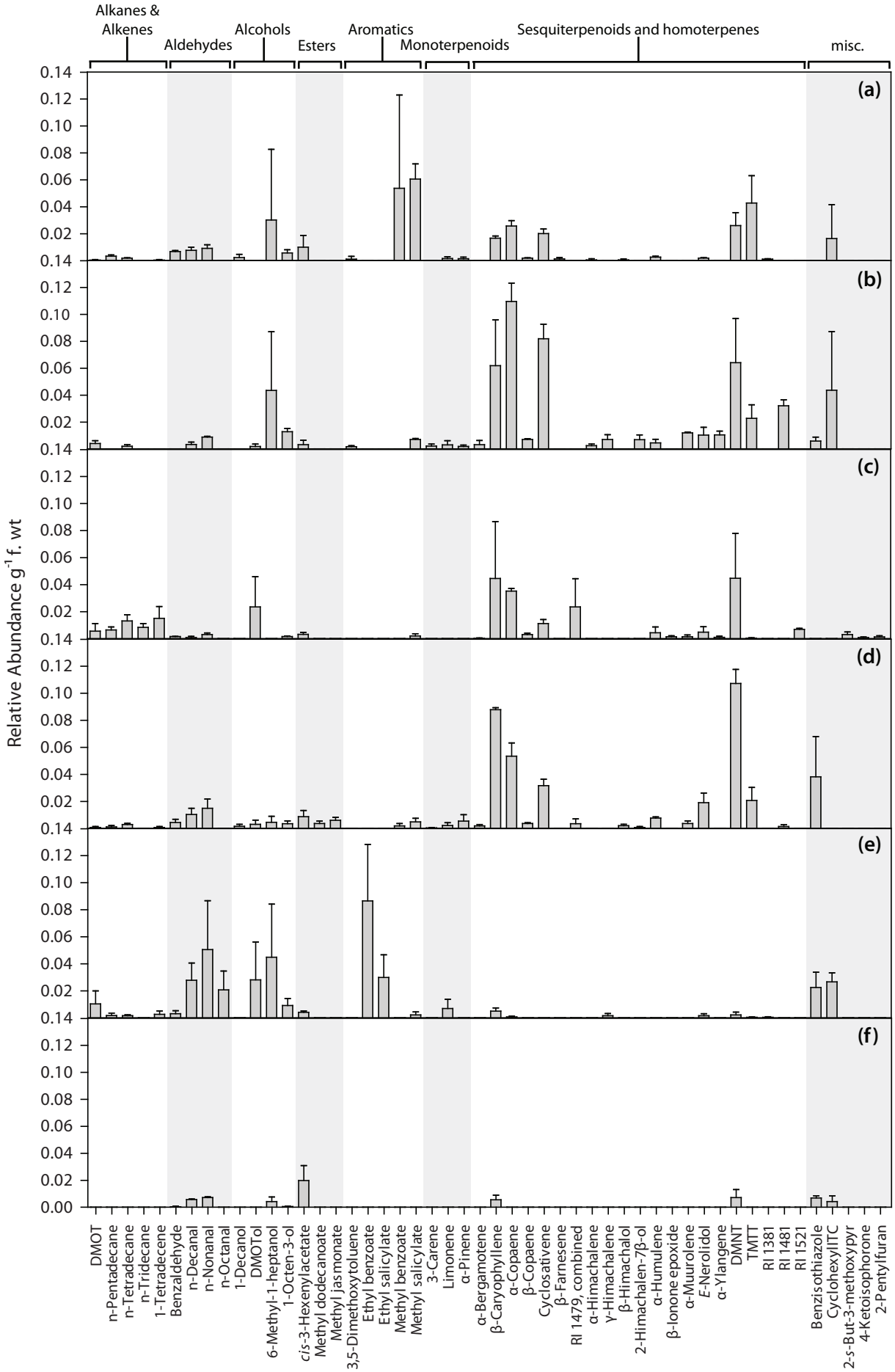


Figure 5-1 Chemical structures of some elicitors. (a) Coronatine and (b) its structural analogue coronalon; (c) jasmonic acid; (d) salicylic acid.

Figure 5-2 (next page) Relative quantification of volatiles emitted by *Medicago truncatula* in response to chemical elicitation. (a) 10 µg ml^{-1} alamethicin; (b) 1 mM copper sulphate; (c) 100 µM coronalon; (d) 1 mM jasmonic acid; (e) 1 mM acetylsalicylic acid; (f) control. n = 3 for all treatments. Abbreviations: DMOT, 2,6-dimethyl-1,3,5,7-octatetraene; DMOTol, 2,6-dimethyl-3,5,7-octatrien-2-ol; CyclohexylITC, cyclohexyl-istothiocyanate; 2-s-But-3-methoxypyr, 2-sec-butyl-3-methoxypyrazine; RI 1479, combined, combined quantification of germacrene D and γ-muurolene. Unidentified sesquiterpenoids are indicated with their respective retention index.


Alkanes & Alkenes
Aldehydes
Alcohols
Esters
Aromatics
Monoterpenoids
Sesquiterpenoids and homoterpenes
misc.
(a)
(b)
(c)
(d)
(e)
(f)
Relative Abundance g-1 f. wt
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
DMOT
n-Pentadecane
n-Tetradecane
n-Tridecane
1-Tetradecene
Benzaldehyde
n-Decanal
n-Nonanal
n-Octanal
1-Decanol
DMOTol
6-Methyl-1-heptanol
1-Octen-3-ol
cis-3-Hexenylacetate
Methyl dodecanoate
Methyl jasmonate
3,5-Dimethoxytoluene
Ethyl benzoate
Ethyl salicylate
Methyl benzoate
Methyl salicylate
3-Carene
Limonene
α-Pinene
α-Bergamotene
β-Caryophyllene
α-Copaene
β-Copaene
Cyclosativene
β-Farnesene
RI 1479, combined
α-Himachalene
γ-Himachalene
β-Himachalol
2-Himachalen-7β-ol
α-Humulene
β-Ionone epoxide
α-Muurolene
E-Nerolidol
α-Ylangene
DMNT
TMTT
RI 1381
RI 1481
RI 1521
Benzisothiazole
Cyclohexylitc
2-s-But-3-methoxypyr
4-Ketoisophorone
2-Pentylfuran

Due to their prior characterisation with regard to induced VOC emission, and their rather distinct patterns of elicitation, the above-mentioned compounds were considered particularly suitable for a more detailed survey of VOCs emitted by *M. truncatula*. The biochemical potential and the putative specificity of VOCs emitted in response to different elicitors were especially interesting.

5.2 Results

All of the compounds tested induced some emission of VOCs (Figure 5-2). Though only three samples per treatment were tested, the effects were consistent. It is striking that every substance tested induced distinct VOC blends, which is already apparent if merely considering compound classes for comparison. Pronounced emission of alkanes and alkenes was induced only by elicitation with coronalon (Figure 5-2, c). Emission of aldehydes was most effectively caused by acetyl-SA (Figure 5-2, e), which altogether produced a VOC pattern obviously different from any other induction treatment. In particular, the highest amounts of aldehydes, alcohols, and aromatics were detected after induction with acetyl-SA. Another potent elicitor of release of

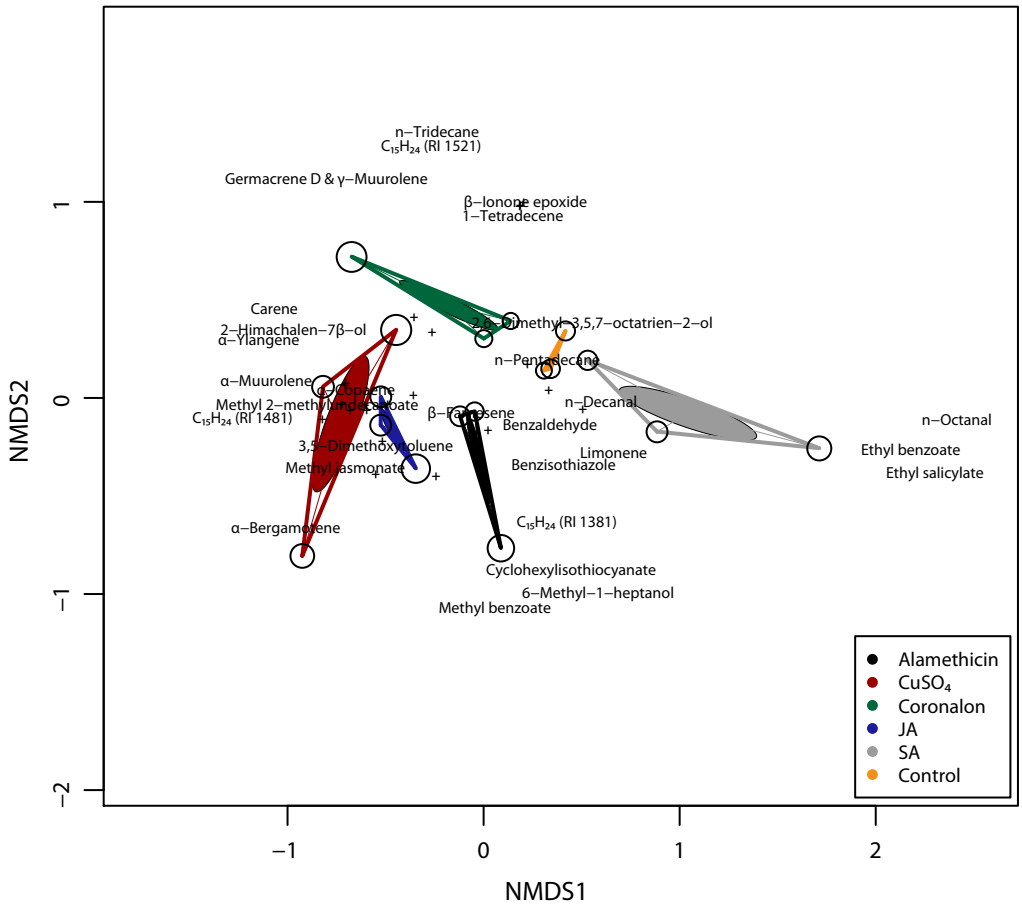


Figure 5-3 Non-metric multidimensional scaling (NMDS) plots of VOC blends emitted by *Medicago truncatula* in response to chemical elicitation using square root transformed data and Euclidean distance as a dissimilarity measure. Stress, 10.47. Open circles indicate the relative location of single volatile samples in ordination space. The size of the circles represents the goodness of the fit into the model for each particular sample. The centroids are given by the intersection of the spiderweb-like lines within each treatment group; the groups' standard errors are given by filled ellipses. The size of the ellipse can be interpreted as a measure of consistency for the respective group. The relative distances between any sample or substance shown represent similarities, and positive (low distances) or negative (high distances) correlations, respectively. Variables are plotted according to their weighted averages.

aromatic compounds was ALA (Figure 5-2, a), though again the evoked patterns were clearly distinct from those observed after acetyl-SA treatment. As for sesquiterpenoids (including homoterpenes), usually the substance class most abundantly present in VOC blends emitted by *M. truncatula*, some characteristics are notable. Some of the compounds were commonly encountered in volatile blends. β-caryophyllene, α-copaene, cyclosativene, and DMNT are emitted after most of the induction treatments tested; this also includes herbivory (cf. Chapter 2), glucan- and Nod-factor treatment (cf. Chapter 3). The only exceptions are elicitation with chitotetraose, acetyl-SA, and of course the control, although even there β-caryophyllene was detected in some cases in minor amounts. Moreover, the above-mentioned compounds were present in the highest quantities. Induction of other terpenoids varied both qualitatively and quantitatively with the respective treatment. It is remarkable, however, that copper sulphate, which induces a very narrow spectrum of volatiles in lima bean, elicited a considerable variety of sesquiterpenoids in *M. truncatula* compared to the other treatments. Looking more closely, it becomes evident that all of the elicitors induced some compounds specifically or at least with only minor overlaps (Table 5-1). Besides these clear qualitative differences, the overall patterns of VOCs induced were very distinctive for each individual treatment (Figure 5-3), as visualised by ordination using Non-metric Multidimensional Scaling (NMDS). Here, Euclidean distance was employed as a dissimilarity measure. Although only three measurements per treatment were available for comparison, separate groups were discernible. With a stress of 10.47 the fit of the model is fairly good. When using binomial NMDS to depict qualitative differences, as introduced in Chapter 3, the picture did not change much (data not shown). This indicates that the dissimilarity observed is mainly due to the qualitative differences, which were indeed very conspicuous.

Table 5-1 Qualitative comparison of volatiles emitted in reaction to different induction treatments.

	Alamethicin	$CuSO_4$	Coronalon	JA	SA
β-Farnesene	x				
Methyl benzoate	x			x	
1-Decanol	x			x	
β-Himachalol	x			x	
$C_{15}H_{24}$ (RI 1381)	x				x
α-Himachalene	x	x			
3,5-Dimethoxytoluene	x	x			
Carene		x		x	
2-Himachalen-7β-ol		x		x	
$C_{15}H_{24}$ (RI 1481)		x		x	
γ-Himachalene		x			x
α-Ylangene		x	x		
2-*sec*-Butyl-3-Methoxypyrazine			x		
2-Pentylfuran			x		
4-Ketoisophorone			x		
β-Ionone epoxide			x		
$C_{15}H_{24}$ (RI 1521)			x		
n-Tridecane			x		
Germacrene D & γ-Muurolene			x	x	
Methyl 2-methylundecanoate				x	
Methyl jasmonate				x	
n-Octanal					x
Ethyl benzoate					x
Ethyl salicylate					x

In summary, each of the tested elicitors caused volatile emission with considerable specificity. Though certain overlaps were detected, particularly concerning induction with ALA, JA, and copper sulphate, the overall compositions of the VOC blends emitted were quite characteristic for the respective elicitation.

Finally, it is noteworthy that oligogalacturonic acid and linolenoyl glutamine were also tested for their ability to induce VOC emission in *M. truncatula*. Strikingly, these elicitors, along with

chitotetraose (cf. Chapter 3), were the only compounds tested in the course of the study that prove to be unable to induce VOC release in *M. truncatula*.

5.3 Discussion

Assessment of VOC emission in reaction to chemical elicitation confirmed the remarkable diversity of compounds emitted by *Medicago truncatula*. Conspicuously, every stimulus applied yielded a different pattern of VOCs emitted. This is in stark contrast to results gained from lima bean. There, not only is the diversity of VOCs emitted much lower, but also the blends do not differ as drastically. For example, as outlined in the introduction, the blends induced by ALA and copper sulphate are highly similar in lima bean, whereas JA and coronalon elicit VOCs comparable to those emitted in response to herbivory. In *M. truncatula*, however, VOCs induced by the above-mentioned compounds are at first glance quite similar. Looked at in more into detail, it becomes clear that all the compounds induce distinct blends of VOCs. It seems not to hold true that ALA and copper sulphate triggered VOC emission is mediated via a crosstalk comparable to that postulated for lima bean (Engelberth, 2000; cf. 5.1 Introduction). Otherwise the comparative likeness of JA treatment with those two stimuli would be hard to explain. Furthermore, acetyl-SA induced a VOC pattern that deviates completely from the other elicitors. Remarkably, the aromatic compounds ethyl benzoate and ethyl salicylate were emitted in considerable amounts, whereas MeSA was released only in minor quantities. The fact that substances structurally related to SA are emitted in reaction to treatment with this phytohormone might indicate a plant's mechanism for eliminating superfluous signalling compounds and regaining physiological balance in this instance. Coming back to the comparison of effects in lima bean and in barrel medic, some parallels are noteworthy: in response to acetyl-SA treatment lima bean also emits ethyl benzoate, ethyl salicylate, and MeSA, though in this case MeSA is the compound released in highest amounts (unpublished data, A. Mithöfer). Moreover, MeSA is emitted in response to ALA treatment in higher abundance than in response to any other treatment. Regarding control of VOC emission by the phytohormones JA and SA, however, the correlations stated by Engelberth *et al.* (2001) did not apply to any of the cases studied, because cutting of the stem of *M. truncatula* increased endogenous levels of SA (cf. Chapter 3), which again is in contrast to results gathered from experiments with lima bean (Engelberth *et al.*, 2001). Hence, the emission of MeSA or changes in VOC patterns can hardly be ascribed to SA. Although phytohormone levels were not determined in reaction to most of the compounds used here, it can be assumed that the response with regard to SA levels may be the same as described in Chapter 3 (confirmed only for coronalon). Taken together, the data indicate that SA neither reduces the diversity of VOC blends emitted nor inhibits VOC release in *M. truncatula*. The notion that increased JA levels are necessarily linked with altered VOC emission patterns in a causal relation can virtually be excluded with respect to the results presented earlier (Chapters 2 & 3). Thus, it still remains to be answered how the formation of those intricate VOC patterns is regulated in this species.

6 General discussion

In the present study, components of direct and indirect defence in reaction to a range of biotic stimuli as well as elements of signal transduction were compared using the model legume *Medicago truncatula*. Defence against different herbivores was assessed along with the impact of microbial oligosaccharides, both of bacterial and fungal origin. Those compounds were derived from organisms both beneficial and detrimental for plant health. Furthermore, the impact of mycorrhization on herbivore-induced VOC emission was monitored, as a step towards the investigation of multiple interactions. As data on direct comparisons of distinct biotic interactions is still sparse in the literature, this provides an opportunity to match several parameters assessed within one species in order to elucidate specificities and general phenomena of plants' responses in biotic interactions.

As *Medicago truncatula* prove to emit numerous VOCs in reaction to the majority of biotic stimuli applied, another aspect of this study aimed to complete the profile by adding the VOC patterns caused by elicitation with abiotic stimuli and exogenous application of phytohormones. Thereby, insight can be gained into the high apparent specificity of VOCs emitted under diverse physiological conditions.

When the qualitative distribution of all parameters assessed throughout this study, i.e. the presence or absence of a certain response, is summarised, the overlaps and distinctions in plants' defence responses can be easily viewed (Table 6-1). Conspicuous resemblance was found in the plants' reactions to different forms of herbivory and elicitation with the pathogen-derived β-glucans. The overlap extended to virtually all parameters assessed, with exception the of SA levels that could not be reliably determined with the induction methods used for elicitation by β-glucans. Though all responses were traceable after these stimuli, the quantitative as well as the spatio-temporal patterns differed considerably. Regarding the other elicitors of biotic origin, more distinctions became clear. Notably, all organisms adverse to plants' health or elicitors thereof induced elevated levels of JA. In contrast, both Nod-factors as well as mechanical wounding (as control) failed to do so. Only the local accumulation of phenolics and of ROS seemed to represent general phenomena. The most dissimilar responses, however, were recorded with regard to VOC emission, accumulation of SA, and overproduction of NO. Possible implications of these discrepancies will be discussed in the following subsections.

Table 6-1 Qualitative comparison of defensive traits recorded in *Medicago truncatula* in reaction to biotic stimuli.

	VOCs	JA	SA	phenolics	ROS	NO
***Spodoptera* spp.**	+	+	+[1]	+	+	+
Tetranychus urticae	+	+	+	+	+	n.d.
β-glucans	+	+	n.d.[3]	+	+	+
Chitotetraose	-	+	n.d.[3]	n.d.	+	-
LCO-IV (C16:2, S)	+/-[2]	-	n.d.[3]	n.d.	+	+
LCO-IV (C16:2)	+/-	-	n.d.[3]	n.d.	+	+
wounding / control	-	-	+[3]	+[4]	-	-

n.d., not determined; [1] transient increase in systemic leaves; [2] highly variable responses; [3] SA accumulation was induced by cutting the stem. SA levels were not further influenced by the addition of any elicitors and are thus referred to as not determined; [4] accumulation only detected late after wounding with clearly differing patterns.

6.1 Volatile emission

As demonstrated throughout the present study, *Medicago truncatula* emits a sizeable number of VOCs in response to various stimuli, with more than 90 compounds found to be differentially emitted. Almost all induction treatments tested led to elevated levels of VOC release, whereas few elicitors failed to evoke this reaction.

VOC emissions can serve multiple functions (Dudareva *et al.*, 2006; cf. 1.6 Plant-derived volatile organic compounds). Regarding the data gathered for VOC patterns in *Medicago truncatula*, the most evident is the direct impact on the attacking organisms. Though reports on the impact of essential oils or VOCs on organisms interacting with plants are quite scarce, literature concerning their medicinal properties abounds. Many uses have been known for ages, e.g. the use of essential oils as insect repellents or as medicine due to their antimicrobial properties, though comparable specifications of the bioactivity of particular oils are wanting (Schneider & Hiller, 1999; Cseke *et al.*, 2006). However, broad range antimicrobial activity has been reported for many compounds, which suggests that the respective substances may exert the same effects *in planta*. In fact, this has been shown for *Phytophthora infestans*, using essential oils of various aromatic plants (Soylu *et al.*, 2006). Interestingly, the antimicrobial impact was stronger when the oomycete was exposed to VOCs (Soylu *et al.*, 2006). Another intriguing point is that combinations of compounds often have more potent levels of bioactivity than do single substances (Muroi & Kubo, 1993; Wink, 2003; Koutsoudaki *et al.*, 2005; Spelman *et al.*, 2006). The use of synergistic effects is substantially aided by multi-product biosynthetic enzymes that offer enormous catalytic flexibility by producing numerous compounds with different functionalities and kinds of bioactivity (Spelman *et al.*, 2006; Tholl, 2006). Taken together, these phenomena may explain the often high variety of compounds present in one plant. By fine-tuning the emitted profile of VOCs in reaction to diverse stimuli, the plant may additionally enhance the effectiveness of its defence. These considerations may help explain the relevance of VOC emission in reaction to pathogen-derived elicitors, such as β-glucans, and to herbivory. But why certain pathogen-derived elicitors (CH4) do not induce VOC release, whereas Nod-factors do, remains to be answered.

Indirect defences, i.e. the attraction of natural enemies of herbivores, might mechanistically be based on the same approach as direct defences. By emitting a multitude of compounds, a plant is more likely to reach a variety of predators and parasitoids, with regard to the sensory capacities of different arthropod species. The synergistic effects of attractants should also be taken into account (Hammack, 2001). These considerations conveniently explain the variety of compounds emitted by *Medicago truncatula*, and furthermore account for the attraction of predatory mites to damaged plants, irrespective of whether they are infested with host or non-host organisms (cf. Chapter 2).

Interestingly, evidence is accumulating that many herbivores locate their host plants not by means of specific compounds, but rather by the perception and integration of combinations of substances with widespread occurrence; the ratio of the compounds emitted seems to play a crucial role for preference and avoidance (Bruce *et al.*, 2005). Since the biochemistry of plants is highly variable, and not only depends on the genetic background but also varies with environmental conditions, this offers an opportunity to selectively choose appropriate host plants (Renwick, 2001). It seems reasonable to deduce that the same might hold true for natural enemies of phytophages.

As certain plant-derived compounds may negatively influence the performance of predators, parasites, or parasitoids (Ode, 2006), they could prefer or avoid certain volatile blends. In the end, varying volatile patterns would be a tremendous way to fine-tune tritrophic interactions. This supports the importance of establishing reliable procedures for pattern recognition in this context; employing such methods would render it possible to correlate changing behaviour of herbivores and / or their natural enemies with specific volatile patterns. This field of research remains enormously challenging, particularly with regard to additional effects linked to specialist or generalist interactions, learning behaviour of herbivores and their natural enemies, and the involvement of more trophic levels such as symbionts or pathogens.
Finally, VOCs may also serve as means of communication between plants or between distant plant parts. This function has been reported in several instances (Bate & Rothstein, 1998; Engelberth *et al.*, 2004; Kessler *et al.*, 2006; Kishimoto *et al.*, 2006; Heil & Bueno, 2007). Regarding the diversity of VOC patterns emitted by *Medicago truncatula* in response to various stimuli, it would be of interest to investigate the respective responses in neighbouring plants. Direct defences may be a major function of VOC emission, and functionality of indirect defence has been ascertained at least for one tritrophic system in *Medicago truncatula*. Studies on interplant communication might contribute to the explanation of VOC functions in connection with symbiosis, i.e. the relevance of Nod-factor-induced volatiles and changed VOC patterns in response to mycorrhization. It would also be of considerable interest to compare the responses not only of neighbouring plants but also of other organisms involved in the trophic network to such subtle changes in volatile patterns.
In summary, *Medicago truncatula* emits VOCs in response to all kinds of stimuli. This response may not be as specific as widely assumed but rather may represent a byproduct of the up-regulation of secondary metabolism in the first place, perhaps with direct defence as the major function. As many substances that are emitted in reaction to pathogen or herbivore attack are *per se* deterrent to or toxic for the adverse organism, this could be a plausible explanation. The multiple functions, particularly the role in communication, can presumably be seen as secondary developments.

The volatile blends detected in *Medicago truncatula* exhibited in part striking specificity depending on the preceding elicitation. Whether these slight differences, compared to the complex natural background, are crucial for overall effects remains to be answered. This implies that those patterns are specific from the chemical point of view, but not necessarily in terms of biological activity. For theoretical considerations, however, this specificity is of considerable interest. As demonstrated in the Chapters 3, 4, and 5, volatile patterns can be visualised and discriminated using multivariate statistical methods. The problem is trivial in instances where substantial differences in the qualitative composition of the blends were recorded (cf. Chapter 5). The problem becomes more complicated if considering that only slight quantitative differences may be decisive for distinction in certain cases, as demonstrated for the effect of mycorrhization on VOC emission (Chapter 4). Though these differences are traceable under controlled conditions in the laboratory, this poses a major obstacle for the applicability in the field, where an enormous "background" of VOCs can be presumed to disturb any classification attempts. Additionally, other environmental factors also influence VOC patterns (Takabayashi *et al.*, 1994). As a result, the variability of VOC blends under natural conditions would have to be taken into account when classification rules are created.

Considering the statistical methods used it can be concluded that NMDS offers a robust and reliable method to depict VOC patterns. As it does not rely on linear responses and can handle data with only few samples, it is an adequate method to analyse VOC patterns. In contrast, the applicability of LDA is not unquestionably optimal. In the first place, it cannot handle multicollinear data, which is quite disadvantageous for volatile profiles that usually consist of highly correlated variables. However, this problem can be overcome if stepwise classification is used. Although this approach disregards the biological importance of complete VOC blends, it can be successfully applied for the determination of compounds that are useful as discriminants (Chapter 4). The results are satisfactory but still can only be seen as approximations, since LDA relies on linear correlations, which are not necessarily given in the response of plants to VOC-inducing stimuli. The advantages of the method lie in its comparatively low computational efforts and plain algorithm. The use of non-parametric methods that can also manage multicollinear data, as for example *k*-nearest neighbour or learning vector quantization, could improve the performance of VOC classification. Further amelioration can be assumed to result from the use of non-linear models, such as neural networks or support vector machines (Venables & Ripley, 2002 and references therein).
Besides methodological considerations, it is promising that volatile blends can be distinguished at all. This might facilitate the elucidation of signalling networks, offering a measure to link differential signalling to specific output in terms of VOC emission. However, the volatile patterns of different plant species have to be stringently evaluated in order to assess whether this high specificity is a general phenomenon. Finally, the physiological and ecological relevance of such distinctive VOC patterns remains to be determined. An accurate description of patterns could help determine how far changing ratios of volatiles emitted (but also of other non-volatile metabolites) influence biotic interactions.

6.2 Phytohormonal changes

The JA content of plants exposed to different herbivores and oligosaccharidic elicitors was determined in the course of this study. Conspicuously, only herbivory and induction with pathogen-derived elicitors caused JA accumulation, whereas Nod-factors did not increase JA levels. However, the patterns of JA accumulation differed, depending on the stimulus applied. These results are not strictly comparable, as it seems that the affected tissue highly influences the reaction. More specifically, wounding the stem seems to produce different effects than leaf damage does. For this reason, the recorded patterns of SA accumulation do not provide much information, except that *Medicago truncatula* accumulates this phytohormone when the stem is wounded. This is in contrast to the situation in lima bean, where cutting the stem has been shown to influence neither SA nor JA levels (Engelberth *et al.*, 2001).

Even though accumulation of JA is manifestly not solely responsible for the VOC profiles detected, it may add to patterns measured. Although it is critical to deduce the endogenous role of a certain compound from the effects of its exogenous application, particularly if the concentrations administered are far beyond the physiological range, JA was found to induce VOCs that are, in the broadest sense, comparable to those found after herbivore feeding (Boland *et al.*, 1995). But as demonstrated in Chapter 5, the effects of exogenous JA applied alone are quite specific in

Medicago truncatula. Regarding the endogenous levels of JA in response to the stimuli applied in the course of this study, this phytohormone alone is clearly not sufficient to explain the outcome in terms of defence responses (cf. Chapter 3). Thus, a correlation between induction of secondary metabolism and JA accumulation could not be unequivocally shown in this study, which is in line with some previous reports calling the essential role of JA in this context into question (Zhao *et al.*, 2005; see 3.3 Discussion for examples).

As aforementioned, the data gathered on the role of SA in plant defence responses are not very conclusive, because SA accumulates in *Medicago truncatula* in response to simple wounding of the stem. This is contrary to the reactions reported for lima bean, where wounding alone did not evoke any changes in the levels of SA (Engelberth *et al.*, 2001). This rise, however, seemed not to interfere with VOC emission; otherwise, in all cases where cut plants were used for the detection of VOCs, patterns comparable to those found in lima bean after elicitation with copper sulphate or alamethicin could be expected (Engelberth, 2000; Schulze, 2005). But completely unlike lima bean, *Medicago truncatula* emits very distinct volatile blends in reaction to each of those elicitors. Thus, it is unlikely that signalling events comparable to those postulated for lima bean mediate VOC emission in *Medicago truncatula*. That VOC emission is suppressed by increased SA levels is especially questionable. On the contrary, SA could be suspected to support VOC emission in this instance. Taking into account that one of the earliest roles described for SA is its function as calorigen in certain flowers, assisting the volatilisation of odorous compounds (Raskin, 1992), it could function similarly in the emission of volatiles by vegetative plant parts. Indeed, in the case of viral infection, local temperature increase has been detected in infected tobacco leaves, a non-thermogenic tissue. Also exogenous application SA can increase leaf temperature in this species (Chaerle, 2000). Moreover, Schmelz *et al.* (2001) demonstrated that in *Zea mays* detached leaves emitted higher amounts of VOCs when induced with JA or volicitin compared to intact plants. Unfortunately, endogenous SA levels were not determined, but it is within the bounds of possibility, though very speculative, that changes in SA levels contribute to this effect. Besides, SA seems to play a role in the regulation of VOC emission in certain plant species (Van Poecke & Dicke, 2004).

Eventually, the emerging picture seems to warn against underrating species specificity in phytohormonal action. As mentioned above, *Medicago truncatula* and lima bean, though both belong to the family of Fabaceae, react differently to the same stimulus. It is worth mentioning that JA accumulates in reaction to the excision of leaves in maize which is not further enhanced by application of volicitin (Schmelz *et al.*, 2003a). Conversely, JA levels do not increase in reaction to wounding in barrel medic (cf. Chapter 3) and only marginally in lima bean (Koch *et al.*, 1999). Additionally, plant species vary in their resting levels of phytohormones (Schmelz *et al.*, 2003b), so that different sensitivities can be assumed, and consequently threshold levels for physiological reactions might also differ.

Clearly, both JA and SA are insufficient to explain the effects observed in terms of VOC emission. Regarding the multifaceted interactions plants are involved in and the high variability of volatile emission in *Medicago truncatula*, it seems unlikely that a two-component system would be adequate to regulate the complex outcome. Extensive profiles of phytohormonal responses and other signalling components would certainly help answer the remaining questions about the induction pathways leading to volatile release.

But in the case of defence against herbivores, *Medicago truncatula* seems to fit the established picture, namely, mounting JA-related defences predominantly in defence against chewing herbivores and both JA- and SA-mediated defences in response to sucking phytophages or cell content feeders, as mirrored by the respective phytohormone levels (Chapter 2).

6.3 Reactive oxygen species and nitric oxide

In the course of this study it could be shown that any stimulus applied induced the accumulation of ROS in *Medicago truncatula*, with exception of mechanical damage. It thus seems to be a general trait in many biotic interactions. But with regard to the temporal development of the reaction, it seems unlikely that its primary role is to be found in signal transduction, at least not in the defence against herbivores. However, the applied detection method may not have been sensitive enough to get a hold of an early, transient increase of ROS. In connection with elicitation by microbial oligosaccharides, a contribution to signal transduction is more imaginable, as enhanced ROS production was detected as early as 15 minutes after application of the respective elicitor (cf. Chapter 3). Nevertheless, it may be reasonable to suspect that ROS play a role in direct defences, i.e. as direct antimicrobial agents, given their widespread occurrence in response to diverse stimuli and their prolonged overproduction in some instances (cf. Chapter 2). This of course does not contradict their additional involvement in signal transduction. However, again certain species specificity can be assumed, as other species, in contrast to *Medicago truncatula*, react with ROS overproduction to mechanical damage as well (Orozco-Cardenas & Ryan, 1999).
A more selective pattern was found for the accumulation of NO. Strikingly, NO accumulation and VOC emission coincided in all instances where both parameters were assessed. NO production was induced by Nod-factors, β-glucans, and also to some extent by caterpillar feeding (data not shown). Only CH4, which also proved to be unable to elicit volatile emission, did not increase NO production, along with controls (mechanical damage). This correlation might be merely incidental, and the data do not support solid causal links, but this point is certainly worth further investigation; particularly regarding the increasing awareness of possible roles of NO in diverse signalling cascades, not only in animals but also in plants (Grün *et al.*, 2006).

6.4 Accumulation of phenolic compounds

The local accumulation of phenolics at the wounding site, as assessed by microscopic means, can be regarded as a direct mode of general defence, as far as can be judged from the measurements conducted in the course of this study. Phenolics have been detected in all instances assessed, including in responses to herbivory, pathogen-derived elicitors, and wounding, though with slightly varying patterns. This response has long been known in connection with defence against pathogens (Dixon & Paiva, 1995; Kuc, 1995; Dixon *et al.*, 2002). In this case, phenolics are used in cell wall fortification, and serve as direct antimicrobial agents. For the action against herbivores, feeding deterrent or toxic properties would be obvious candidates for an explanation of the function. Regarding mechanical damage and the late accumulation of phenolics, it can be argued

that protection against secondary infections by opportunistic pathogens is the main function. A major drawback of the data gathered in this study is the lack of analytical determination of the compounds involved. The spatial patterns of accumulation clearly differed depending on the inducing stimulus, and also the intensity of fluorescence, i.e. the amounts of phenolics accumulated, seemed to be different. A thorough chemical analysis of the compounds accumulated, with respect to qualitative and quantitative composition, would substantially aid the interpretation of this phenomenon. Superficially, the reaction seems to be a general trait of direct defence against a broad range of organisms, but certain specificities might be revealed by more detailed analysis. Particularly, the spatial differences, i.e. the enhanced fluorescence located on the cells or on the cell walls, suggest distinct compounds involved in the different interactions.

6.5 Comparing induced defences in biotic interactions

On a larger scope, this study aimed to find overlaps and divergences between responses to diverse biotic stimuli. For single parameters, this comparison was done in the previous subsections; here, some conclusions based on the observed patterns are to be summarised.

Considering only qualitative traits, i.e. presence or absence of a certain monitored responses, substantial overlaps between the reactions to diverse stimuli were detected (Table 6-1). If the spatio-temporal patterns are also regarded, the resulting configurations gained certain distinctiveness. More specifically, none of the overall patterns recorded in reaction to any of the applied stimuli was identical with another. Thus, it may eventually be concluded that the defence responses are made up of a variable range of possible outcomes, using the same components that are slightly modified depending on the inducing agent, rather than of distinct and specific responses. The manifold signalling nets, of which only a glimpse was caught of in the present study, still leave sufficient capacities to make the overall reaction seemingly unique. The tendency to interpret those differences as specific or targeted responses might be somewhat misleading and disregards biological and physiological gradients.

Plants dispose of an arsenal of defensive weapons that are active against a broad range of organisms. This applies to generalist herbivores and non-host pathogens, but general defences might fail to detain specialised attackers. Yet, the idea that general defence responses of plants resemble innate immunity in animals seems quite plausible (Nürnberger *et al.*, 2004). Attributing all the observed reactions to simple general defence responses would still oversimplify the problem. Some reactions appear to be common traits of pants' defensive systems. The patterns of these responses, however, vary depending on the eliciting stimulus. It remains to be seen how far the slight variations of common responses contribute to the overall success of defence, and how these divergences are regulated.

Comprehensive data supporting the notion of innate immunity in plants were gathered in the course of this study for one model species. The profit thus clearly lies in one comparable system, examples of which are still sparse in literature. The data partially contradict previously published investigations, presumably due to species-specific differences. Such a discrepancy in results indicates the urgent need to consider several plant species among multiple families before stating general phenomena and mechanisms, if this is possible at all. Physiological effects can easily vary given the genetic diversity of plants. For primary investigations, model species are indispensable.

But based on such studies, conclusions leading to universally valid mechanisms might be somewhat precipitate. Hence, the discrepancies may be attributed to an under-representation of interspecific comparisons. These might finally lead to a deeper understanding of common phenomena in the plant kingdom and physiological disparities due to genetic and environmental variation. Reductionist approaches are clearly necessary to get a first idea of certain mechanisms but definitely will fail to reveal the complexity underlying plants' impressive defensive capacities.

7 Material and Methods

7.1 Biological material and induction methods used

7.1.1 *Medicago truncatula*

Medicago truncatula Gaertn. cv. Jemalong A17 seeds were provided by Dr. J. M. Prosperi (INRA-SGAP, Montpellier, France) and Dr. T. Huguet (INRA, Toulouse, France). Seeds were allowed to germinate in the dark for four days, then the seedlings were grown in the greenhouse at 18°C – 23°C with a light period from 7 a.m. to 9 p.m. Humidity was kept at 60 – 70%.

7.1.2 *Spodoptera* spp.

In all experiments either larvae of *Spodoptera littoralis* (Boisduval, 1833) or *Spodoptera exigua* (Hübner, 1808) were used.
The larvae were kept on an artificial diet (500 g chopped beans, 9 g ascorbic acid, 9 g 4-ethylbenzoic acid, 0.7 g vitamin E and 4 ml formaldehyde per litre water are mixed with approximately 650 ml of a 7.5% agar-solution) at 23°C with a light period from 7 a.m. to 9 p.m.

7.1.3 *Tetranychus urticae*

For experiments on volatile emission, determination of phytohormone levels, and detection of ROS and phenolics, *Tetranychus urticae* Koch (two-spotted spider mites) were reared on *Medicago truncatula* plants under the same conditions as described for the plant growth.
The mites used for volatile induction in the behavioural studies (olfactometer experiments) were reared on lima bean plants (*Phaseolus lunatus* L. cv. Sieva) at 25 ± 5 °C, 50 – 70% humidity and a 16 h / 8 h light-dark rhythm.

7.1.4 *Phytoseiulus persimilis*

The predatory mites (*Phytoseiulus persimilis* Athias-Henriot) were reared on lima bean leaves infested with *T. urticae* at 23 ± 1 °C, 50 – 70% humidity, and permanent light.
For all experiments adult females were used. Prior to an experiment, they were kept individually in 1.5 ml reaction tubes for 2 h.

7.1.5 Mycorrhization

Mycorrhized *M. truncatula* plants were supplied by Dr. B. Hause (Leibniz-Institut für Pflanzenbiochemie, Halle/Saale, Germany). Two different batches of seeds were used in the experiments. One was obtained from AustraHort Pty Ltd (Cleveland, Australia) with a mixture of cultivars, including *Medicago truncatula* cv. Jemalong (without further specification of the line) and cv. Paraggio. The other one was *M. truncatula* cv. Jemalong A17. Seeds were sterilised in H_2SO_4, rinsed several times in water, transferred to moist filter paper, and kept at 4°C for 3 days. Afterwards, they were allowed to germinate in the dark for one day before being transferred to the greenhouse at 22°C. After five days, the seedlings were piqued and inoculated with *Glomus intraradices* Schenck & Smith.

Thereafter, plants were kept at 23 – 25°C with a light-dark rhythm of 16 h / 8 h. Four weeks after inoculation, the degree of mycorrhization was approximately 36% and 59% for the mixed cultivars and *M. truncatula* cv. Jemalong A17, respectively.

7.1.6 Plant treatments, induction

7.1.6.1 Elicitors

β-Glucan elicitors were prepared from mycelia cell walls of the oomycete *Phytophthora sojae* Kaufmann & Gerdemann as described (Schmidt & Ebel, 1987) and applied in a concentration of 200 µg ml^{-1}. *N*,*N*',*N*'',*N*'''-tetraacetylchitotetraose (CH4) was purchased from Sigma, Germany, and used at 100 µM. Nod-factors, the lipo-chitooligosaccharides LCO-IV (C16:2,S) and LCO-IV (C16:2), were synthesised as described (Rasmussen *et al.*, 2004) and added at 10 µM. Alamethicin (Sigma, Germany) was applied at 10 µg ml^{-1}. Jasmonic acid (obtained from its methyl ester, which was provided by Dr. R. Kaiser, Givaudan Company, Dübendorf, Switzerland, via saponification with K_2CO_3), acetylsalicylic acid (Sigma, Germany), and copper sulphate (Sigma, Germany) treatments were done with a concentration of 1 mM; Coronalon (synthesised as described by Schüler *et al.*, 2001; available in our department) was added at a concentration of 100 µM.
All substances were dissolved in water except for alamethicin (stock solution in methanol), LCO-IV (C16:2) (stock solution in DMSO), and coronalon (stock solution in DMSO). Dilution to the respective concentration for the treatments was done with tap water. Solvent controls using methanol and DMSO did not reveal any influence on volatile emission.
In all experiments involving chemical elicitation, 5 ml of the respective elicitor solution were used. Within about 24 h, these 5 ml were completely taken up by the plant, which was thereafter supplied with tap water.

7.1.6.2 Herbivore infestation

In all experiments dealing with *Spodoptera* spp. infestation, 10 – 15 larvae were allowed to feed on *M. truncatula* plants for a period of time as indicated in the results section.
The sampling times after infestation with *Tetranychus urticae* were judged according to symptom development in all experiments on volatile induction, determination of phytohormone levels, and detection of ROS and phenolics, in order to obtain comparable damage levels after caterpillar feeding and spider mite infestation. Symptoms were related to early and late stages of infestation. Samples representing the early stages of damage were collected after the appearance of yellowish spots; samples representing the late stages of damage were collected when initially infested leaves yellowed.
For behavioural tests using the Y-tube olfactometer, 50 spider mites were placed on one plant and were allowed to feed on it for 48 h prior to the Y-tube experiments. Fifteen *S. littoralis* larvae were placed on four plants and equally allowed to feed for 48 h on the plants, which were continuously kept under the conditions described above.

7.2 Analysis of VOC emission

7.2.1 Closed-loop stripping and GC-MS analysis

Volatiles were collected over a period of 48 h using the closed-loop stripping method as described by Donath & Boland (1995). Plants were enclosed in desiccators and connected to a circulation pump (Fürgut, Aitrach, Germany) containing a charcoal trap (1.5 mg of charcoal, CLSA-filter, Le Ruisseau de Montbrun, Daumazan sur Arize, France). By providing air circulation, emitted volatiles were continuously collected on charcoal filters. Finally, desorption was done using methylene chloride (2 x 20 µl) containing 100 µg ml^{-1} n-bromodecane (or 200 µg ml^{-1} in some experiments) as internal standard; the volatiles were analysed using GC-MS (TRACE 2000 series, Finnigan, U.K.).
Plant treatments were done as described above (7.1.6 Plant treatments, induction). As controls both undamaged plants and plants cut and placed into tap water were used. Wounding plants by cutting did not induce elevated levels of volatile emission. Equally, for experiments on herbivore-induced VOC release, a comparison of the emission pattern of cut and intact plants did not reveal any apparent differences.

7.2.2 Identification of VOCs and determination of retention indices

The analysis of VOCs was done on a GC-MS (TRACE 2000 series, Finnigan, U.K.) equipped with an EC-5 capillary (15 m x 0.25 mm x 0.25 µm; Alltech, Unterhaching, Germany). Helium was used as carrier gas at a constant flow of 1.5 ml min^{-1}. Automatic injection of the samples (1 µl) was done with a split ratio of 1:10, the temperature of the injector was set to 220°C. The separation of compounds was achieved with an oven temperature program from 40°C for 2 min, and then increased at 10°C min^{-1} to 200°C, and finally at 30°C min^{-1} to 280°C, which was held for 1 min.
Compounds were identified according to their fragmentation pattern (MS) and by calculation and comparison of retention indices on an EC-5 capillary. For some compounds, retention indices were also determined on a second column with different polarity (DB 225MS, WiCom, Heppenheim, Germany) in order to confirm the prior identification (data not shown).
To compare mass spectra, the NIST/EPA/NIH Mass Spectral Library (Version 1998) and MassFinder (V 3.5; Dr. D. Hochmuth, Hamburg, Germany) were used.
The linear retention index (*I*) of all compounds detected was calculated according to the formula

$$I = 100\left[\frac{t_{Ri} - t_{Rz}}{t_{R\,(z+1)} - t_{Rz}} + z\right]$$

where t_{Ri} refers to the retention time of the compound of interest measured under conditions of temperature programming. *z* is the number of carbon atoms of the n-alkane eluting before the compound in question; accordingly, *(z+1)* is the number of carbon atoms of the n-alkane eluting after substance *i*, and *t* refers to the respective retention times.
The resulting values were compared either with those calculated from pure reference compounds or with literature data (Adams, 2001; Linstrom & Mallard, 2005 and references therein; retention indices of the compound in question measured under comparable conditions, i.e. temperature ramp, equivalent GC column etc.). Deviations of ± 2 for reference compounds and ± 5 for literature data were accepted for identical compounds in accordance with Hochmuth (2004).

7.2.3 Relative quantification of volatiles

In the case of chemically elicited plants, relative quantification of the compounds emitted was done by relating the respective peak areas to the peak area of the internal standard (100 µg ml^{-1} n-bromodecane) and to the fresh weight of the plants. In instances, where 200 µg ml^{-1} n-bromodecane were added, the data were divided by two to yield comparable values.
Volatile emission by mycorrhized and non-mycorrhized plants was normalised only by the internal standard, as the fresh weight of the plants that were not detached could not be determined prior to the experiments. However, all plants tested were of the same age and thus the variation in biomass can be assumed to be rather low.

7.2.4 Statistics

The first line of statistical analysis was done using one-way ANOVA combined with the Newman-Keuls *post hoc* test to compare the levels of single compounds emitted after the different treatments using square root transformed data.
In order to achieve exploratory mappings of different treatment effects in a mathematical space as defined by the volatile blends, a Non-metric Multidimensional Scaling (NMDS) ordination was carried out (Kruskal, 1964). NMDS is a method of multivariate statistics that is employed to analyse the structure of similarity or dissimilarity of data in multidimensional feature space. NMDS represents data as distances between points in a geometric space of low dimensionality (three or less). Therefore, only the configuration of points (samples) and their *interitem* distances count, i.e. points close to each other are likely to share some intrinsic properties, whereas distant points bear little or no similarity. NMDS maps observed dissimilarities non-linearly onto ordination space and can effectively handle non-linear responses of any shape. Therefore, the method does not rely on any particular relationship between variables and is commonly regarded as one of the most robust ordination methods in exploratory multivariate analysis, especially in ecology where data are often noisy and / or sparse (Minchin, 1987; Legendre & Legendre, 1998; Borg & Groenen, 2005). The analysis was performed in the way recommended by (Minchin, 1987), as implemented in the R package VEGAN (Oksanen *et al.*, 2006).
Prior to analysis, the data were standardised using square root transformation and tested for proper standardisation using the Shapiro-Wilk test of normality (Royston, 1982). In the first line of analysis by NMDS, Euclidean distance was selected as dissimilarity measure. In order find out, if volatile blends can be separated in ordination using only binary responses (i.e. presence or absence of a particular compound in the sample), and hence disregarding any effect of concentration, data were reduced to the binomial form in some instances. A binary dissimilarity index was used in this respect.
The overall goodness of fit of the models was measured by the stress statistic, the correlation between fitted values and ordination distances (Venables & Ripley, 2002). This statistic gives the proportion of data not ideally depicted in the ordination.
As volatile blends proved to exhibit rather distinct patterns in NMDS, further approaches aimed to test whether certain treatments can be diagnosed by their respective volatile profiles.
Linear Discriminant Analysis (LDA) aims to find linear transformations of the variables that yield maximal separation of the given groups by maximizing between-class variance and minimizing within-class variance (McLachlan, 1992). In doing so, it helps to describe differential features of

observations, to sort objects into labelled classes, and to assign new observations to previously defined classes. For simple LDA, multicollinearity poses a major problem. This term refers to multiple variables, which carry basically the same information (cf. Appendix III, Glossary of statistical terms). This obstacle can be overcome by using stepwise selection of variables for classification. Here, forward variable selection was performed using the Wilk's lambda criterion, as executed by the R package klaR (Mardia *et al.*, 1979; Weihs *et al.*, 2005). The value of Wilk's lambda, which can range from zero to one, indicates whether the means of variables are different between groups. The smaller this value is, the more likely it is that the group means differ. The method used selects variables that minimize the Wilk's lambda as long as the *p*-value still indicates statistical significance at the 0.9 significance level.

The quality of the resulting models is usually given by the estimated error of misclassification. This parameter was tested by 10-fold cross-validation. For that purpose, the actual measurements were split into a training set and a test set. The first is used to build up a classification rule, which in turn is used to predict the items in the test set, i.e. to allocate them to the appropriate group. This procedure is performed on 10 different subsets (Venables & Ripley, 2002); in order to achieve numerical stability, this test was repeated 99 times. The overall results, which give the proportions of classification and the error rates of the model in question, were summarised in a confusion matrix. Visual inspection of the model can be done by plotting the first few linear discriminants, i.e. the transformed variables.

7.3 Analysis of phytohormone levels

7.3.1 Extraction and quantification by GC-MS

Salicylate and jasmonate levels were determined according to the protocol for jasmonate quantification of Koch *et al.* (1999) with minor modifications. Briefly, plants were weighed and immediately frozen in liquid nitrogen. After the addition of 30 ml acetone:50 mM citric acid (7:3, v/v) and 150 ng [9,10-$^2H_2$]-9,10-dihydro-JA and 500 ng [3,4,5,6-$^2H_4$]-SA as internal standards, plants were homogenised using an ultra-turrax. The acetone was allowed to evaporate overnight at room temperature. Samples were cleared by filtration and subsequently extracted with 3 x 10 ml diethyl ether. Extracts were then loaded on solid-phase extraction cartridges containing 500 mg aminopropyl (Chromabond, Macherey-Nagel, Germany). After washing with 5 ml chloroform: isopropanol (2:1, v/v), bound acids were eluted using 12 ml diethyl ether:formic acid (98:2, v/v). After the solvents had evaporated, the residues were methylated using excess diazomethane. The final sample volume was adjusted to 50 µl with dichloromethane and analysis was performed using GC-MS (TRACE 2000 series, Finnigan, U.K.) in the selective ion mode. The fragment ions were monitored at m/z = 120, 124 and 83 for SA, [3,4,5,6-$^2H_4$]-SA and JA and [9,10-$^2H_2$]-9,10-dihydro-JA, respectively. The GC was equipped with an EC-5 capillary (15 m x 0.25 mm x 0.25 µm; Alltech, Unterhaching, Germany). Helium was used as carrier gas at a constant flow of 1.5 ml min^{-1}. Automatic injection of the samples (1 µl) was done with a split ratio of 1:10, the temperature of the injector was set to 260°C. For the separation of compounds, the oven was operated with a temperature program starting at 80°C for 2 min, increasing at 8°C min^{-1} to 127°C, held for 5 min, then heated at 30°C min^{-1} to 280°C, and held for 3 min.

The endogenous concentrations of salicylate and jasmonate were calculated from the peak areas of the respective substance and its standard using calibration curves. [3,4,5,6-$^2H_4$]-SA and [9,10-$^2H_2$]-9,10-dihydro-JA were synthesised as described by Engelberth *et al.* (2001) and Koch *et al.* (1999), respectively, and were readily available in our department.

7.3.2 Statistics

Statistics were done using the Student's *t*-test. All comparisons were done between treatments and the respective controls.

7.4 Microscopy

7.4.1 Detection of reactive oxygen species

7.4.1.1 Iodine-starch stain

To detect reactive oxygen species (ROS) in entire leaves, an iodine-starch stain was conducted using the method described by Olson & Varner (1993) with slight modifications. The samples were covered with a staining solution containing 4% (w/v) starch and 0.1 M NaI, and incubated for 15 – 30 min at room temperature. Samples were then rinsed with water and bleached overnight in ethanol (abs.). Slides were prepared using 70% ethanol and were viewed and documented using a light microscope (Axioskop, Zeiss, Germany) equipped with a digital imaging system (Spot, Visitron Systems, Germany).

7.4.1.2 3,3'-Diaminobenzidine

As an alternative method for the detection of hydrogen peroxide with light microscopic methods, staining with DAB was applied (Orozco-Cardenas & Ryan, 1999).
Branches of the plants were placed in a solution of 1 mg ml^{-1} 3,3'-Diaminobenzidine (DAB; Sigma-Aldrich), pH 3.8. The vessels with the plants were then put into desiccators that were slightly evacuated and incubated overnight under constant light. After induction treatments the plants were further incubated in DAB until sampling. After 48 h leaves were cut into pieces of adequate size and incubated for another 30 minutes in DAB solution. Afterwards, samples were bleached for 10 min in boiling ethanol (abs.) and then left in ethanol for 4 h. To prepare the slides, samples were transferred to 80% lactic acid.
Slides were viewed and documented using a Nikon Eclipse E400 light microscope equipped with a Nikon coolpix 4500 digital camera.

7.4.1.3 Nitroblue tetrazolium

For the detection of $O_2^{\cdot-}$ nitroblue tetrazolium (NBT; Sigma-Aldrich) was used. A droplet of NBT solution (6 mM in deionised water) was spread on a microscope slide; fresh hand-cut cross-sections *of M. truncatula* stems were put onto the liquid and covered with a cover slip. After 10 – 15 min of incubation the sections were rinsed by sucking water through the preparation by means of filter paper.
The slides were viewed and documented using a light microscope (Axioskop, Zeiss, Germany) equipped with a digital imaging system (Spot, Visitron Systems, Germany).

7.4.2 Detection of nitric oxide

To detect NO by microscopic means, the method described by Foissner *et al.* (2000) was applied with minor modifications.

As a probe, 4,5-diaminofluorescein diacetate (DAF-2 DA; Sigma-Aldrich) was used. A droplet of a 10 µM solution (in 10 mM Tris/HCl, pH 7.2) was spread on a slide; fresh hand-cut cross-section of stems were put onto the liquid, covered with a cover slip, and left for about 10 min in the dark. Objects were washed by sucking buffer through the preparation with filter paper.

Staining can be viewed by means of fluorescence microscopy. The microscope (Axioskop, Zeiss, Germany) was operated with two different filters. For the visualisation of the fluorescein signal, a 450 – 490 nm excitation filter and a 515 – 565 nm emission filter were used. For viewing chlorophyll autofluorescence, a band pass 546 (ex) / long pass 590 (em) filter was used. A digital imaging system was used for documentation (Spot, Visitron Systems, Germany). For the sake of comparability, exposure times were kept constant within one row of experiments.

Alternatively, entire leaves were tested for the suitability for NO-detection. Leaves were incubated in the staining solution described above for 30 min in the dark. Subsequently, samples were washed in fresh buffer to remove excess dye.

For time courses with low duration, intact leaves were stained and treated immediately prior to microscopic analysis. For measurements over a longer period of time, inducing treatments were conducted prior to the staining procedure.

The microscope used was an Olympus (Tokyo) FLUOview confocal scanning laser microscope operated with a Krypton / Argon laser at 488 nm and 568 nm. Images generated by the FLUOView software were analysed using the public domain NIH Image program (available on the Internet at http://rsb.info.nih.gov/ij/).

7.4.3 Autofluorescence

For the detection of phenolic compounds, whole leaves were used without further preparation. The fluorescence microscope (Axioskop, Zeiss, Germany) was operated with a 365 nm band pass excitation filter and a 397 nm long pass emission filter. A digital imaging system was used for documentation (Spot, Visitron Systems, Germany).

7.5 Behavioural studies – Y-tube olfactometer experiments

In all experiments, the responses of the predatory mites were tested in a Y-tube olfactometer. As an odour source, 4 plants per treatment were put into the respective vial. Constant air flow was provided at 4 l min^{-1}.

Individual predatory mites were observed in the olfactometer. Mites that did not choose one branch of the olfactometer within 5 min or did not reach the end of one branch within 10 min were recorded as "no choice".

Experiments were repeated on several days with new odour sources and new groups of about 20 mites in order to randomise individual variations in volatile emission and searching behaviour, respectively.

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Curriculum vitae

Personal data

Name	Margit Leitner
Date and place of birth	11th February 1979 in Bruck an der Mur, Austria
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Education

1985 – 1989	Elementary school in Kapfenberg, Styria
1989 – 1997	Grammar school in Kapfenberg, Styria (BG Kapfenberg)
1997 – 2003	Study of Biology at the University of Salzburg, Salzburg; Branch of study: Botany
May 2003	Graduation (Mag. rer. nat.)
since September 2003	PhD student at the Max-Planck-Institute for Chemical Ecology in Jena, in co-operation with the Friedrich-Schiller-Universität, Jena, Germany

Employment

September 1999	Short-time employment as student laboratory assistant, University of Salzburg, Institute for Plant Physiology
July – August 2000	Short-time employment as student laboratory assistant, University of Salzburg, Institute for Plant Physiology
November 2000 – January 2003	Biological technical assistant, University of Salzburg, Institute for Plant Physiology

Research activities

Diploma thesis	"Programmierter Zelltod in *Chenopodium rubrum* L. Suspensionskulturen" (programmed cell death in *Chenopodium rubrum* L. suspension cultures)
PhD thesis	Comparing induced responses of *Medicago truncatula* to biotic challenges: common themes, varying patterns

Research activities abroad

31st May – 13th June 2004	Italy, University of Turin, Department of Plant Biology (group of M. Maffei): "Signaling mediating volatile release in *Medicago truncatula* after insect herbivory" (ESF-VOCBAS short visit grant)
August 2004	Italy, University of Turin, Department of Plant Biology (group of M. Maffei): continued studies on: "Signaling mediating volatile release in *Medicago truncatula* after insect herbivory"
18th May – 2nd June 2006	The Netherlands, University of Wageningen, Laboratory of Entomology (group of M. Dicke): "Specific attraction of natural enemies of herbivores by different volatile blends emitted by *Medicago truncatula*" (ESF-VOCBAS short visit grant)

13th – 22nd September 2006	The Netherlands, University of Wageningen, Laboratory of Entomology (group of M. Dicke), continued studies on: "Specific attraction of natural enemies of herbivores by different volatile blends emitted by *Medicago truncatula*"

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Publications

Leitner M., Boland W., Mithöfer A. (2005) Direct and indirect defences induced by piercing-sucking and chewing herbivores in *Medicago truncatula*. New Phytologist 165: 597 - 606

Conference contributions:

Oral presentations:

Doktorandenworkshop: „Naturstoffe: Chemie, Biologie und Ökologie", Halle/Saale (Germany), 4th May 2005: Vergleich induzierter Verteidigung in *Medicago truncatul*a gegen Herbivoren mit unterschiedlichem Fraßverhalten

Kurt-Mothes-Doktoranden-Workshop, Halle/Saale (Germany), 5th – 7th October 2005: Comparison of defences induced by piercing-sucking and chewing herbivores in *Medicago truncatula*.

Tagung: „Molekularbiologie der Pflanzen", Dabringhausen (Germany), 7th – 10th March 2006: Abwehrmechanismen von *Medicago truncatula* gegen verschiedene biotische Stressoren.

1st VOCBAS status seminar (ESF workshop), Jülich (Germany), 13th – 15th June 2006: *Medicago truncatula* – a new model for plant herbivore interactions?

Joint International Workshop: "PR-Proteins" and "Induced Resistance against Pathogens and Insects", Doorn (The Netherlands), 10th – 14th May 2007: The impact of microbial oligosaccharides on *Medicago truncatula*: Differential induction of volatile emission and components of signal transduction.

Poster presentations:

Botanikertagung in Freiburg i. Br. (Germany), 22nd – 27th September 2002: Leitner M., Pfeiffer W.; Programmed cell death in *Chenopodium rubrum* L. cell cultures.

Botanikertagung in Braunschweig (Germany), 5th – 10th September 2004: Leitner M., Boland W., Mithöfer A.; Knocking on *Medicago*'s door: The host's responses to pathogens and herbivores.

International Botanical Congress in Vienna (Austria), 17th – 23rd July 2005: Leitner M., Boland W., Mithöfer A.; Comparison of defences induced by piercing-sucking and chewing herbivores in *Medicago truncatula*.

Erklärung

Ich erkläre hiermit, daß mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität bekannt ist.

Ich habe die vorliegende Dissertation selbständig verfaßt und alle Hilfmittel, persönlichen Mitteilungen und Quellen in der Arbeit angegeben.

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Ich habe die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Ferner habe ich nicht versucht, die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation einzureichen.

Jena, Juni 2007

Margit Leitner

Appendix I
Retention indices of volatiles detected in *Medicago truncatula*

The volatiles emitted by *M. truncatula* are listed in the order of their retention index (temperature programmed, EC-5). This list does not claim completeness; compounds detected only in trace amounts or found only randomly in a few samples were excluded.

Table A-1 Volatiles emitted by *Medicago truncatula* with retention indices (temperature programmed EC-5) measured in the samples, from pure reference compounds, and from literature data.

compound	I_s	I_{lit}	I_{ref}	comments
α-Pinene	931	939	931	
Benzaldehyde	955	960	955	
β-Pinene	972	973		
1-Octen-3-ol	981	979	982	
3-Octanone	986	984		
6-Methylhept-5-en-2-one	986	986		
2-Pentylfuran	991	993		
6-Methyl-1-heptanol	992			tentative identification; MS
n-Decane	1000	1000	1000	
Ethyl hexanoate	1000	998		
n-Octanal	1001	999	1003	
Carene	1005	1002	1006	
cis-3-Hexenylacetate	1007	1005	1007	
p-Methylanisol	1016	1019		
Cymene	1020	1023		
Limonene	1024	1029	1025	
RI 1038; M 142, BP 109	1038			unidentified compound
E-β-Ocimene	1047	1050	1048	
Cresol, *o*-	1057	1056		
1-Octanol	1074	1072		
Cresol, *p*-	1078	1077		
Methyl benzoate	1090	1091		
Linalool	1096	1097	1098	
n-Undecane	1100	1100	1100	
n-Nonanal	1103	1101	1105	
β-Phenylethanol	1109	1107		
RI 1112; M 198, BP 123	1112			unidentified compound
4,8-Dimethylnona-1,3,7-triene (DMNT)	1116		1116	
2,6-Dimethyl-1,3,5,7-octatetraene	1127			tentative identification; MS
4-Ketoisophorone	1140	1145		
Ethyl benzoate	1166	1168		
2-*sec*-Butyl-3-methoxypyrazine	1171		1172	
Methyl salicylate	1193	1192	1193	
Ethyl octanoate	1196	1197		
n-Dodecane	1200	1200	1200	
n-Decanal	1205	1202	1206	
2,6-Dimethyl-3,5,7-octatriene-2-ol	1207			tentative identification; MS
1,2-Benzisothiazole	1215			tentative identification; MS
β-Cyclocitral	1216	1218		

Table A-1, continued

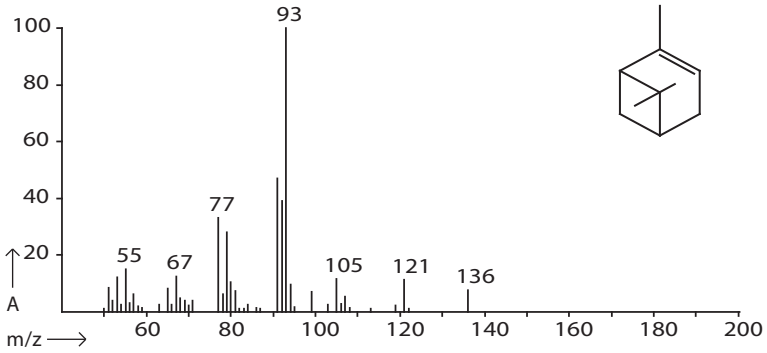
compound	I_s	I_{lit}	I_{ref}	comments
Cyclohexylisothiocyanate	1226		1226	
3,5-Dimethoxytoluene	1263	1264	1264	
Ethyl salicylate	1265	1270		
1-Decanol	1271	1270		
n-Tridecane	1301	1300	1300	
n-Undecanal	1303	1307		
7α*H*-Silphiperfol-5-ene	1321	1322		
7β*H*-Silphiperfol-5-ene	1340	1344		
Cyclosativene	1362	1371	1364	
Longicyclene	1366	1374	1366	
α-Ylangene	1370	1375	1370	
α-Copaene	1374	1377	1374	
RI 1381; M204, BP 161	1381			unidentified sesquiterpene
β-Cubebene	1387	1388		
1-Tetradecene	1389	1389		
Ethyl decanoate	1393	1396		
n-Tetradecane	1400	1400	1400	
α-Gurjunene	1405	1410	1407	
E-β-Caryophyllene	1417	1419	1418	
RI 1417; M 204, BP 147; presumably a Pacifigorgiane	1417			
β-Copaene	1427	1432	1428	
α-Bergamotene	1432	1435		
α-Himachalene	1447	1451	1446	
α-Humulene	1451	1455	1451	
Geranylacetone	1452	1455		
RI 1457; M 204, BP 204	1457			δ-Patchoulene or Valerena-4,7(11)-diene
β-Farnesene	1458	1457	1458	
allo-Aromadendrene	1460	1460	1460	
γ-Himachalene	1476	1475	1476	
γ-Muurolene	1478	1480	1478	
Germacrene D	1479	1485	1478	
RI 1481; M 204, BP 91	1481			unidentified sesquiterpene
β-Ionone epoxide	1483			tentative identification; MS
β-Ionone	1488	1489	1489	
α-Muurolene	1500	1500	1500	
n-Pentadecane	1500	1500	1500	
RI 1521; M 204, BP 109	1521			Trichodiene or β-Bazzanene
Ethyl 4-ethoxybenzoate	1524			tentative identification; MS
Dihydroactinidiolide	1524	1525		
Methyl dodecanoate	1525			tentative identification; MS
RI 1532; M 196, BP 68	1532			unidentified compound
E-Nerolidol	1564	1563	1566	
(3*E*,7*E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene (TMTT)	1578	1579	1579	
Caryophyllene oxide	1579	1583	1579	
RI 1587; M 222, BP 109	1587			Viridiflorol or Globulol
2-Himachalen-7β-ol	1637		1637	
β-Himachalol	1645		1647	
Methyl jasmonate	1643	1638		
α-Santalol	1669	1675		tentative identification
α-Bisabolol	1679	1681	1686	tentative identification
RI 1806; M 272, BP 191	1806			unidentified diterpene
RI 1876; M 272, BP 191	1876			unidentified diterpene
RI 1983; M 272, BP 189	1983			unidentified diterpene

I_s, linear retention index of compounds in VOC samples; I_{lit}, retention index of the respective compound measured under comparable conditions, as found in the literature (Adams, 2001; Linstrom & Mallard, 2005 and references therein); I_{ref}, retention index of authentic reference compounds measured under the same conditions as the volatile samples. Deviations of ± 2 for reference compounds and ± 5 for literature data were still accepted as identity in accordance with Hochmuth (2004).

Appendix II

Mass spectra of volatiles detected in *Medicago truncatula*

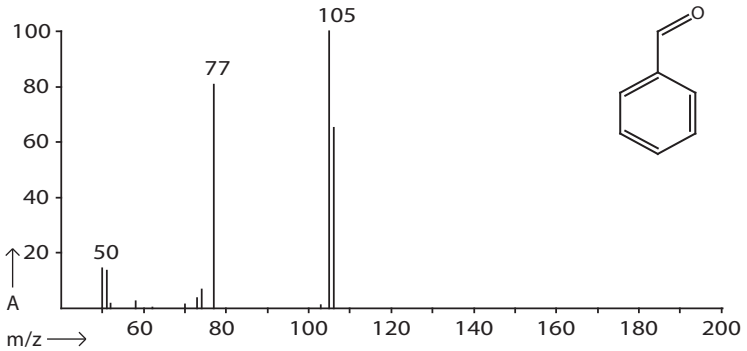
The mass spectra of VOCs found to be emitted by *M. truncatula* are listed in the order of their retention index (temperature programmed, EC-5). This list does not claim completeness; compounds detected only in trace amounts or found only randomly in a few samples were excluded.



α-Pinene
RI 931

CAS 80-56-8
$C_{10}H_{16}$
FW 136

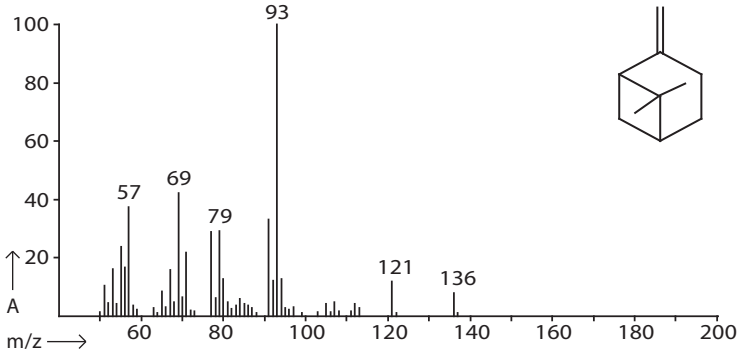
m/z (%) = 93 (100), 77 (33), 55 (15), 67 (13), 105 (11), 121 (11), 136 (8)



Benzaldehyde
RI 955

CAS 100-52-7
C_7H_6O
FW 106

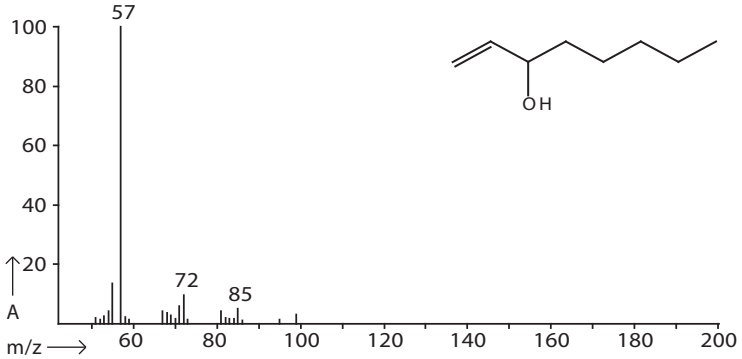
m/z (%) = 105 (100), 77 (81), 50 (15)



β-Pinene
RI 972

CAS 127-91-3
$C_{10}H_{16}$
FW 136

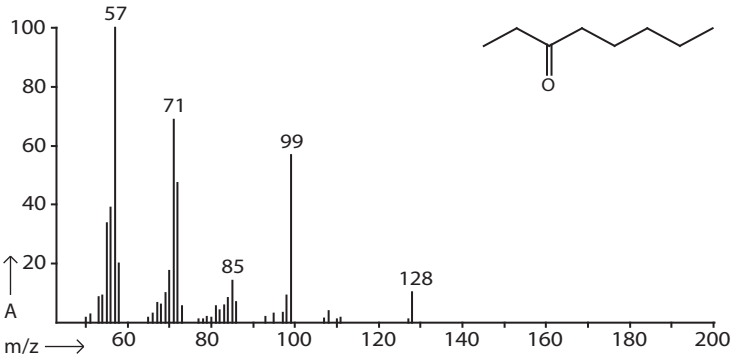
m/z (%) = 93 (100), 69 (42), 57 (37), 79 (29), 121 (12), 136 (8)



1-Octen-3-ol
RI 980

CAS 3391-86-4
$C_8H_{16}O$
FW 128

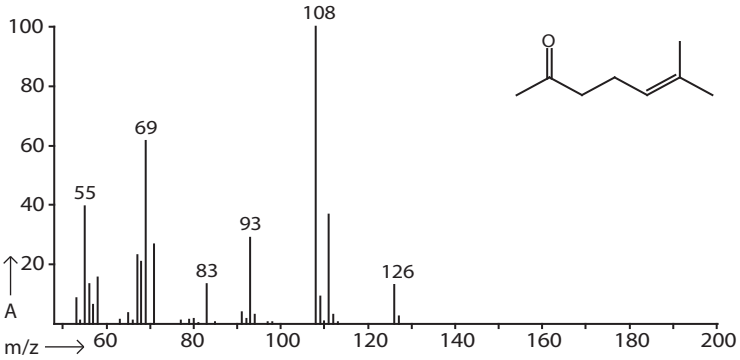
m/z (%) = 57 (100), 72 (10), 85 (5)



3-Octanone
RI 986

CAS 106-68-3
$C_8H_{16}O$
FW 128

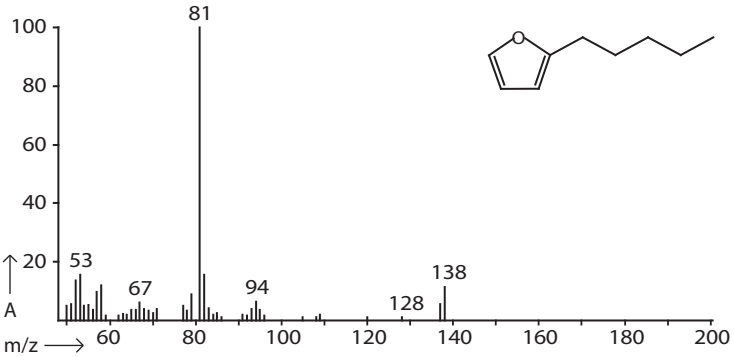
m/z (%) = 57 (100), 71 (69), 99 (57), 85 (14), 128 (10)



6-Methylhept-5-en-2-one
RI 986

CAS 110-93-0
$C_8H_{14}O$
FW 126

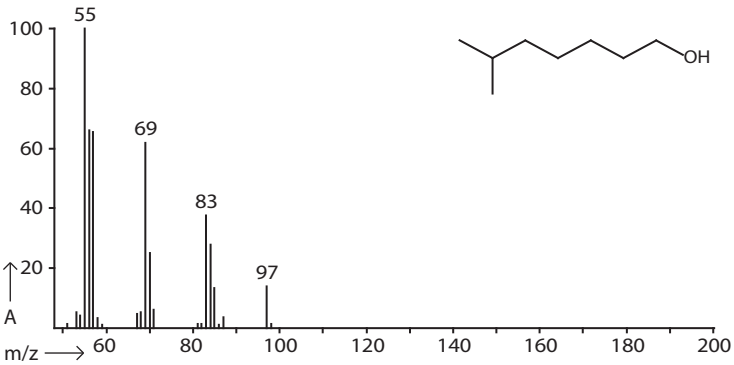
m/z (%) = 108 (100), 69 (62), 55 (40), 93 (29), 83 (13), 126 (13)



2-Pentylfuran
RI 991

CAS 3777-69-3
$C_9H_{14}O$
FW 138

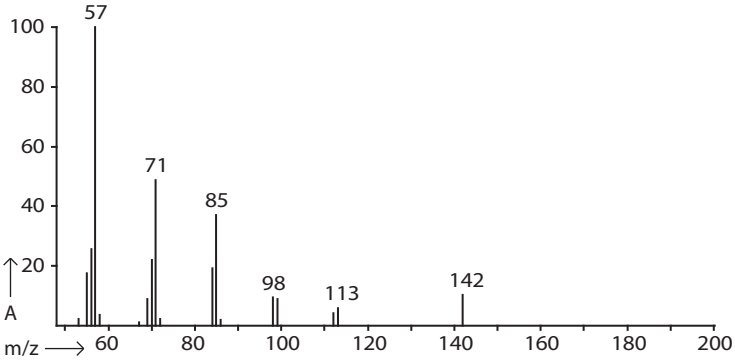
m/z (%) = 81 (100), 53 (16), 138 (11), 94 (6), 67 (6), 128 (1)



6-Methyl-1-heptanol
RI 992

CAS 1653-40-3
$C_8H_{18}O$
FW 130

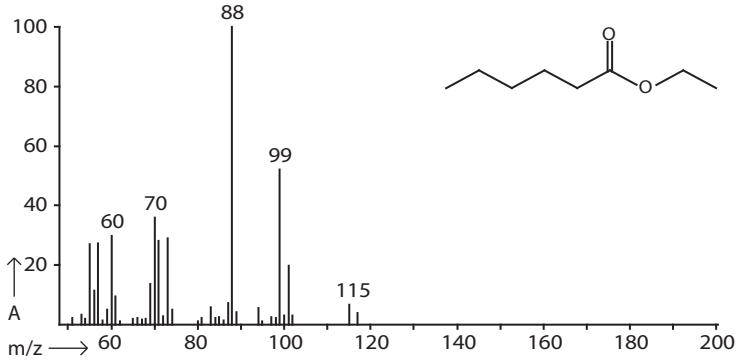
m/z (%) = 55 (100), 69 (62), 83 (38), 97 (14)



n-Decane
RI 1000

CAS 124-18-5
$C_{10}H_{22}$
FW 142

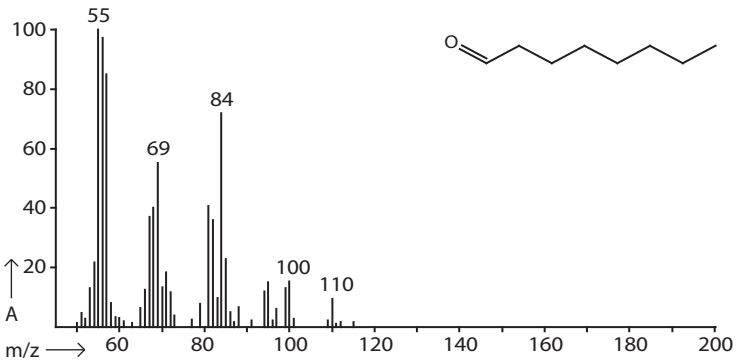
m/z (%) = 57 (100), 71 (49), 85 (37), 142 (10), 98 (10), 113 (6)



Ethyl hexanoate
RI 1000

CAS 123-66-0
$C_8H_{16}O_2$
FW 144

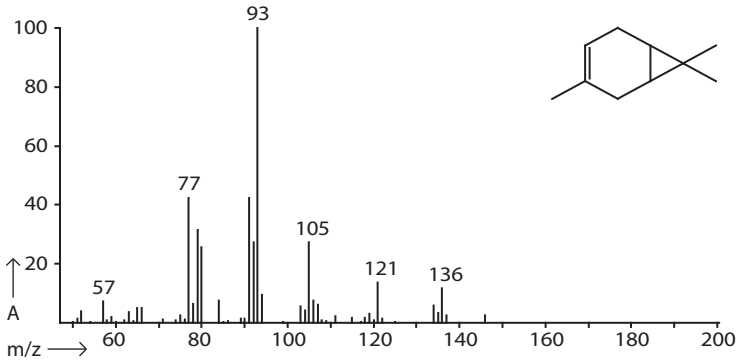
m/z (%) = 88 (100), 99 (52), 70 (36), 60 (30), 115 (7)



n-Octanal
RI 1001

CAS 124-13-0
$C_8H_{16}O$
FW 128

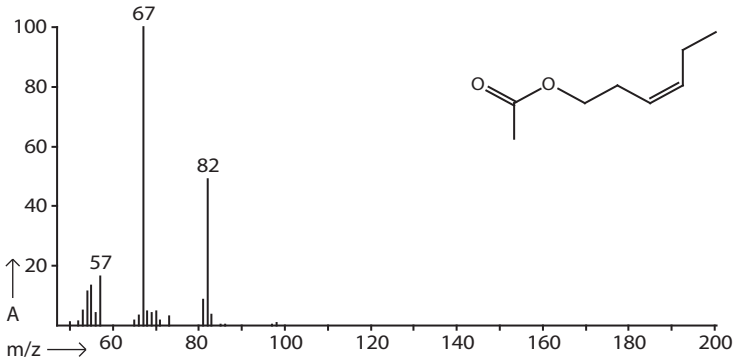
m/z (%) = 55 (100), 84 (72), 69 (55), 100 (15), 110 (10)



3-Carene
RI 1005

CAS 13466-78-9
C_8H_{16}
FW 136

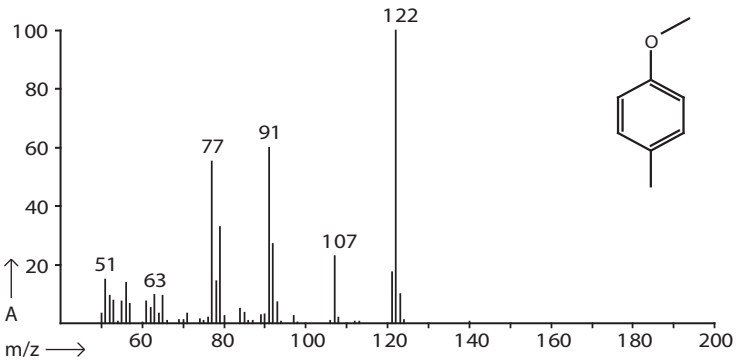
m/z (%) = 93 (100), 77 (42), 105 (27), 121 (14), 136 (12), 57 (7)



***cis*-3-Hexenylacetate**
RI 1006

CAS 3681-71-8
$C_8H_{14}O_2$
FW 142

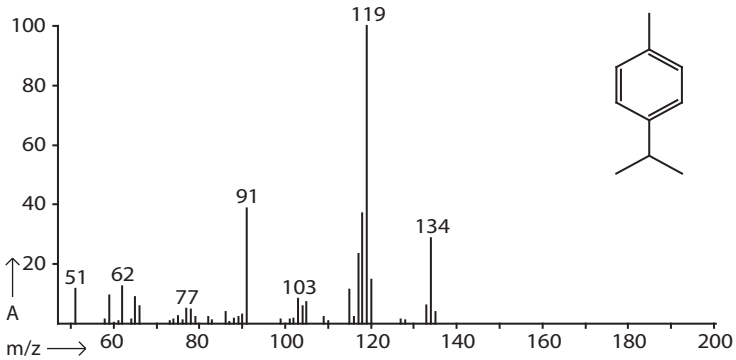
m/z (%) = 67 (100), 82 (49), 57 (17)



***p*-Methylanisol**
RI 1016

CAS 104-93-8
$C_8H_{10}O$
FW 122

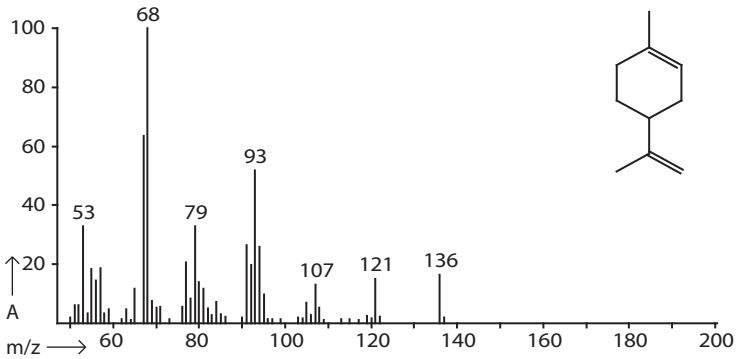
m/z (%) = 122 (100), 91 (60), 77 (55), 107 (23), 51 (15), 63 (10)



_p_-Cymene
RI 1020

CAS 99-87-6
$C_{10}H_{14}$
FW 134

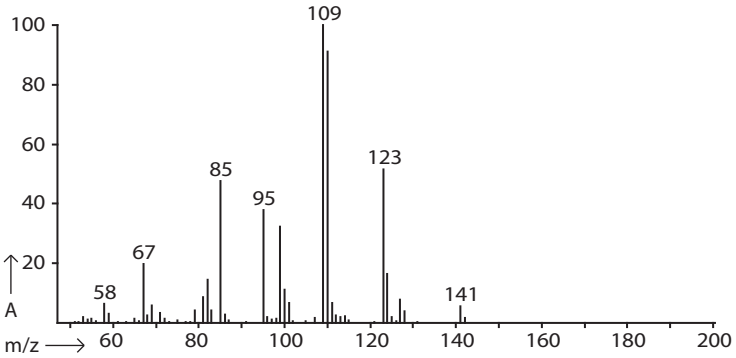
m/z (%) = 119 (100), 91 (39), 134 (29), 62 (13), 51 (12), 103 (8), 77 (5), 127 (1)



Limonene
RI 1024

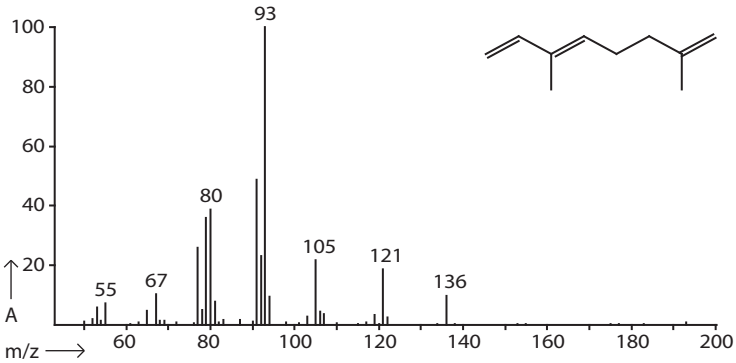
CAS 138-86-3
$C_{10}H_{16}$
FW 136

m/z (%) = 68 (100), 93 (52), 79 (33), 53 (33), 136 (17), 121 (15), 107 (13)



unidentified compound
RI 1038

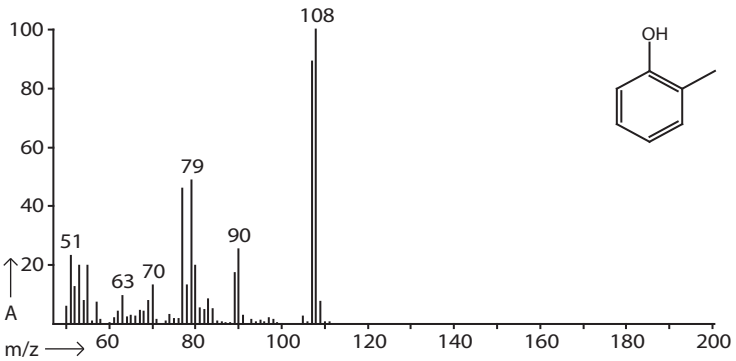
m/z (%) = 109 (100), 123 (52), 85 (48), 95 (38), 67 (20), 58 (6), 141 (6)



_E_-β-Ocimene
RI 1047

CAS 502-99-8
$C_{10}H_{16}$
FW 136

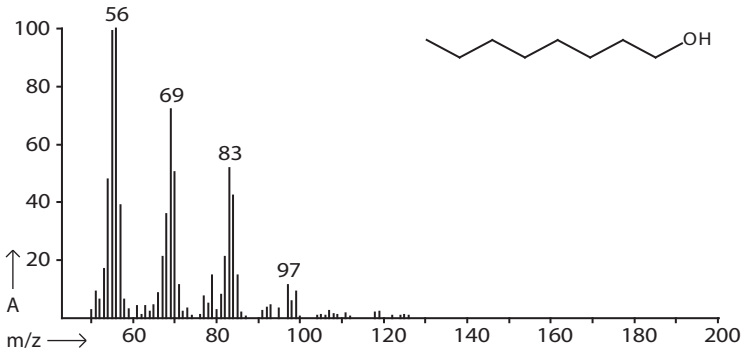
m/z (%) = 93 (100), 80 (39), 105 (22), 121 (19), 67 (10), 136 (10), 55 (7)



_o_-Cresol
RI 1057

CAS 95-48-7
C_7H_8O
FW 108

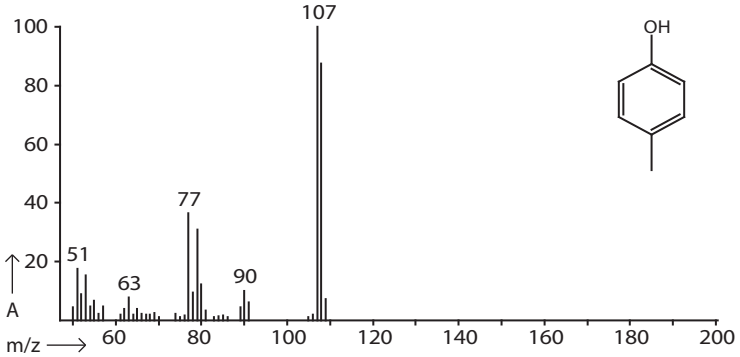
m/z (%) = 108 (100), 79 (49), 90 (25), 51 (23), 70 (13), 63 (10)



1-Octanol
RI 1074

CAS 111-87-5
$C_8H_{18}O$
FW 130

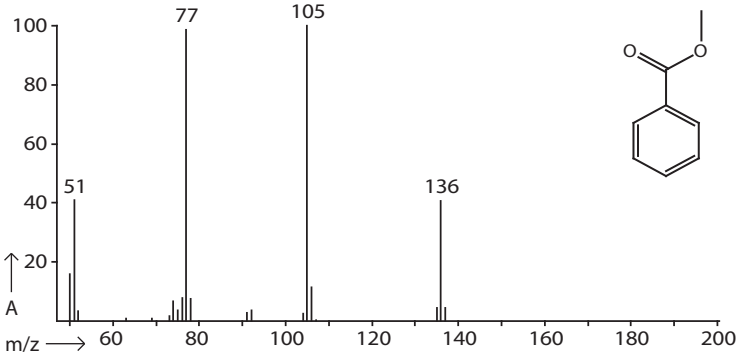
m/z (%) = 56 (100), 69 (72), 83 (52), 97 (11)



***p*-Cresol**
RI 1078

CAS 106-44-5
C_7H_8O
FW 108

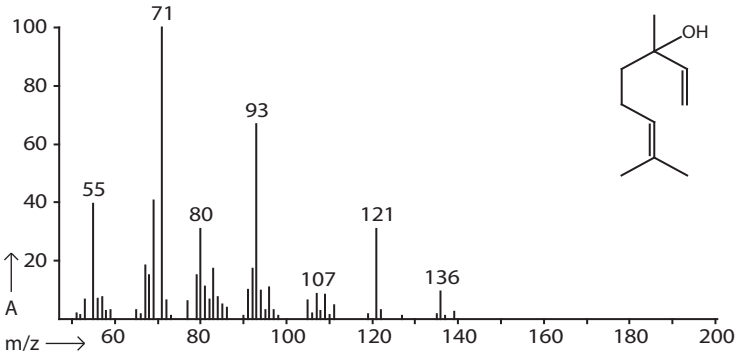
m/z (%) = 107 (100), 77 (36), 51 (18), 90 (10), 63 (8)



Methyl benzoate
RI 1090

CAS 93-58-3
$C_8H_8O_2$
FW 136

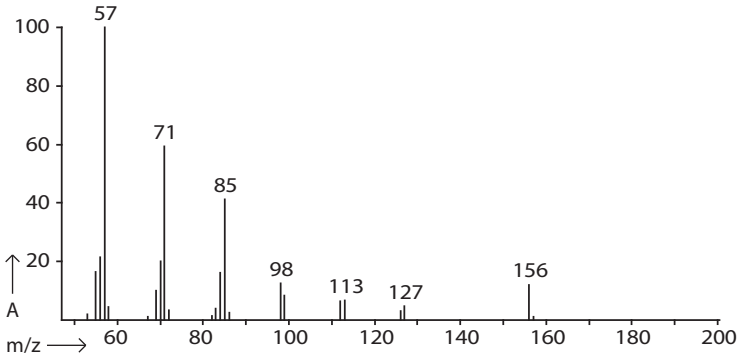
m/z (%) = 105 (100), 77 (99), 51 (41), 136 (41)



Linalool
RI 1096

CAS 78-70-6
$C_{10}H_{18}O$
FW 154

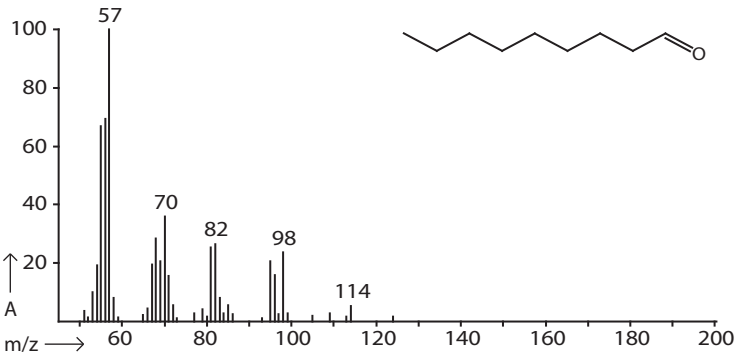
m/z (%) = 71 (100), 93 (67), 55 (40), 121 (31), 80 (31), 136 (9), 107 (9)



n-Undecane
RI 1100

CAS 1120-21-4
$C_{11}H_{24}$
FW 156

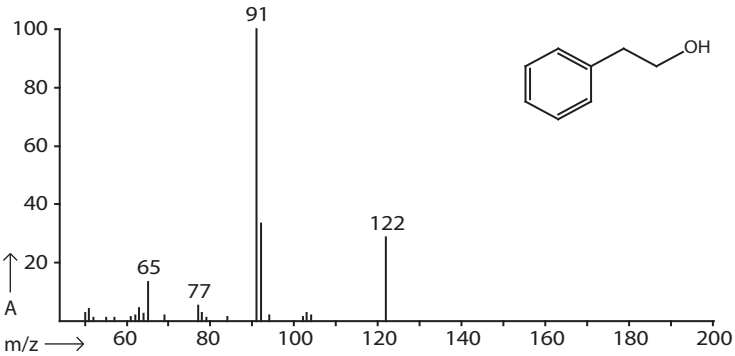
m/z (%) = 57 (100), 71 (59), 85 (41), 98 (13), 156 (12), 113 (7), 127 (5)



n-Nonanal
RI 1103

CAS 124-19-6
$C_9H_{18}O$
FW 142

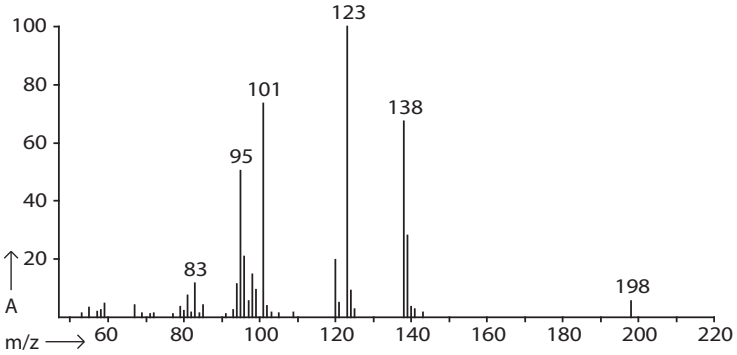
m/z (%) = 57 (100), 70 (36), 82 (26), 98 (24), 114 (5)



β-Phenylethanol
RI 1109

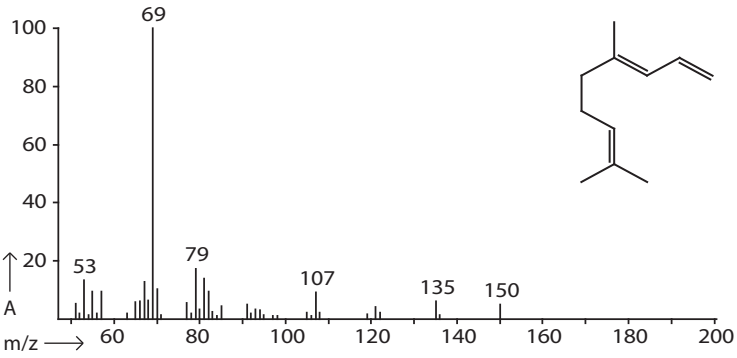
CAS 60-12-8
$C_8H_{10}O$
FW 122

m/z (%) = 91 (100), 122 (29), 65 (13), 77 (5)



unidentified compound
RI 1112

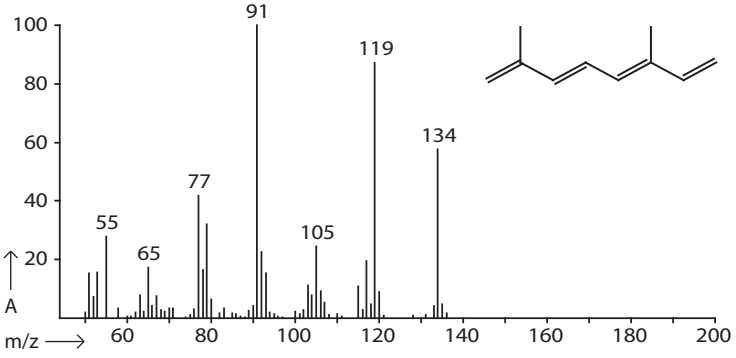
m/z (%) = 123 (100), 101 (73), 138 (67), 95 (50), 83 (12), 198 (6)



4,8-Dimethylnona-1,3,7-triene (DMNT)
RI 1116

CAS 51911-82-1
$C_{11}H_{18}$
FW 150

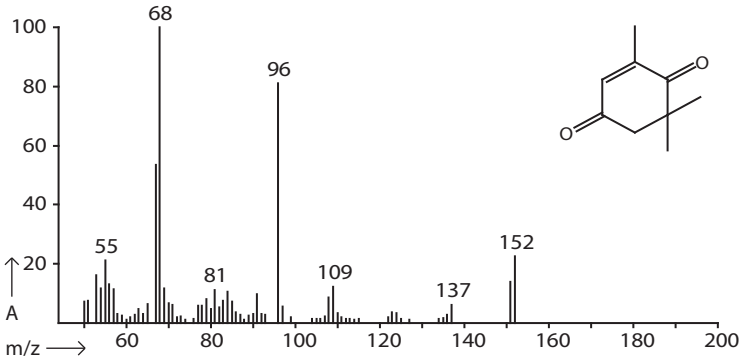
m/z (%) = 69 (100), 79 (17), 53 (13), 107 (9), 135 (6), 150 (5)



2,6-Dimethyl-1,3,5,7-octatetraene
RI 1127

CAS 460-01-5
$C_{10}H_{14}$
FW 134

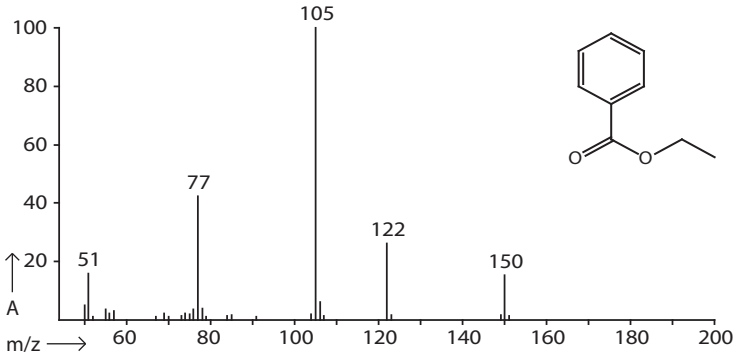
m/z (%) = 91 (100), 119 (87), 134 (58), 77 (42), 55 (28), 105 (24), 65 (17)



4-Ketoisophorone
RI 1140

CAS 1125-21-9
$C_9H_{12}O_2$
FW 152

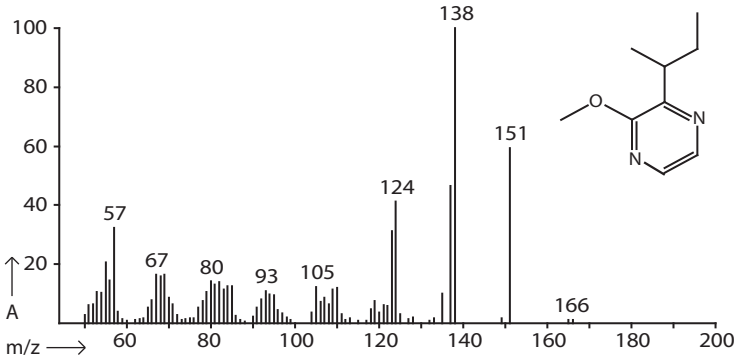
m/z (%) = 68 (100), 96 (81), 152 (23), 55 (21), 109 (12), 81 (11), 137 (6)



Ethyl benzoate
RI 1166

CAS 93-89-0
$C_9H_{10}O_2$
FW 150

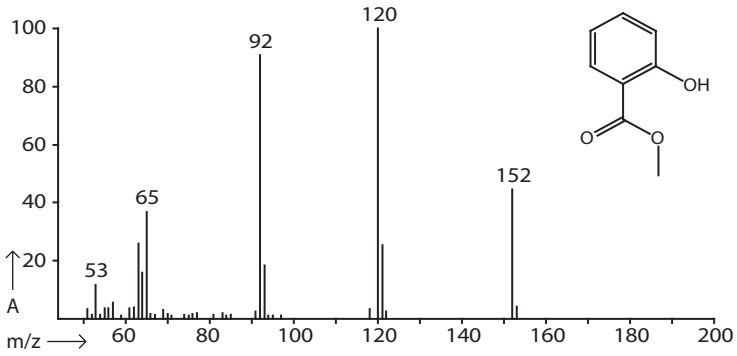
m/z (%) = 105 (100), 77 (42), 122 (26), 51 (16), 150 (15)



2-*sec*-Butyl-3-methoxypyrazine
RI 1170

CAS 24168-70-5
$C_9H_{14}N_2O$
FW 166

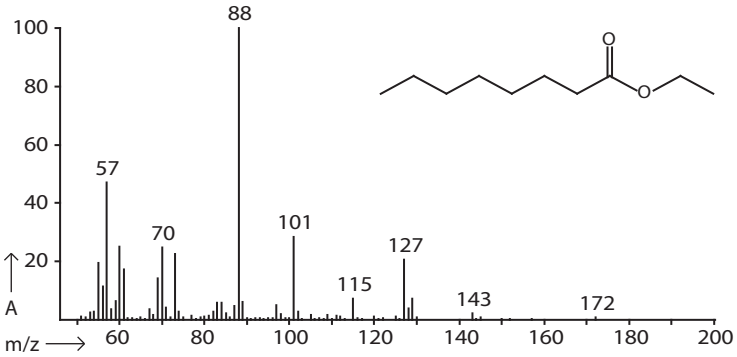
m/z (%) = 138 (100), 151 (59), 124 (41), 57 (32), 67 (16), 80 (14), 105 (12), 93 (11), 166 (1)



Methyl salicylate
RI 1193

CAS 119-36-8
$C_8H_8O_3$
FW 152

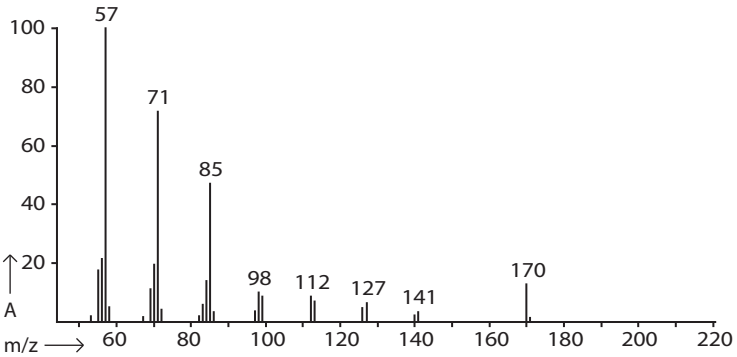
m/z (%) = 120 (100), 92 (91), 152 (45), 65 (37), 53 (12)



Ethyl octanoate
RI 1196

CAS 106-32-1
$C_{10}H_{20}O_2$
FW 172

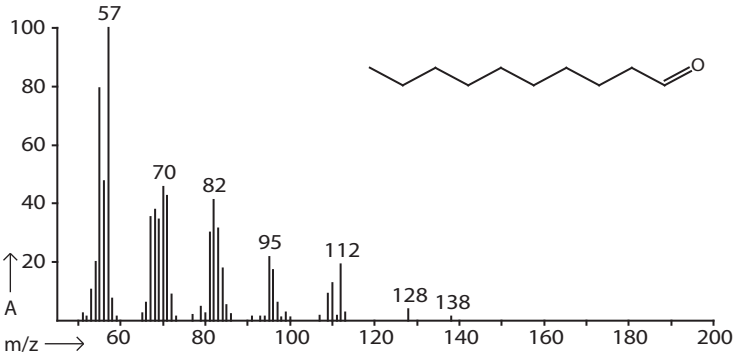
m/z (%) = 88 (100), 57 (47), 101 (28), 70 (25), 127 (21), 115 (7), 143 (2), 172 (1), 116 (0)



n-Dodecane
RI 1200

CAS 112-40-3
$C_{12}H_{26}$
FW 170

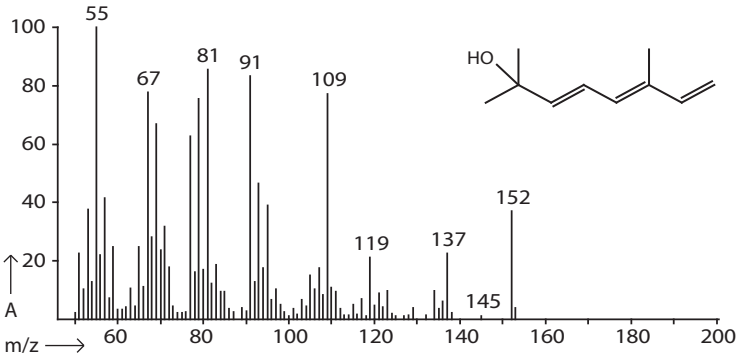
m/z (%) = 57 (100), 71 (71), 85 (47), 170 (13), 98 (10), 112 (9), 127 (6), 141 (3)



n-Decanal
RI 1204

CAS 112-31-2
$C_{10}H_{20}O$
FW 156

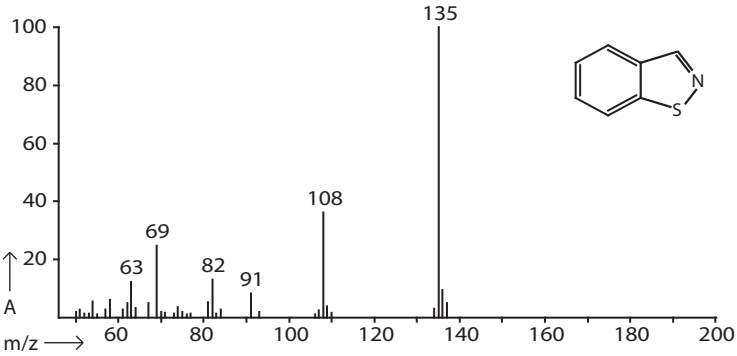
m/z (%) = 57 (100), 70 (46), 82 (41), 95 (22), 112 (19), 128 (4), 138 (1)



2,6-Dimethyl-3,5,7-octatrien-2-ol
RI 1207

$C_{10}H_{16}O$
FW 152

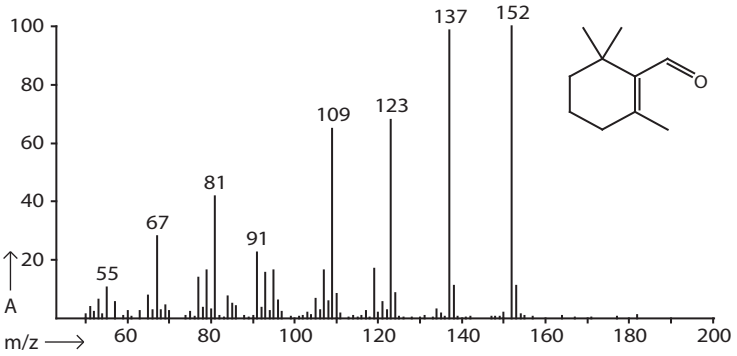
m/z (%) = 55 (100), 81 (86), 91 (83), 67 (78), 109 (77), 152 (37), 137 (22), 119 (21), 207 (1), 145 (1)



1,2-Benzisothiazole
RI 1215

CAS 272-16-2
C_7H_5NS
FW 135

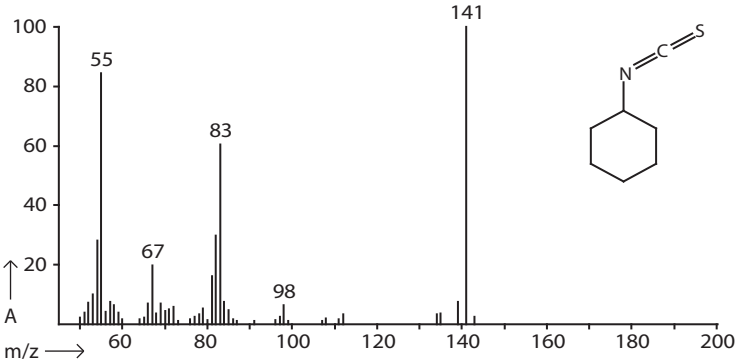
m/z (%) = 135 (100), 108 (36), 69 (25), 82 (13), 63 (12), 91 (8)



β-Cyclocitral
RI 1216

CAS 432-25-7
$C_{10}H_{16}O$
FW 152

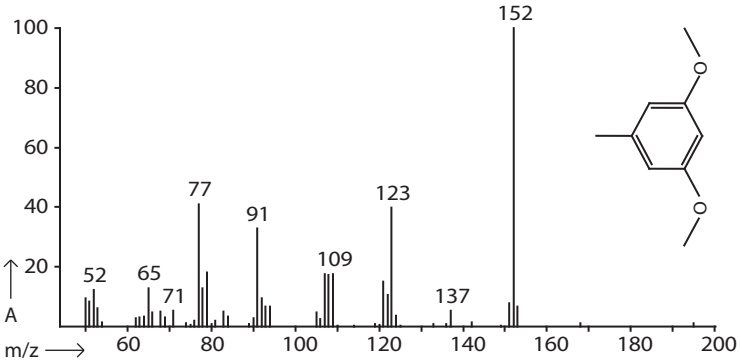
m/z (%) = 152 (100), 137 (99), 123 (68), 109 (65), 81 (42), 67 (28), 91 (22), 55 (11)



Cyclohexylisothiocyanate
RI 1223

CAS 1122-82-3
$C_7H_{11}NS$
FW 141

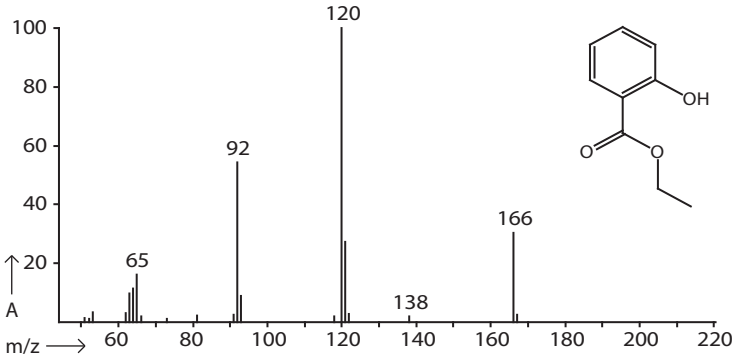
m/z (%) = 141 (100), 55 (84), 83 (60), 67 (20), 98 (7)



3,5-Dimethoxytoluene
RI 1263

CAS 4179-19-5
$C_9H_{12}O_2$
FW 152

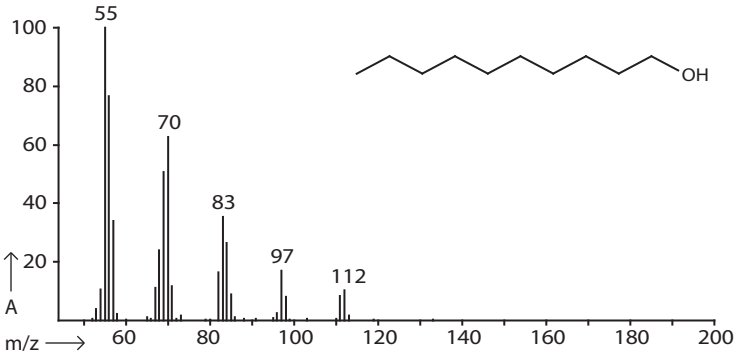
m/z (%) = 152 (100), 77 (41), 123 (40), 91 (33), 109 (18), 65 (13), 52 (12), 71 (5), 137 (5)



Ethyl salicylate
RI 1265

CAS 118-61-6
$C_9H_{10}O_3$
FW 166

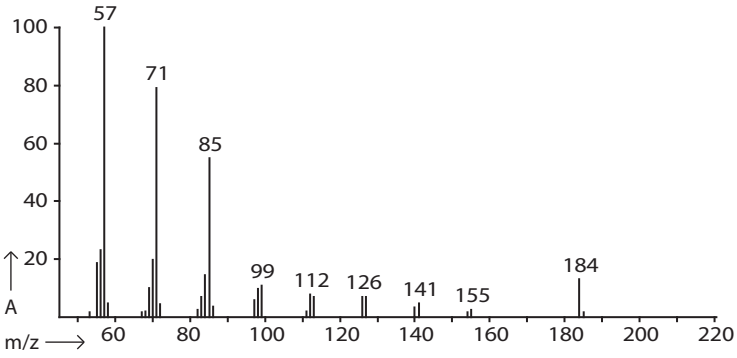
m/z (%) = 120 (100), 92 (54), 166 (30), 65 (16), 138 (2)



n-Decanol
RI 1271

CAS 112-30-1
$C_{10}H_{22}O$
FW 158

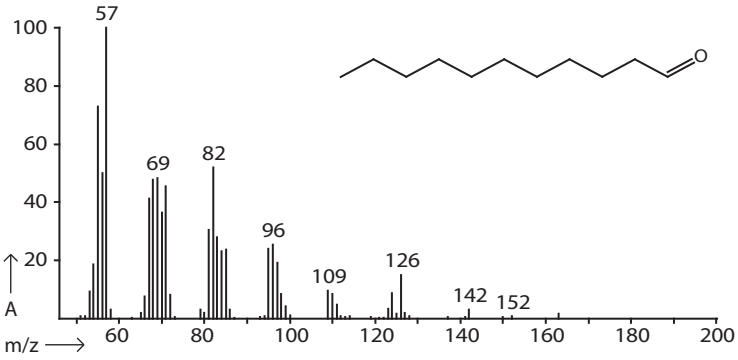
m/z (%) = 55 (100), 70 (63), 83 (35), 97 (17), 112 (10)



n-Tridecane
RI 1301

CAS 629-50-5
$C_{13}H_{28}$
FW 184

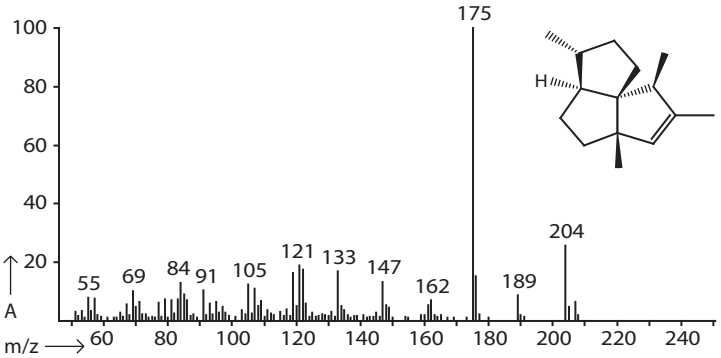
m/z (%) = 57 (100), 71 (79), 85 (55), 184 (13), 99 (11), 112 (8), 126 (7), 141 (5), 155 (2)



n-Undecanal
RI 1303

CAS 112-44-7
$C_{11}H_{22}O$
FW 170

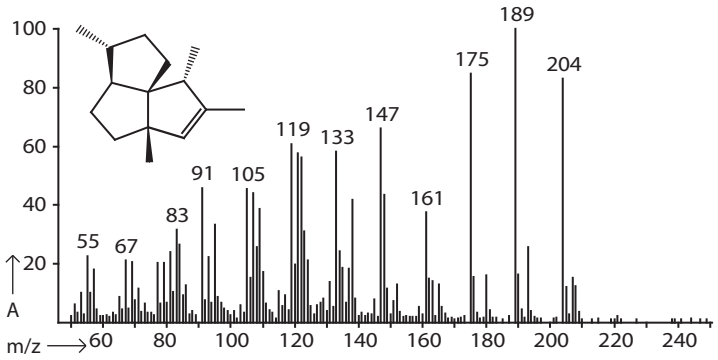
m/z (%) = 57 (100), 82 (52), 69 (48), 96 (26), 126 (15), 109 (10), 142 (3), 163 (2), 152 (1)



7α*H*-Silphiperfol-5-ene
RI 1321

$C_{15}H_{24}$
FW 204

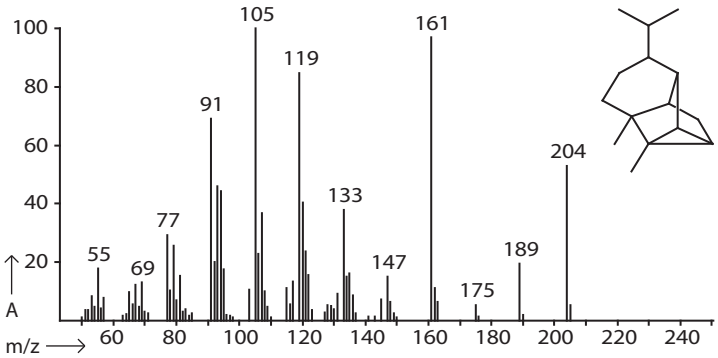
m/z (%) = 175 (100), 204 (26), 121 (19), 133 (17), 147 (13), 84 (13), 105 (12), 91 (10), 69 (10), 189 (9), 55 (8), 162 (7)



7β*H*-Silphiperfol-5-ene
RI 1340

$C_{15}H_{24}$
FW 204

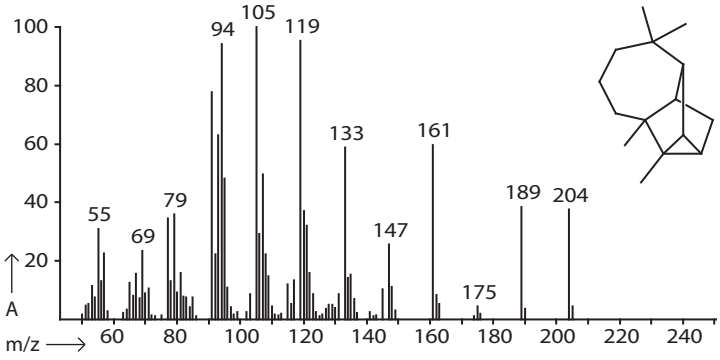
m/z (%) = 189 (100), 175 (85), 204 (83), 147 (66), 119 (61), 133 (58), 91 (46), 105 (46), 161 (38), 83 (32), 55 (22), 67 (21)



Cyclosativene
RI 1362

CAS 22469-52-9
$C_{15}H_{24}$
FW 204

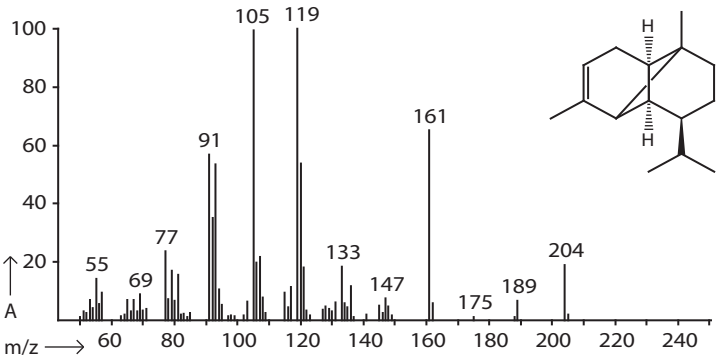
m/z (%) = 105 (100), 161 (97), 119 (85), 91 (69), 204 (53), 133 (38), 77 (29), 189 (20), 55 (18), 147 (15), 69 (13), 175 (5)



Longicyclene
RI 1366

CAS 1137-12-8
$C_{15}H_{24}$
FW 204

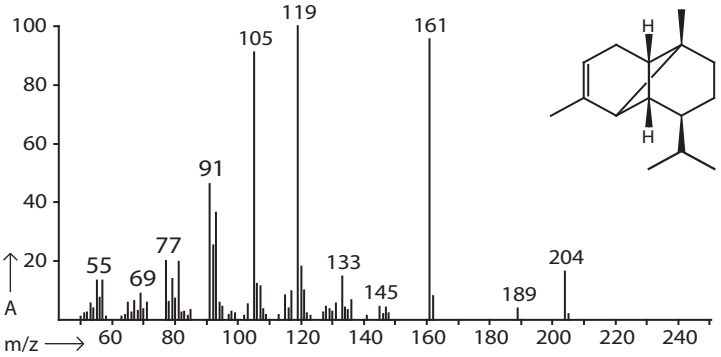
m/z (%) = 105 (100), 119 (95), 94 (94), 161 (60), 133 (59), 189 (39), 204 (38), 79 (36), 55 (31), 147 (26), 69 (23), 175 (4)



α-Ylangene
RI 1303

CAS 14912-44-8
$C_{15}H_{24}$
FW 204

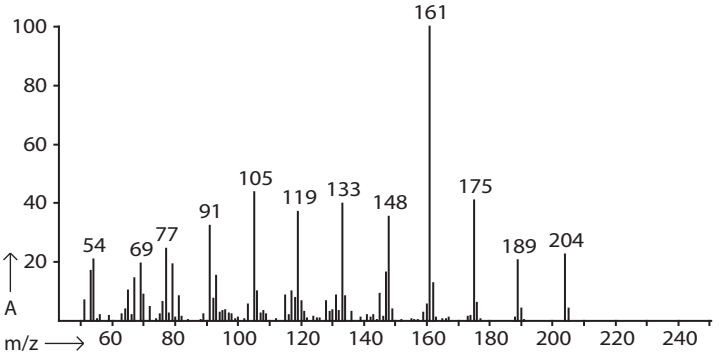
m/z (%) = 119 (100), 105 (99), 161 (65), 91 (57), 77 (24), 204 (19), 133 (18), 55 (14), 69 (9), 147 (8), 189 (7), 175 (1)



α-Copaene
RI 1374

CAS 3856-25-5
$C_{15}H_{24}$
FW 204

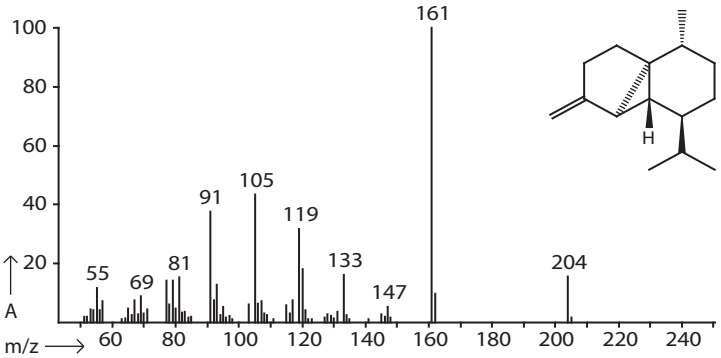
m/z (%) = 119 (100), 161 (96), 105 (91), 91 (46), 77 (20), 204 (16), 133 (15), 55 (13), 69 (9), 145 (4), 189 (4)



unidentified sesquiterpene
RI 1381

$C_{15}H_{24}$
FW 204

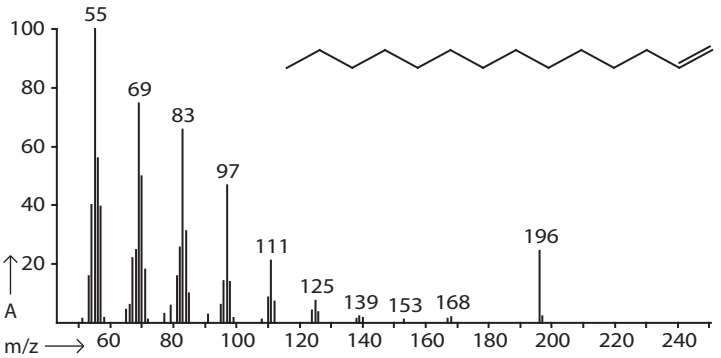
m/z (%) = 161 (100), 105 (44), 175 (41), 133 (40), 119 (37), 148 (35), 91 (32), 77 (25), 204 (23), 54 (21), 189 (21), 69 (20)



β-Cubebene
RI 1387

CAS 13744-15-5
$C_{15}H_{24}$
FW 204

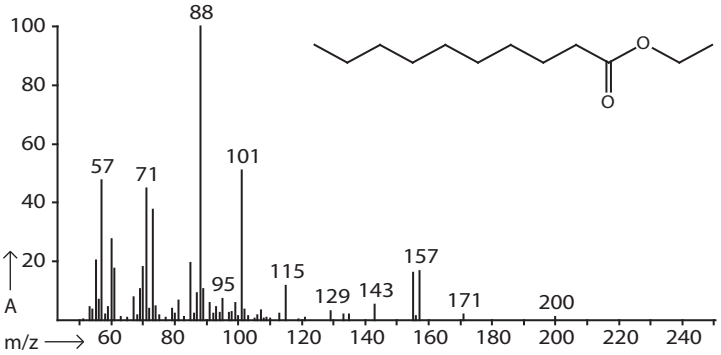
m/z (%) = 161 (100), 105 (44), 91 (38), 119 (32), 133 (16), 204 (16), 81 (15), 55 (12), 69 (9), 147 (5)



1-Tetradecene
RI 1389

CAS 1120-36-1
$C_{14}H_{28}$
FW 196

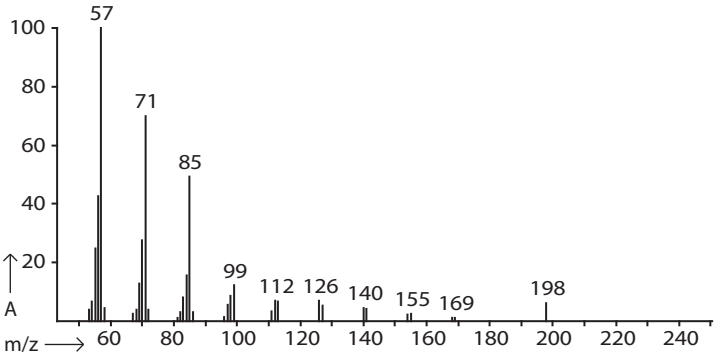
m/z (%) = 55 (100), 69 (75), 83 (66), 97 (47), 196 (25), 111 (21), 125 (7), 139 (2), 168 (2), 153 (1)



Ethyl decanoate
RI 1393

CAS 110-38-3
$C_{12}H_{24}O_2$
FW 200

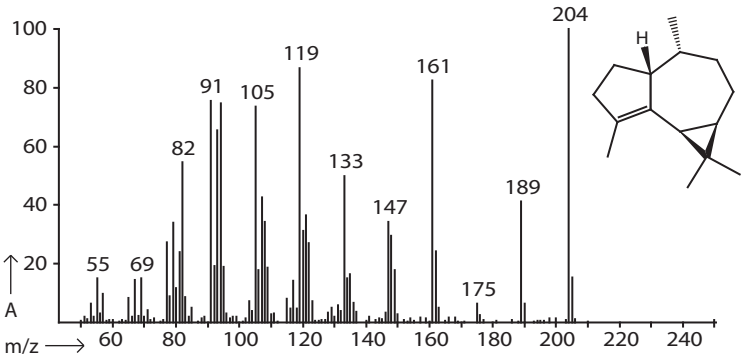
m/z (%) = 88 (100), 101 (51), 57 (48), 71 (45), 157 (17), 115 (12), 95 (7), 143 (5), 129 (3), 171 (2), 200 (1)



n-Tetradecane
RI 1400

CAS 629-59-4
$C_{14}H_{30}$
FW 198

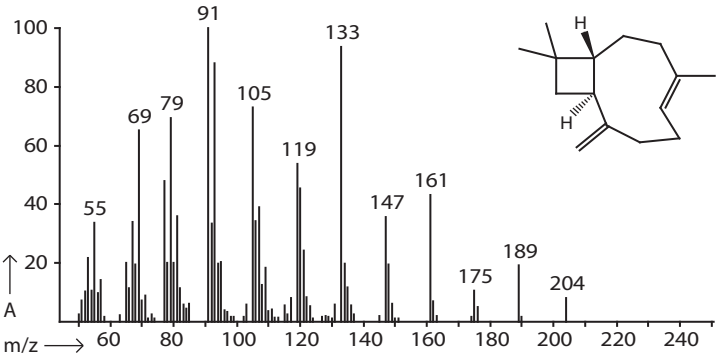
m/z (%) = 57 (100), 71 (70), 85 (49), 99 (12), 126 (7), 112 (7), 198 (6), 140 (4), 155 (3), 169 (1)



α-Gurjunene
RI 1405

CAS 489-40-7
$C_{15}H_{24}$
FW 204

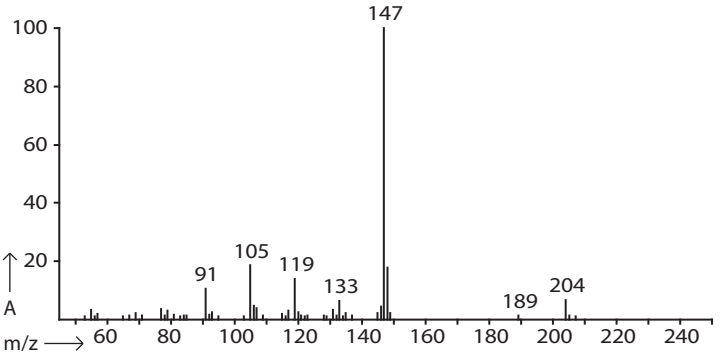
m/z (%) = 204 (100), 119 (87), 161 (83), 91 (75), 105 (73), 82 (55), 133 (50), 189 (41), 147 (34), 55 (15), 69 (15), 175 (6)



β-Caryophyllene
RI 1417

CAS 87-44-5
$C_{15}H_{24}$
FW 204

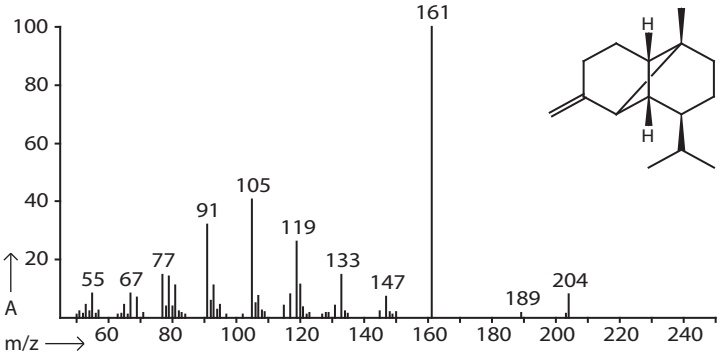
m/z (%) = 91 (100), 133 (94), 105 (73), 79 (69), 69 (65), 119 (54), 161 (43), 147 (36), 55 (34), 189 (19), 175 (11), 204 (8)



unidentified sesquiterpene
(presum. a Pacifigorgiane)
RI 1417

$C_{15}H_{24}$
FW 204

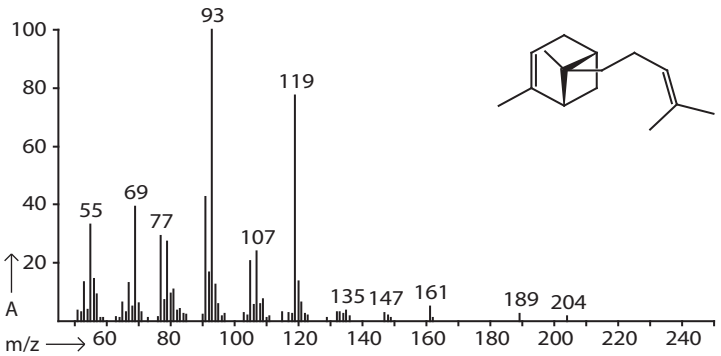
m/z (%) = 147 (100), 105 (19), 119 (14), 91 (11), 204 (7), 133 (6), 189 (1)



β-Copaene
RI 1427

CAS 18252-44-3
$C_{15}H_{24}$
FW 204

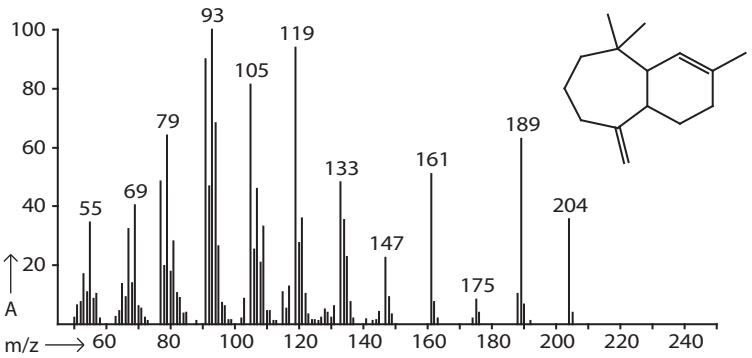
m/z (%) = 161 (100), 105 (41), 91 (32), 119 (26), 133 (15), 77 (15), 67 (8), 55 (8), 204 (8), 147 (7), 189 (2)



α-Bergamotene
RI 1432

CAS 13474-59-4
$C_{15}H_{24}$
FW 204

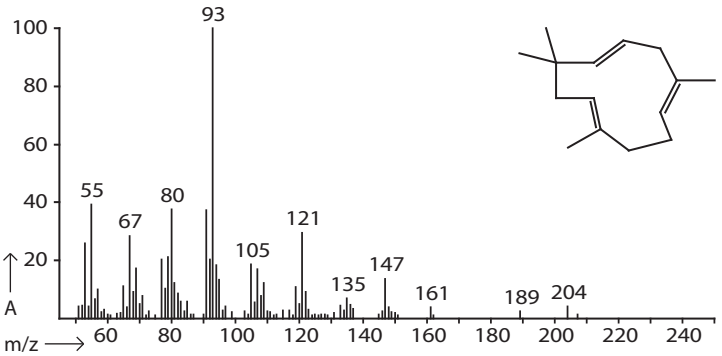
m/z (%) = 93 (100), 119 (77), 69 (39), 55 (33), 77 (29), 107 (24), 161 (5), 135 (4), 147 (3), 189 (3), 204 (2)



α-Himachalene
RI 1447

CAS 3853-83-6
$C_{15}H_{24}$
FW 204

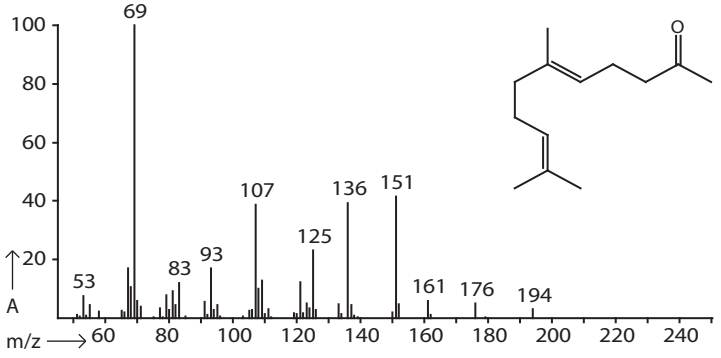
m/z (%) = 93 (100), 119 (94), 105 (81), 79 (64), 189 (63), 161 (51), 133 (48), 69 (40), 204 (36), 55 (35), 147 (23), 175 (8)



α-Humulene
RI 1451

CAS 6753-98-6
$C_{15}H_{24}$
FW 204

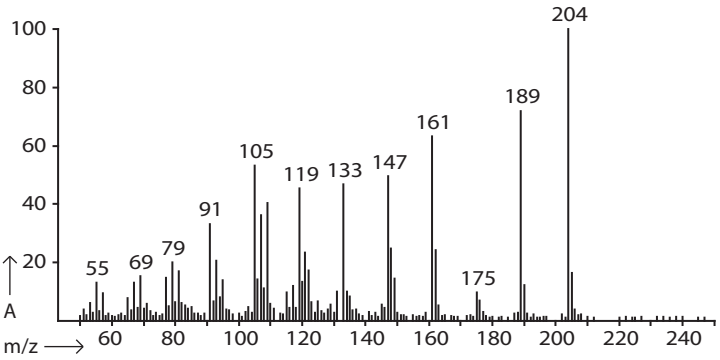
m/z (%) = 93 (100), 55 (39), 80 (38), 121 (30), 67 (29), 105 (19), 147 (14), 135 (7), 204 (4), 161 (4), 189 (3)



Geranylacetone
RI 1452

CAS 3796-70-1
$C_{13}H_{22}O$
FW 194

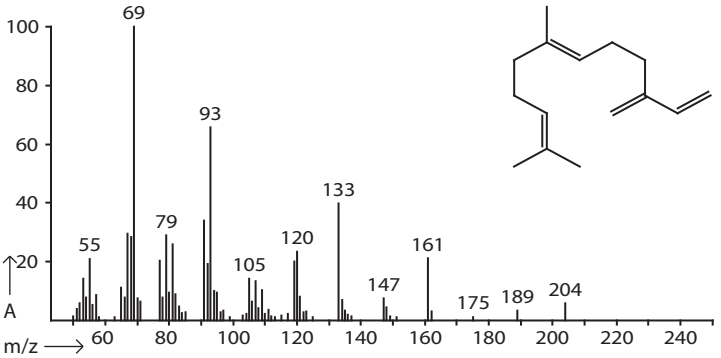
m/z (%) = 69 (100), 151 (41), 136 (39), 107 (39), 125 (23), 93 (17), 83 (12), 53 (8), 161 (6), 176 (5), 194 (3)



unidentified sesquiterpene
RI 1457

$C_{15}H_{24}$
FW 204

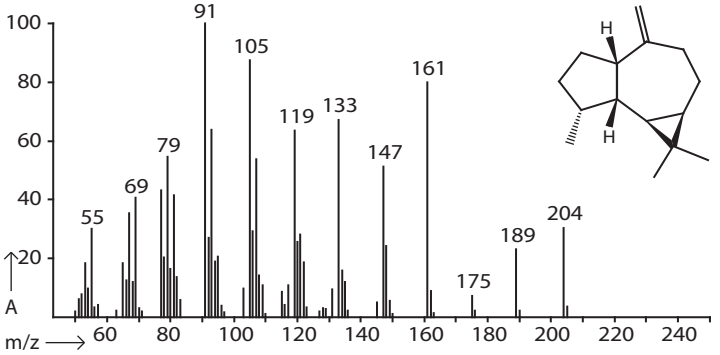
m/z (%) = 204 (100), 189 (72), 161 (63), 105 (53), 147 (50), 133 (47), 119 (46), 91 (33), 79 (20), 69 (15), 55 (13), 175 (10)



β-Farnesene
RI 1458

CAS 18794-84-8
$C_{15}H_{24}$
FW 204

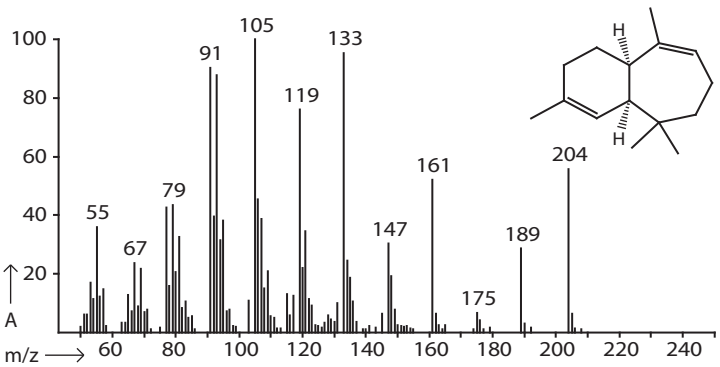
m/z (%) = 69 (100), 93 (66), 133 (40), 79 (29), 120 (23), 161 (21), 55 (21), 105 (14), 147 (7), 204 (6), 189 (3), 175 (1)



***allo*-Aromadendrene**
RI 1460

CAS 25246-27-9
$C_{15}H_{24}$
FW 204

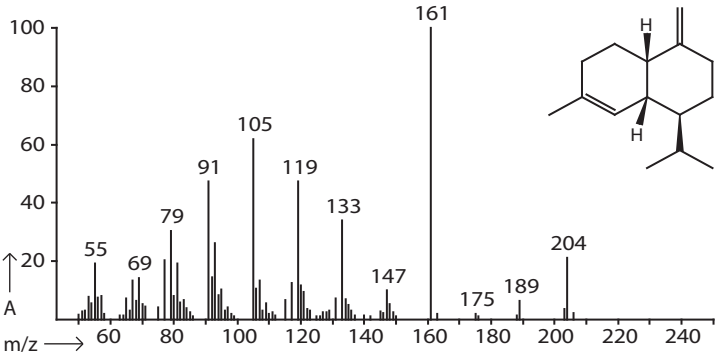
m/z (%) = 91 (100), 105 (87), 161 (80), 133 (67), 119 (64), 79 (55), 147 (51), 69 (41), 204 (30), 55 (30), 189 (23), 175 (7)



γ-Himachalene
RI 1476

CAS 53111-25-4
$C_{15}H_{24}$
FW 204

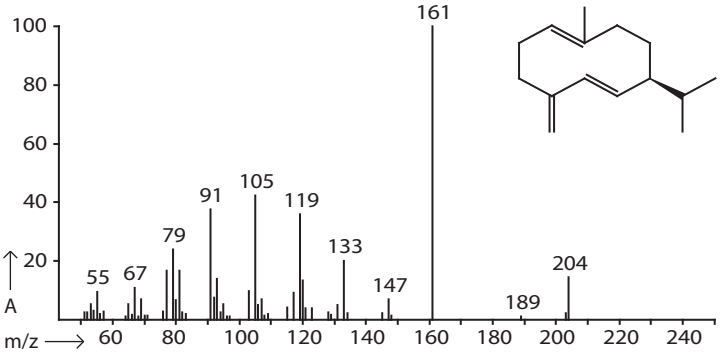
m/z (%) = 105 (100), 133 (95), 91 (90), 119 (76), 204 (56), 161 (52), 79 (43), 55 (36), 147 (30), 189 (29), 67 (24), 175 (7)



γ-Muurolene
RI 1478

CAS 30021-74-0
$C_{15}H_{24}$
FW 204

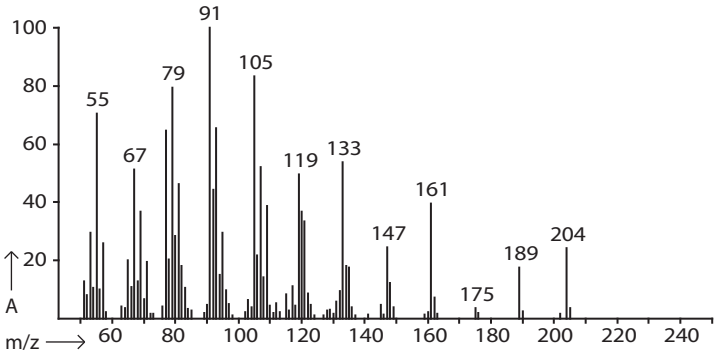
m/z (%) = 161 (100), 105 (62), 119 (47), 91 (47), 133 (34), 79 (30), 204 (21), 55 (19), 69 (14), 147 (10), 189 (6), 175 (2)



Germacrene D
RI 1479

CAS 23986-74-5
$C_{15}H_{24}$
FW 204

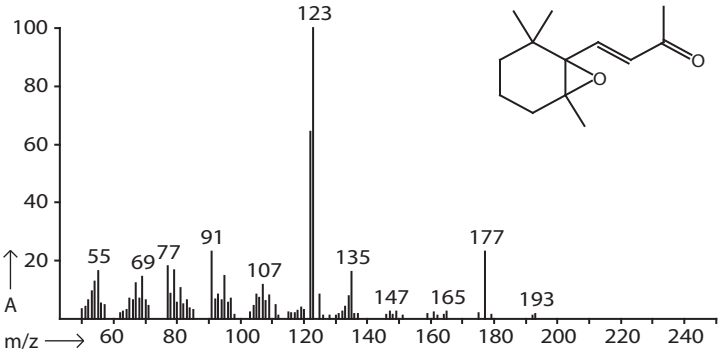
m/z (%) = 161 (100), 105 (42), 91 (38), 119 (36), 79 (24), 133 (20), 204 (15), 67 (11), 55 (10), 147 (7), 189 (1)



unidentified sesquiterpene
RI 1481

$C_{15}H_{24}$
FW 204

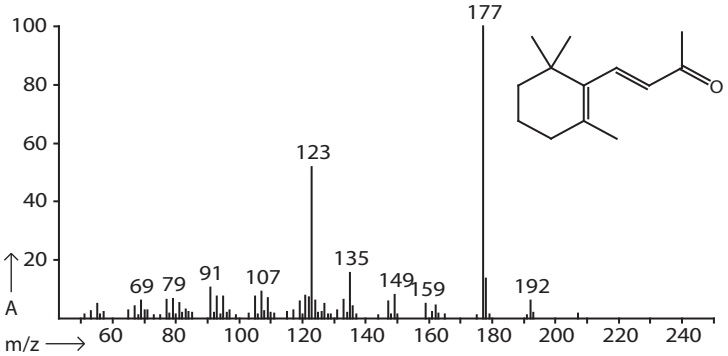
m/z (%) = 91 (100), 105 (83), 79 (79), 55 (71), 133 (54), 67 (51), 119 (50), 161 (40), 147 (24), 204 (24), 189 (18), 175 (4)



β-Ionone epoxide
RI 1483

CAS 23267-57-4
$C_{13}H_{20}O_2$
FW 208

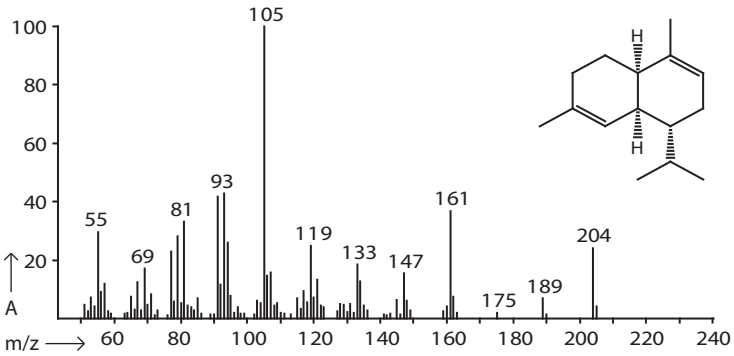
m/z (%) = 123 (100), 177 (23), 91 (23), 77 (18), 55 (17), 135 (16), 69 (14), 107 (12), 147 (3), 165 (3), 193 (2)



β-Ionone
RI 1488

CAS 79-77-6
$C_{13}H_{20}O$
FW 192

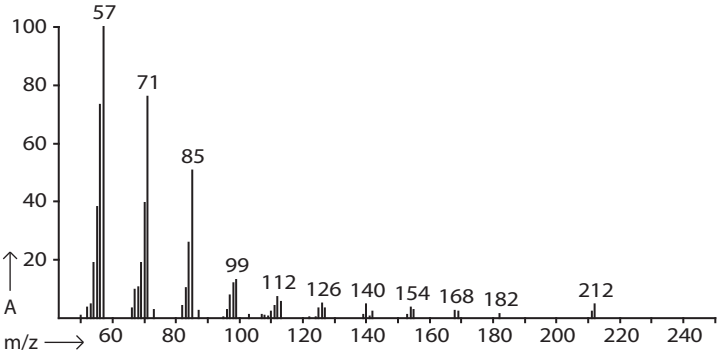
m/z (%) = 177 (100), 123 (52), 135 (16), 91 (11), 107 (9), 149 (8), 79 (7), 192 (6), 69 (6), 159 (5)



α-Muurolene
RI 1500

CAS 31983-22-9
$C_{15}H_{24}$
FW 204

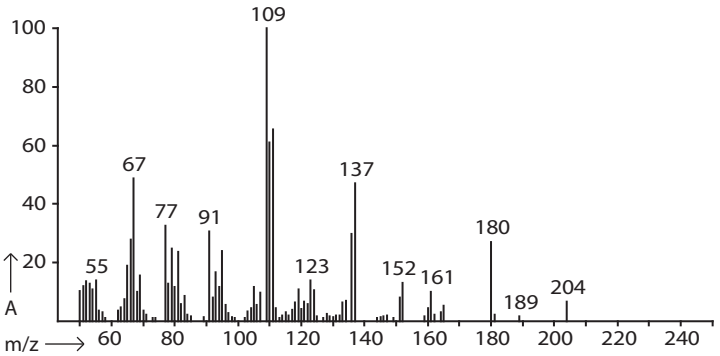
m/z (%) = 105 (100), 93 (43), 161 (37), 81 (33), 55 (30), 119 (25), 204 (24), 133 (18), 69 (17), 147 (15), 189 (7), 175 (2)



n-Pentadecane
RI 1500

CAS 629-62-9
$C_{15}H_{32}$
FW 212

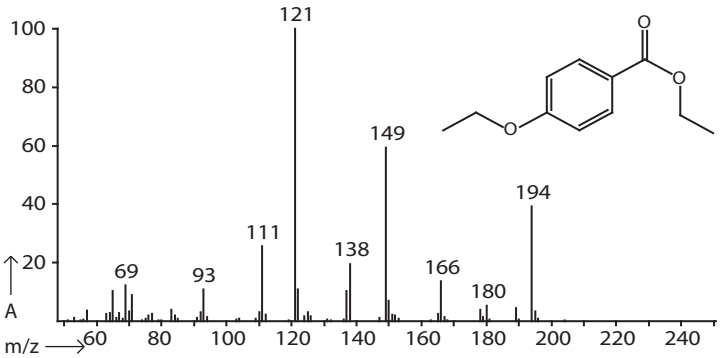
m/z (%) = 57 (100), 71 (76), 85 (51), 99 (13), 112 (7), 126 (5), 140 (5), 212 (5), 154 (4), 168 (3), 182 (1)



unidentified sesquiterpene
Trichodiene or β-Bazzanene
RI 1521

$C_{15}H_{24}$
FW 204

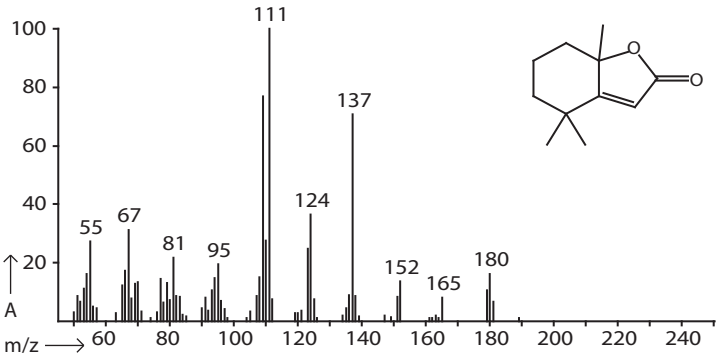
m/z (%) = 109 (100), 67 (49), 137 (47), 77 (33), 91 (31), 180 (27), 55 (14), 123 (14), 152 (13), 161 (10), 204 (7), 189 (2)



Ethyl 4-ethoxybenzoate
RI 1524

CAS 23676-09-7
$C_{11}H_{14}O_3$
FW 194

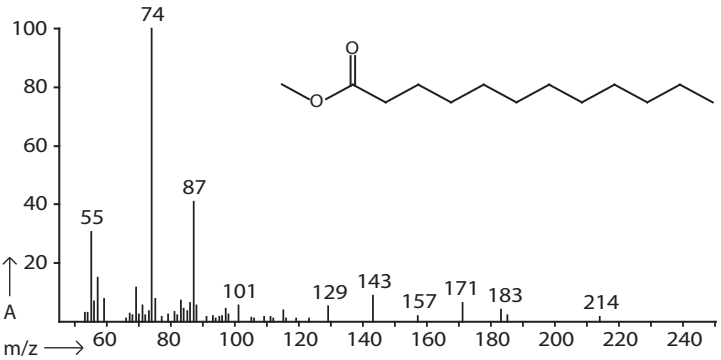
m/z (%) = 121 (100), 149 (59), 194 (39), 111 (26), 138 (19), 166 (14), 69 (12), 93 (11), 180 (5)



Dihydroactinidiolide
RI 1524

CAS 17092-92-1
$C_{11}H_{16}O_2$
FW 180

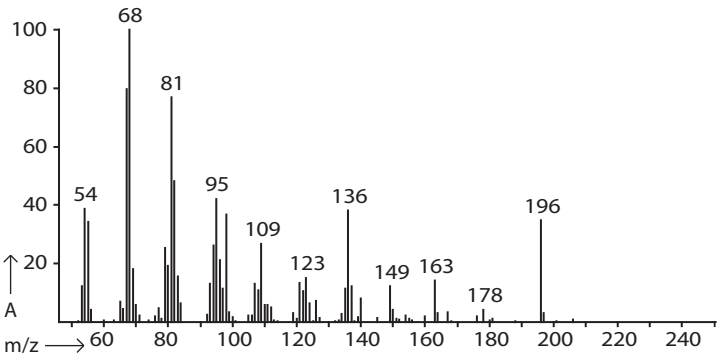
m/z (%) = 111 (100), 137 (71), 124 (37), 67 (31), 55 (27), 81 (22), 95 (19), 180 (16), 152 (14), 165 (8)



Methyl dodecanoate
RI 1525

$C_{13}H_{26}O_2$
FW 214

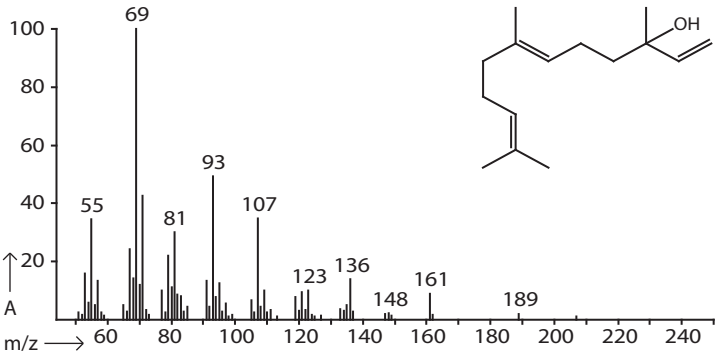
m/z (%) = 74 (100), 87 (41), 55 (31), 143 (9), 171 (7), 101 (6), 129 (5), 183 (4), 157 (2), 214 (2)



unidentified compound
RI 1532

FW 196

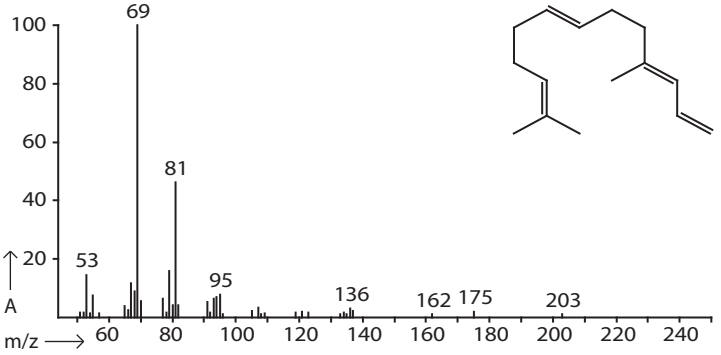
m/z (%) = 68 (100), 81 (77), 95 (42), 54 (39), 136 (38), 196 (35), 109 (27), 123 (15), 163 (14), 149 (12), 178 (4)



***E*-Nerolidol**
RI 1564

CAS 40716-66-3
$C_{15}H_{26}O$
FW 222

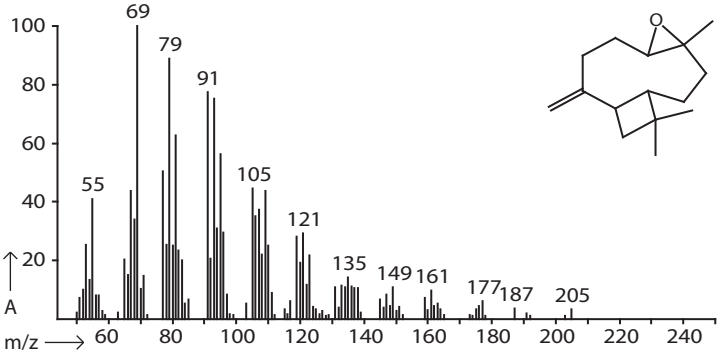
m/z (%) = 69 (100), 93 (49), 107 (35), 55 (35), 81 (30), 136 (14), 123 (10), 161 (9), 148 (2), 189 (2)



4,8,12-Trimethyltrideca-1,3,7,11--tetraene (TMTT)
RI 1578

CAS 62235-06-7
$C_{16}H_{26}$
FW 218

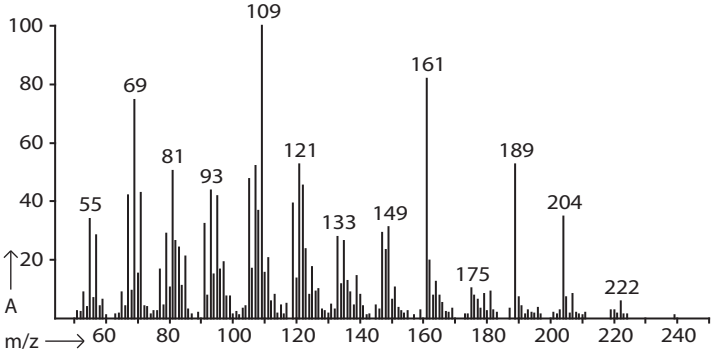
m/z (%) = 69 (100), 81 (46), 53 (14), 95 (8), 136 (3), 175 (2), 203 (1), 162 (1)



Caryophyllene oxide
RI 1579

CAS 1139-30-6
$C_{15}H_{24}O$
FW 220

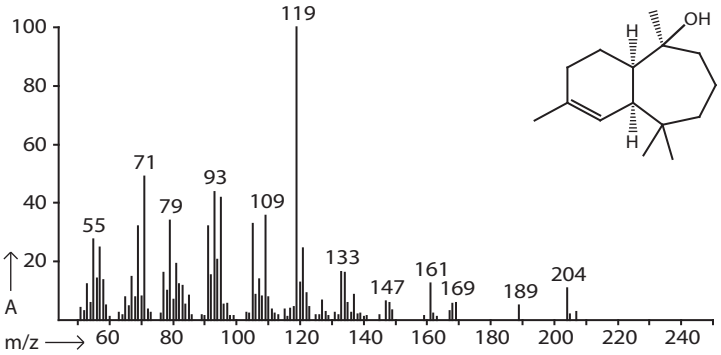
m/z (%) = 69 (100), 79 (89), 91 (78), 105 (45), 55 (41), 121 (29), 135 (14), 149 (11), 161 (10), 177 (6), 187 (4), 205 (3)



unidentified sesquiterpenoid
Viridiflorol or Globulol
RI 1587

$C_{15}H_{26}O$
FW 222

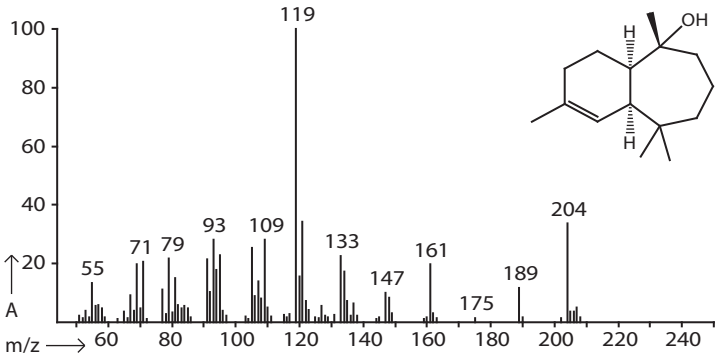
m/z (%) = 109 (100), 161 (82), 69 (75), 189 (53), 121 (53), 81 (50), 93 (44), 204 (35), 55 (34), 149 (31), 133 (28), 175 (10), 181 (9), 222 (6)



2-Himachalen-7β-ol
RI 1637

$C_{15}H_{26}O$
FW 222

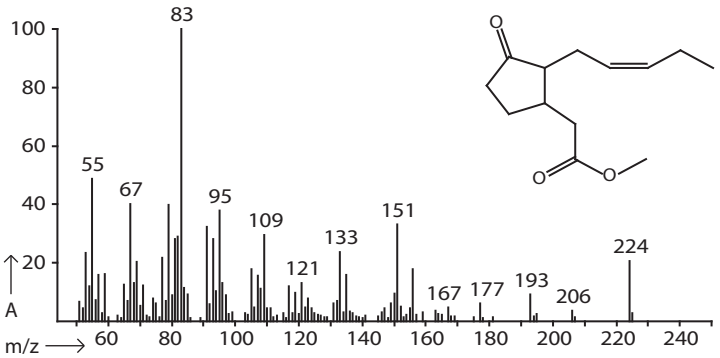
m/z (%) = 119 (100), 71 (49), 93 (44), 109 (36), 79 (34), 55 (28), 133 (16), 161 (12), 204 (11), 147 (6), 169 (6), 189 (5)



β-Himachalol
RI 1645

CAS 1891-45-8
$C_{15}H_{26}O$
FW 222

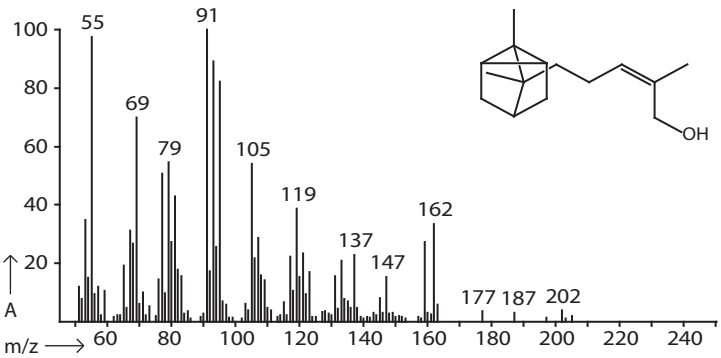
m/z (%) = 119 (100), 204 (34), 109 (28), 93 (28), 133 (23), 79 (22), 71 (21), 161 (20), 55 (13), 189 (12), 147 (10), 175 (1)



Methyl jasmonate
RI 1643

CAS 1211-29-6
$C_{13}H_{20}O_3$
FW 224

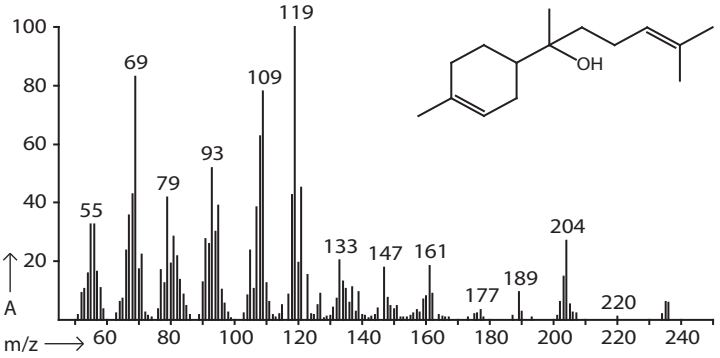
m/z (%) = 83 (100), 55 (49), 67 (40), 95 (38), 151 (33), 109 (29), 133 (24), 224 (21), 121 (13), 193 (9), 177 (6), 167 (5), 206 (4)



α-Santalol
RI 1669

CAS 115-71-9
$C_{15}H_{24}O$
FW 220

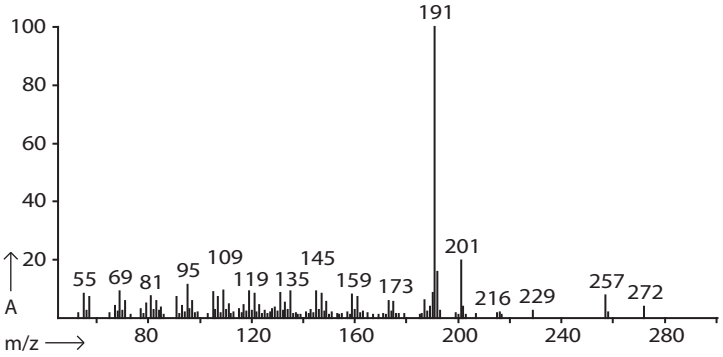
m/z (%) = 91 (100), 55 (98), 69 (70), 79 (55), 105 (54), 119 (39), 162 (33), 137 (23), 147 (15), 202 (4), 177 (4), 187 (3)



α-Bisabolol
RI 1679

CAS 515-69-5
$C_{15}H_{26}O$
FW 222

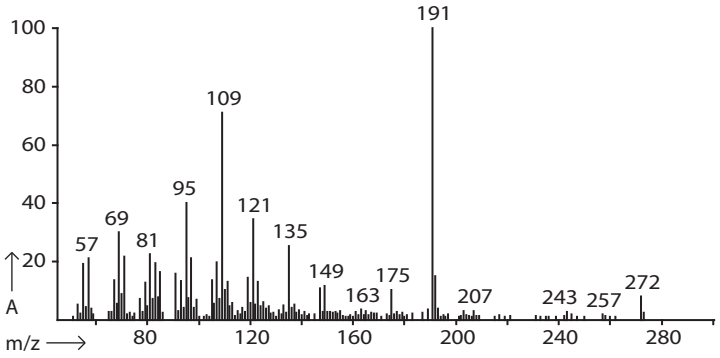
m/z (%) = 119 (100), 69 (83), 109 (78), 93 (52), 79 (42), 55 (33), 204 (27), 133 (20), 161 (18), 147 (18), 189 (9), 177 (3), 220 (1)



unidentified diterpene
RI 1806

$C_{20}H_{32}$
FW 272

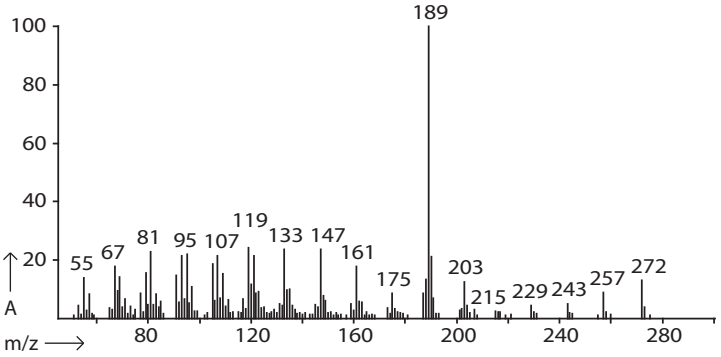
m/z (%) = 191 (100), 201 (20), 95 (11), 109 (10), 119 (9), 69 (9), 145 (9), 135 (9), 55 (8), 159 (8), 257 (8), 81 (7), 173 (6), 272 (4), 229 (2), 216 (2)



unidentified diterpene
RI 1876

$C_{20}H_{32}$
FW 272

m/z (%) = 191 (100), 109 (71), 95 (40), 121 (35), 69 (30), 135 (25), 81 (22), 57 (21), 149 (12), 175 (10), 272 (8), 163 (4), 155 (3), 207 (3), 243 (3), 257 (2), 217 (2), 231 (1)



unidentified diterpene
RI 1983

$C_{20}H_{32}$
FW 272

m/z (%) = 189 (100), 119 (24), 147 (24), 133 (24), 81 (23), 95 (22), 107 (21), 161 (18), 67 (18), 55 (14), 272 (13), 203 (13), 257 (9), 175 (9), 243 (5), 229 (4), 215 (3), 313 (3)

Appendix III
Glossary of statistical terms

Terms in *italics* indicate cross-references

ANOVA (Analysis of Variance) serves the comparison of two or more normally distributed groups with equal variance. By comparing the variances, the test tells whether the group means are significantly different or not. It does not indicate which group is different from which group. For this, *post hoc tests* have to be applied.

Binomial tests are used to assess the probability of a result, if two mutually exclusive outcomes are to be compared, i.e. whether the outcome is strictly random or whether there is discrimination in favour of one of them.

Confusion matrices can be used to summarise the predictive accuracy of a *Linear Discriminant Analysis* as estimated by *cross-validation*. It specifies the proportion of cases in which a sample was correctly or falsely classified.

Contingency tables are tables of frequencies. They are used to summarise categorical data, revealing relationships between variables. Significance can be tested using *Fisher's exact test*.

Cross-validation is a method to test the predictive accuracy of a model. A "learning set", consisting of actually measured samples, is used to build up a model. Then, new samples, the "test set", are allocated to the groups by the classification rules of the model in question. The results give the proportion of correct classification, which can be summarised in a *confusion matrix*.

Fisher's exact test. *Contingency tables* can be analysed using Fisher's exact test in order to tell whether the association between different variables is merely random or not. Small *p*-values indicate that the result is unlikely to be caused by chance.

Linear Discriminant Analysis (LDA) can be used both as exploratory method and for classification. It aims to determine which variables discriminate between given groups. To do so, multivariate observations are linearly combined and transformed, such that the derived populations are separated as much as possible.

Multicollinearity refers to cases in which one or more variables are linear functions of other variables, i.e. variables are linearly correlated. In such instances, it cannot be determined which of the variables accounts for the variance of the dependent variable (group).

Multidimensional Scaling (MDS) is an exploratory method that aims to map objects (samples) in a low-dimensional space so that the resulting representation reproduces the original distances or similarities as closely as possible. The distances between any data point in the *ordination* thus indicate the respective similarities or correlations.

Multivariate data analysis. Altogether, methods of multivariate data analysis aim to extract relevant information from complex data. Thereby, hidden dynamics underlying the process might be revealed and represented in a simplified manner. The number of measurement types or variables is the dimension of the data set. Multivariate data analysis can be used for data reduction or structural simplification. This aims to find a representation as simple as possible without losing valuable information, which should make the interpretation easier. Furthermore, groups of similar objects or variables can be created based on measured characteristics. This also includes the investigation of dependencies among variables and mutual interrelationships of variables. Certain methods, moreover, allow predictions to be made, and thus facilitate hypothesis construction and testing. Basically, multivariate data analysis serves three purposes: exploratory methods provide a basis for visualisation of complex patterns; classification algorithms aim to find specific distinctions between given groups; such algorithms can also be used for allocation, that is, to assign new samples to previously defined groups.

Non-metric Multidimensional Scaling (NMDS) is a variant of *Multidimensional Scaling*. Instead of using original similarities, rank orders are used for scaling.

Ordination is a low-dimensional plot representing multivariate data.

***post hoc* tests** are performed after significant differences of means have been found by *ANOVA*. All combinations of groups are compared in order to find which groups actually differ from each other. Several tests are available with varying degrees of stringency.

Shapiro-Wilk test is applied to assess normal distribution of data.

Stress statistics give a numerical measure for the closeness of the fit in *Multidimensional Scaling*. It indicates the proportion of the original data that is not ideally depicted in the calculated scale. The smaller the value, the better the fit.

***t*-test** is a common test for the difference of means between two groups.

Type I error (α) occurs, if the null hypothesis is rejected when it is actually true. The type I error is defined by the significance level. It sets the probability of falsely rejecting the null hypothesis. The α-error is inversely related to the *type II error*. The smaller α gets, the larger is the chance for a type II error.

Type II error (β) occurs, if the null hypothesis is not rejected even though it is false. It depends on the sample number, the degree of differences between groups, and the power of the statistical test applied.

Wilk's lambda is a statistical test in multivariate analysis to determine whether there are differences of means between combinations of variables for specified groups.

Literature

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