The Effect of Root Volatiles on the Orientation Behaviour of Cockchafer Larvae in the Soil

Dissertation

In Partial Fulfilment of the Requirements for the Degree

Doctor of Forest Sciences (Dr. forest.)

of the Faculty of Forest Sciences and Forest Ecology

Georg-August-University Göttingen

submitted by

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Göttingen, 2010

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Date of oral examination: 03.12.2010

Summary

The present study investigates the effect of root volatiles on the orientation behaviour of cockchafer larvae *Melolontha hippocastani* in the soil. Cockchafer larvae are known as severe pests in different crops and forests (e.g. reforestations of oaks), vineyards, orchards, grassland, pastures and meadows. In order to contribute to a better understanding of the mechanisms of food choice belowground, the present study adressed three relevant aspects of their chemical ecology.

In the first part, volatile organic compounds (VOCs) of several potential host plant roots (*Quercus* sp. *-Q. rubra and Q. petraea-*, *Aesculus hippocastanum*, *Daucus carota, Daucus carota* ssp*. sativus, Solanum tuberosum, Achillea millefolium, Cirsium arvense, Plantago lanceolata, Taraxacum officinale and Calamagrostis sp.*) and shoots (*Quercus* sp., *A. hippocastanum*, *Daucus carota* ssp*. sativus,* and *Solanum tuberosum*) were investigated and analysed by using gas chromatography – mass spectrometry (GC-MS). Additionally, the roots of *Quercus* sp., *A. hippocastanum, Daucus carota* ssp*. sativus and Solanum tuberosum* were mechanically damaged by a pair of scissors, or damaged by larval feeding during several days. The obtained volatile patterns of the damaged roots differ clearly from the undamaged ones.

In a second step, electrophysiological methods were employed to record sensory reactions of the detached larval antennae to several compounds identified in the first step. Volatile emissions of *Quercus* sp. (undamaged, mechanically damaged or damaged by larval feeding) were investigated in detail. By using electroantennography (EAG), changes in the receptor potential elicited by odour stimuli were recorded. However, reproducible results could be obtained only in the autumn- and winter-months. The following compounds elicited reproducible dose-response curves in 2007 and 2008 as a result of odour stimuli based on a silicon oil dilution series in concentrations ranging from 10^{-7} to 10^{-2}: anisol, (1R)-camphor, (1S)-camphor, 1.8-cineol, β-caryophyllene, furanoid-trans-linalooloxide, 3-octanone and terpinolene. To identify biologically active compounds, the electro-antennographic detection (EAD) was applied as one appropriate method in chemical ecology. In the study presented here, this method did not provide any

reproducible results throughout one season. However, over some period of time, a small part of the tested antennae showed similar responses to special compounds, which were anisol, 1.8-cineol, (1R)-camphor, 3-octanone and furanoid trans-linalooloxid.

The third part of this study tested the behavioural response of cockchafer larvae on selected compounds. A dual choice test setup was designed to study the behavioural orientation of the belowground living larvae. The compounds, which were able to attract the larvae of *M. hippocastani* were anisol, 1,8-cineol, and terpinolene. A repellent effect was elicited by acetone (as a representative of the compounds with a high vapour pressure) and β-caryophyllene.

General results:

- The emitted shoot volatiles of *Quercus* sp. and *A. hippocastanum* differ clearly from the emitted root volatiles.
- Electrophysiologically active compounds could (in a reproducible manner) only be detected in special time periods during the season, which suggests that seasonal and maybe also circadian rhythms play an important role in the complex system of impulse processing in cockchafer larvae.
- Distinct orientation behaviour of the larvae of *M. hippocastani* could be observed in dual choice tests with several compounds found in root volatile samples of *Quercus* sp. damaged by larval feeding. Attractive and repellent compounds could be identified.
- The antennal lobes (ALs, first central processing unit for olfactory information processing in the insect brain) of 3rd instar of *M. hippocastani* contain a high number of glomeruli, which are regarded as the functional subunits of odour discrimination. Therefore, a highly developed odour discrimination ability of the cockchafer larvae is indicated.

These findings are, to our knowledge, the first proof that larvae of *M. hippocastani* are able to perceive several volatiles emitted by roots of their host plants *Quercus* sp. in electrophysiological and behavioural tests. As a consequence, in dual choice tests they react with attractive or repellent behaviour. As a practical approach, these results could be used as a basis for semi-field studies towards pest control through attractive or repellent volatile compounds. However, in this context, additional investigations concerning the compatibility of the volatile composition (either single

compounds or volatile mixtures) and the carrier substance on floral and faunal organisms activity (especially on non-target organisms) are necessary.

Zusammenfassung

In der vorliegenden Dissertation wurde der Einfluss von Wurzelvolatilen auf das Verhalten von unterirdisch lebenden Waldmaikäferengerlingen *Melolontha hippocastani* untersucht. Diese Larven sind als ernstzunehmende Schadorganismen in Wäldern (z.B. Aufforstungsflächen von Eichenbeständen) sowie in verschiedenen Kulturen wie Wein-und Obstgärten, Wiesen und Weiden gefürchtet. Drei Aspekte der Chemischen Ökologie wurden näher beleuchtet, um zu einem besseren Verständnis der Verhaltensmechanismen bei der Wirtsfindung unterirdisch lebender Maikäferengerlinge beizutragen.

Im ersten Teil wurden volatile Verbindungen (VOCs, Volatile Organic Compounds) von Wurzeln verschiedener potentieller Wirtspflanzen wie *Quercus* sp. (*Q. rubra* und *Q. petraea*), *Aesculus hippocastanum*, *Daucus carota, Daucus carota* ssp*. sativus, Solanum tuberosum, Achillea millefolium, Cirsium arvense, Plantago lanceolata, Taraxacum officinale* und *Calamagrostis sp.* sowie die Sprossvolatile von *Quercus* sp., *A. hippocastanum*, *Daucus carota* ssp*. sativus* und *Solanum tuberosum* gesammelt und mittels Gaschromatographie – Massenspektrometrie (GC-MS) analysiert und identifiziert. Zusätzlich wurden die Wurzeln von *Quercus* sp., *A. hippocastanum, Daucus carota* ssp*. sativus* und *Solanum tuberosum* mit einer Schere bzw. mit einem Messer zerschnitten oder den Maikäferengerlingen für mehrere Tage zum Fraß angeboten. Die Volatilenmuster der verletzten Wurzeln unterschieden sich deutlich von jenen der unverletzten.

Im zweiten Teil wurde mittels elektrophysiologischer Methoden die Reaktion der isolierten Maikäfer-Engerlingsantenne auf die identifizierten Wurzelvolatile ermittelt. Die Duftmuster von Eichenwurzeln *Quercus* sp. (unverletzt, mechanisch verletzt, oder von den Engerlingen angefressen) wurden genauer untersucht. Mittels Elektroantennographie können olfaktorische Reaktionen der Insektenantenne durch Ableitung elektrischer Signale aufgezeichnet werden. Reproduzierbare Ergebnisse konnten jedoch ausschließlich in den Herbst- und Wintermonaten erzielt werden. Folgende Substanzen lieferten 2007 und 2008 in Verdünnungsreihen (10^{-7} to 10^{-2}, als Lösungsmittel wurde Silikonöl verwendet) reproduzierbare Antennenreaktionen auf Duft-Stimuli: Anisol, (1R)-Campher, (1S)-Campher, 1,8-Cineol, β-Caryophyllen, die furanoide Form von trans-Linalooloxid, 3-Octanon und Terpinolen. Zur

Ermittlung biologisch aktiver Substanzen kann die Insektenantenne als elektroantennographischer Detektor (EAD) genutzt werden. Nur ein kleiner Teil der getesteten Maikäfer-Engerlingsantennen lieferte jedoch ähnliche Ergebnisse für Anisol, 1,8-Cineol, (1R)-Campher, 3-Octanon und für die furanoide Form von trans-Linalooloxid, und das jeweils nur ausschließlich während bestimmter Zeitfenster.

Im dritten Teil wurde die Verhaltensreaktion der Maikäferengerlinge auf ausgewählte Substanzen untersucht. Ein dualer Versuchsaufbau wurde entwickelt um das Verhalten unterirdisch lebender Larven zu untersuchen. Anisol, 1,8-Cineol und Terpinolen übten eine anziehende Wirkung auf die Engerlinge aus, während Aceton und β-Caryophyllen abstoßend wirkten.

Allgemeine Ergebnisse:

- Die emittierten Sprossvolatile von *Quercus* sp. und *A. hippocastanum* unterscheiden sich deutlich von den entsprechenden Wurzelvolatilen.
- Reproduzierbare Ergebnisse in elektrophysiologischen Versuchen konnten ausschließlich in den Herbst- und Wintermonaten erzielt werden. Diese Tatsache legt nahe, dass im komplexen neuronalen Reiz-Verarbeitungssystem der Maikäferlarven saisonale und vielleicht auch circadiane Rhythmen eine wichtige Rolle spielen könnten.
- In Verhaltensexperimenten konnte ein deutliches Orientierungsverhalten der Larven von *M. hippocastani* in Reaktion auf verschiedene Wurzelvolatile von angefressenen *Quercus* sp. beobachtet werden. Attraktive und repellente Substanzen konnten identifiziert werden. Die Antennalloben (ALs, erste zentrale Verarbeitungseinheiten für die olfaktorische Informationsverarbeitung im Insektengehirn) von *M. hippocastani* im 3. Larvenstadium beherbergen eine große Anzahl olfaktorischer Glomeruli. Diese werden als die funktionellen Untereinheiten in der Geruchsunterscheidung angesehen. Daraus ergibt sich eine hoch entwickelte Fähigkeit zur Geruchsunterscheidung und -erkennung bei den Maikäferengerlingen.

Nach unserem Kenntnisstand liefern diese Ergebnisse den ersten Nachweis, dass Maikäferengerlinge in der Lage sind, verschiedene Wurzelvolatile ihrer Wirtspflanzen *Quercus* sp. in elektrophysiologischen und verhaltensbiologischen Versuchen wahrzunehmen. In letzteren konnten sowohl attraktive als auch repellente Substanzen ermittelt werden.

In einer praktischen Anwendung könnten diese Ergebnisse als Basis für Freilandversuche in der ökologischen Schädlingsbekämpfung dienen. Zusätzliche Untersuchungen etwa in Form von Biokompatibilitätstests sind jedoch nötig, um das eventuelle Schadpotenzial der volatilen Substanzen auf Nicht-Zielorganismen abschätzen zu können.

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CHAPTER 1

General Introduction

1.1 Introduction

Each year significant parts of crop yields of orchards and in forestry are being destroyed by insect pests. Several phytophagous beetles are known as pests on crops, forests, and stored products. Moreover, they can act as vectors of fungi and viral plant diseases. As carnivores and detritivores many species have beneficial functions by feeding on herbivorous insects (Francke & Dettner 2005).

In the meeting “International Organisation of Biological Control (IOBC)” 1995 it was decided that the larvae of certain scarab beetles should be considered to be important soil pests in forest and agriculture (Keller et al. 1997). The major damage is caused by the two cockchafer beetles also called “maybugs“ *Melolontha hippocastani* and *M. melolontha*, the summer chafer beetle *Amphimallon solstitialis* and the garden chafer beetle *Phyllopertha horticola.* Mainly the cockchafer larvae are known as pests in different crops: forest areas (e.g. Christmas trees plantations), vineyards, orchards, grassland, pastures and meadows (Schwerdtfeger 1970, Berlese 1901, Hill 1987, Brauns 1991, Pötsch et al. 1997).

Recently, the organophosphate insectizide Dimethoate was applied twice by helicopter in “Klingsackertanne”, a part of the urban forest of Pfungstadt (Germany, mostly *Pinus* with several intermediated *Quercus*, rarely *Fagus*) because of the very high density of larvae in the soil (about 70 specimen per square metre, application on May 6th and 27th 2010). This pesticide belongs to the neurotoxic substances and of course the application provoked very controversial discussions (Echo online 2010).

Growing awareness of climate change and environmental pollution makes us to look more sceptical at chemical pest control than during the last decades of the twentieth century.

Especially the cockchafer larvae, exceedingly feeding on plant roots, are now being looked at more in detail. If predilections of feeding and oviposition environments were better understood, one probably could influence insect

behaviour and omit pesticides, some of which already are banned in many countries.

Current research suggests that volatile organic compounds determine the insects search for feeding and oviposition grounds.

This study, therefore, will inquire into the living conditions and behavioural predispositions of cockchafer larvae as one of the most important kind of the scarab beetle.

1.2 Volatile Organic Compounds (VOCs)

Volatile organic compounds (VOCs) can be of natural or synthetic origin and are released into the environment in high amounts during biogenic and anthropogenic processes. Organisms (humans, animals, plants, microorganisms), natural soil and water habitats represent natural sources, whereas agriculture, industry (including solvents) and traffic belong to the important anthropogenic ones.

The definitions of volatile organic compounds are not consistent, also they differ from country to country.

One of the recent definitions is from Grossmannova et al. 2007: „VOCs (volatile organic compounds) are organic chemical compounds that have high enough vapour pressures under normal conditions to significantly vaporize and enter the atmosphere.“ The Council of the European Community defines VOCs as „any organic compound having a vapor pressure of 0.01 kPa or more at 293.15K or having a corresponding volatility under the particular conditions of use“ (CEC, 1999). Another definition could be read in Jones 1999: „Any chemical compound that contains at least one carbon and a hydrogen atom in its molecular structure is referred to as an organic compound. Organic compounds can be further classified into various categories which include volatile organic compounds (VOCs), semi-volatile organic compounds (SVOCs) and non-volatile organic compounds (NVOCs).“ Volatile organic compounds are also defined to have a lower boiling point between 50°C and 100°C and an upper boiling point between

240 °C and 260°C (Maroni et al. 1995). However, a definition based on the temperature only makes sense, if also the pressure is given. These definitions show that is quite possible to emphasize specific aspects without attempting to give a complete view on all parameters.

Guenther et al. (1995) developed a global model to estimate emissions of volatile organic compounds from natural sources (NVOC). The chemical species are grouped into four categories: isoprene, monoterpenes, other reactive VOC (ORVOC), and other VOC (OVOC). The annual global VOC flux is estimated to be 1100 Tg C (1 teragram = 10^{12} gram) composed of 44% isoprene, 10% monoterpenes, 23% other reactive VOC, and 23% other VOC. For each of these estimates exist large uncertainities and particularly for the other reactive VOCs and the other VOCs. About half of all global natural VOC emissions originate from tropical woodlands (rain forest, seasonal, drought-deciduous, and savanna). Isoprenes and monoterpenes are known as the predominant VOCs emitted by plants.

Gases, volatiles and solids can be distinguished as organic compounds in air. Volatiles are usually liquid and have a boiling point distribution similar to benzine. In our environment, gases and volatiles derive from different origins, including plant life (Holzer et al. 1977).

VOCs are ubiquitous indoors. Indoor concentrations are mostly below the threshold of human olfactory perception, but often exceed outdoor levels by up to a factor of five (Wallace 1991). Usually, in the urban atmosphere the concentration of volatiles is 10 to 500 times higher than in rural areas, due to anthropogenic sources (Holzer et al. 1977).

Concerning the medical dangers, many VOCs, also those deriving from natural sources are typically not acutely toxic but can have chronic effects. Several of them can cause sick-building-syndrome, trigger allergies or are known as human carcinogens (*e.g.* Jones 1999, Bernstein et al. 2008).

1.3 Plant Chemicals and Insect Olfaction

The chemicals produced and released by plants during the active growth contain a wide variety of short chain alcohols, aldehydes, ketones, esters, aromatic phenols, lactones, and also mono- and sesquiterpenes (Bernays and Chapman 1994). Especially trees, have long been suspected of being emitters of large quantities of reactive species, mainly terpenes. „Total terpene concentrations in the coniferous forest air usually vary from 3.5 to 35 µg/m^3. Strong influence of meteorological conditions on the emission rate and terpene concentrations in the air under the forest canopy, has been noted“ (Isidorov et al. 1985). Quantity and identity of natural hydrocarbons, however, has been the subject of controversy (Holzer et al 1977). Oak foliar mass is estimated as the major source of isoprene emission in investigated forests (Guenther 1997). Terpenes and homoterpenes are known to be produced by plants in response to herbivory.
The volatile plant chemicals can be classified according to their effects on the behaviour of insects. Dethier et al. (1960) used the following terms:

- **Attractants**: Chemicals that cause an insect to make oriented movements towards the source of the stimulus.
- **Repellents**: Chemicals that cause an insect to make oriented movements away from the source. This definition applies only to oriented responses at short distances from the source, relying on chemotaxis (straight migration) and chemokinesis (random migration). The activity of repellents is restricted to close range (Visser 1983).
- **Feeding** or **oviposition stimulants**: Chemicals that elicit feeding or oviposition („Feeding stimulant“ is synonymous with „phagostimulant“).
- **Feeding deterrents** or **„antifeedants“**: Chemicals that inhibit feeding or oviposition. In contrast to repellents, deterrents do not cause oriented locomotion away from the stimulus source, but simply inhibit feeding and oviposition. Thus, females may avoid oviposition if they do not find appropriate host plants. In the extreme the insect may even starve to death.

Besides attraction, host plant location is also associated with active avoidance of inappropriate or non-host plants. The perception of the host plant volatiles is essential for phytophagous insects and rather depends on ratios of plant volatiles than simply on detection of presence or absence of special compounds (Bruce et al. 2005). In our environment volatiles in the air as well as in the soil do never occur in pure and single form but they occur in compositions of different single chemicals, which as blends are more attractive than the single compounds (Natale et al. 2003). In between these numerous different compounds and their mixtures insects have to be able to detect and process the appropriate blends and maybe also some single compounds. It is not yet clear, how insect mechanisms work to recognize the appropriate volatiles (Bruce et al. 2005). This to understand is one incentive for this study.

Insect world is highly diverse (Stork 2007, Bruyne & Baker 2008) and beetles (Coleoptera) present the most species-rich order therein. More than 350,000 species have been described till 2005; this may be about 10% of the estimated actual number. About 122,000 species are estimated as herbivorous (Schoonhoven et al. 2005). During the holometabolous development beetles pass several larval instars sometimes with biting mouthparts (Francke & Dettner 2005) and in the case of *Melolontha* with well developed antennae (figure 1.1).

Larvae of *Melolontha* species, for instance, have strong mandibles. They help the larvae to feed on lignified root parts, but, if available, they prefer the softer parts. In 1982, Wildbolz recorded the infestation of *M. melolontha* larvae on roots of apple trees. The larvae fed mainly on the soft bark of the primary root and only rarely they also damaged the more lignified, wooden part. The plant is able to survive at least for a certain period by building a callus around the wounded part.

The roots differ from the aboveground parts of the plant in composition of the secondary metabolites and in texture (*e.g.* Kovalenko et al. 2004). The roots of many perennials are permanently available and serve as a food source for soil living larvae. Even if the nutrient level is rather low during the season, at the end of the season the storage nutrient level can be very high (Shepperd et al. 2004 and references therein).

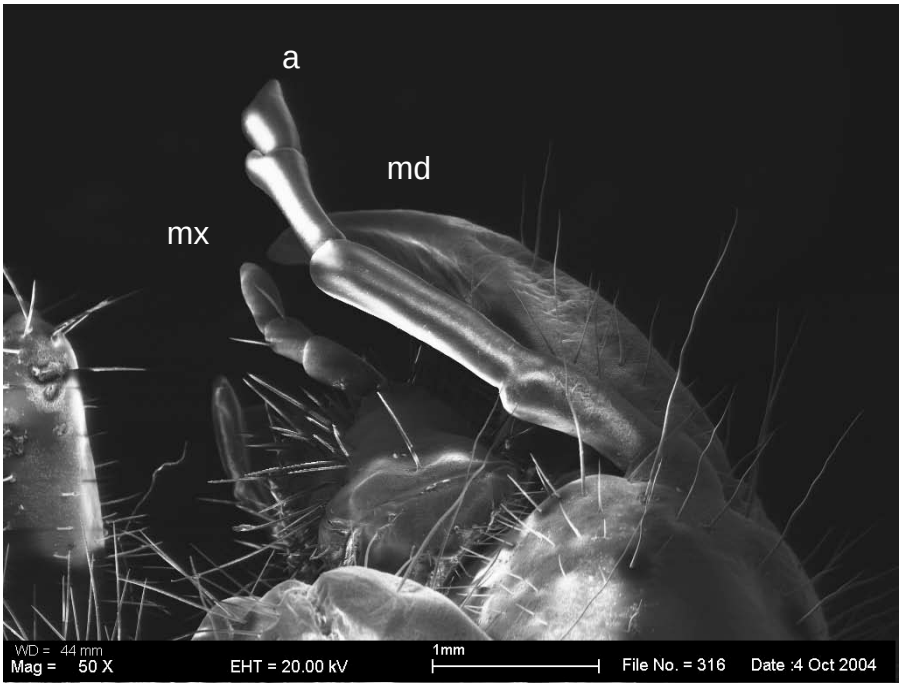

Figure 1.1: Scanning electron micrograph of the ventral mouthpart of *M. hippocastani* showing the maxillae (mx), the sklerotized mandibles (md) and the antenna (a) with 4 segments.

Insect antenna are multifunctional sensory appendages containing sensory neurons responsible for the perception of odours but contain also contact chemoreceptors, mechanoreceptors and receptors for the detection of humidity and temperature (e.g. Altner & Prillinger 1980; Altner & Loftus 1985; Rospars 1988).

Sensory hairs, the so called sensilla, are distributed all over the insect body (Hartenstein 1993). For host location, selection and acception, insect sensilla are equipped with sensory receptors enabling them to perceive visual, olfactory, gustatory and tactile stimuli as well as humidity and light intensity (Städler 1976, Bernays & Chapman 1994). Sensilla can be multimodal, which means that they can house more than one type of receptor. Sensilla that house olfactory receptor cells, may also contain thermo-, hydro- and mechanoreceptors (Shields & Hildebrand 1999).

Chemosensory sensilla can be divided into olfactory (detection of volatile chemicals) and gustatory (detection of dissolved or solid chemicals) sensilla. In

neopteran insects, typically most of the sensilla on the antennae serve olfactory perception (Rospars 1988). Gustatory sensilla are predominantly located in the preoral cavern (*e.g.* the epipharyngeal sensilla) and on mouthparts, antennae, tarsi and even on the ovipositor (Schoonhoven et al. 2005). Gustatory sensilla are uniporous with the pore located at the tip of the sensilla (diameter 200 to 400 nm), whereas the sensilla walls of olfactory sensilla are perforated by up to 1000 pores (diameter about 10 to 15 nm each), with dendrites, which are often branched (Steinbrecht 1997). Olfactory sensilla are predominantly concentrated on the antennae but can also occur on maxillary and labial palps and even on the ovipositor. The number of olfactory sensilla and the associated olfactory receptor cells differ between species. They can morphologically be classified in *sensilla trichoidea* (hair-shape, see figure 1.2 c), *s. basiconica* (peg-shape), *s. coeloconica* (peg-shape, recessed in a pit, see figure 1.2 d), *s. ampullacea* (with a long internal duct), *s. placodea* (also known as areae porosae, pore plate organs or glandular areas) and several other types. *S. placodea* house several neurons and are common *e.g.* in bumblebees (Agren & Hallberg 1996), honeybees (Brockmann et al. 1998) and Thysanoptera (Mound 2009). In *Helicoverpa armigera* pore plates on the maxillary palps are supposed to be possible CO_2 receptors (Keil 1996).

Antennae of the larvae of the family Scarabaeidae always have a distinct apical group of trichoid and basiconic sensilla. *M. hippocastani* has 9 basiconic sensilla and 1 trichoid one (Alekseev et al. 2006, see figure 1.2 b below). However, only few sensilla are located on the antennae of *Melolontha* larvae.

Most olfactory receptors are functionally adapted to respond to airborne volatiles and are located on the antennae. Relatively few of these sensilla are found on other head appendages such as the maxillary palps of lepidopterous larvae (Schoonhoven & Dethier 1966, Schoonhoven 1973, Hanson & Dethier 1973, Roessingh et al. 2007), coleopterous larvae (Alekseev et al. 2006), locust nymphs (Blaney 1977) and Drosophila (Singh & Nayak 1985, Riesgo-Escovar et al. 1997, de Bruyne et al. 1999, de Bruyne & Baker 2008).

The cuticula is composed of many molecular layers (Steinbrecht 1997).

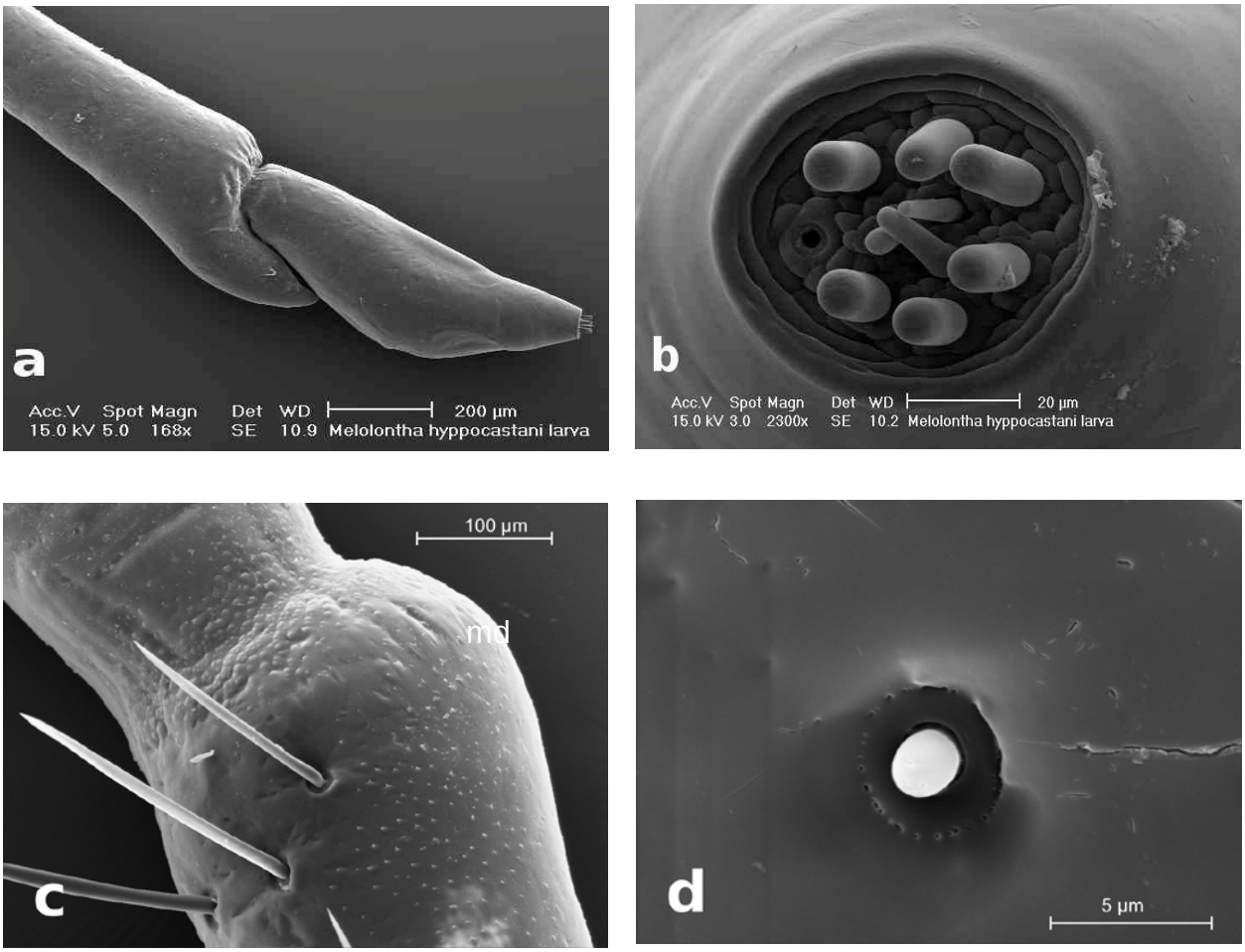

Figure 1.2: Antennae of *M. hippocastani* (3rd larvae instar)*:* a) The first two segments of an antenna with two slightly subsided poreplates (pp) on the dorsal and the ventral part of the first segment; b) View of the distal end of the antenna; c) Long *sensilla trichoidea* at the distal part of the 1st segment; d) Frontal view of a *sensillum coeloconicum* on the distal part of the 2nd segment (identification of the sensillum: personal communication by Kaissling 2004). Preparation and photographs of a) and b): Roberto Romani, fellow researcher at the University of Perugia, Italy.

The general structure of an insect olfactory sensillum is showed in figure 1.3 a. It consits of a cuticula, olfactory receptor neurons, and three sensory neuron-surrounding support or accessory cells at the sensillum base (thecogen, trichogen and tormogen cells).

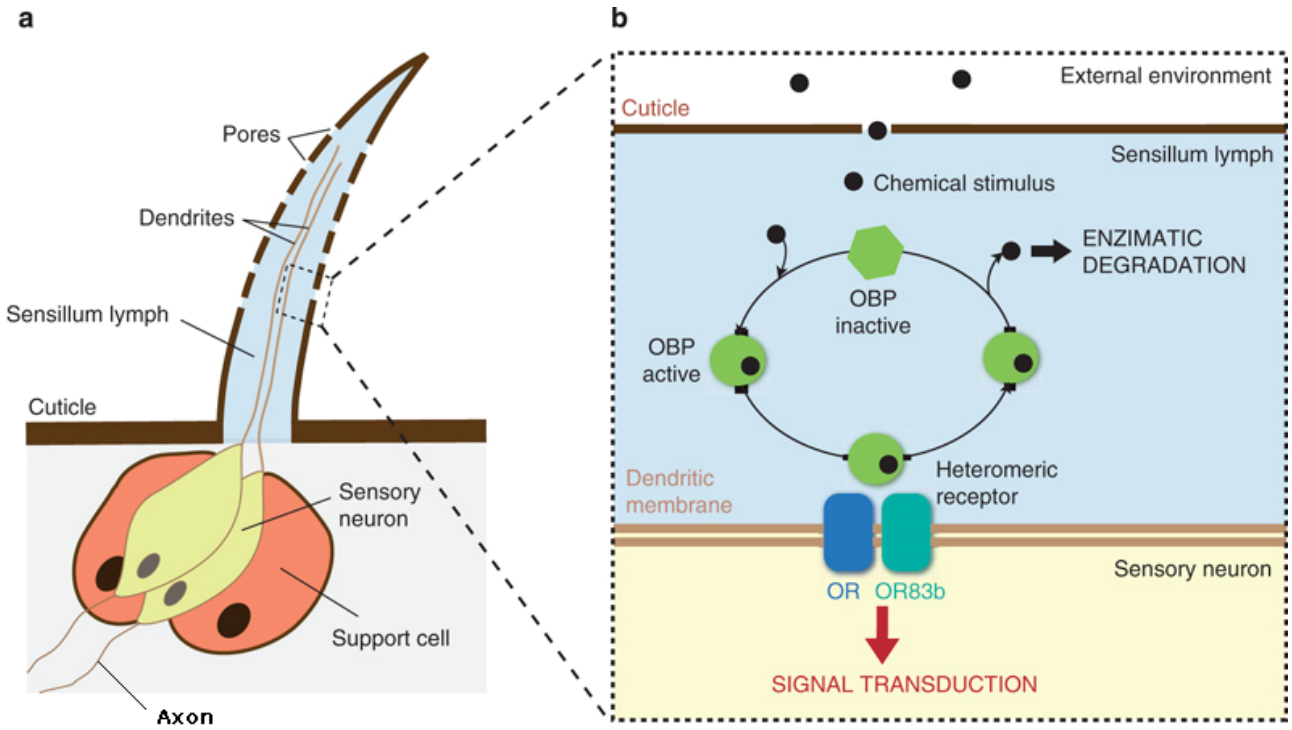

Figure 1.3 a) Schematic representation of the general structure of an insect olfactory sensillum. Gustatory sensilla have only a single pore at the top of the sensory hair. b) The first molecular steps of the insect chemosensory signalling transduction pathway. This figure shows a simplified functional scheme (according to Vogt 2005).

Olfactory insect sensilla contain several olfactory sensory neurons (OSNs, also called olfactory receptor neurons, ORNs, mostly bipolarly innervated) that encode an immense variety of odours and respond very specifically to odours (Hansson 1995). Each OSN typically contains one specific and one unspecific OR expressed in the cell membrane (Sato et al. 2008; Wicher et al. 2008). The number varies from 2 to 200 (Galizia & Rössler 2010). Typically there are 2 to 5 neurons located in one olfactory sensillum (Chapman 1982). Each OSN send a neurite into the sensilla lymph and an axon into the antennal lobe (AL), the first integration center for odour information in the brain. Small acidic soluble proteins (13 to 16 kDa), the so called odourant-binding proteins (OBPs, first discovered in 1981 by Vogt and Riddiford), occur in high amounts in the sensillum lymph. They are responsible for the transport of the hydrophobic odour molecules through the

aqueous matrix, called sensillum lymph towards the olfactory receptors (OR) of the OSNs (figure 1.3 b). ORs are transmembrane proteins, expressed by the OSNs, which belong to the family of G-protein coupled receptors (GPCRs) but have in contrast to their vertebrate counterparts and classical G-proteins a reversed membrane topology (for reviews see Nakagawa & Vosshall 2009; Kaup 2010). Specific ORs are either very broadly tuned to a variety of related odours (up to 200) or very specifically tuned to a certain odour e.g. sex pheromones (for a review see Galizia & Rössler 2010). The unspecific ORs are forming ion gated channels after odourant binding responsible for fast signal transduction, while second messengers may be responsible for longer lasting effects and modulation of the signaling (Wicher et al. 2008; Nakagawa & Vosshall 2009). After odour stimulation, action potentials are conducted via the axonal membrane to the paired ALs. The ALs of insects share their principal organization with the primary integration centers for olfactory information in the brain of vertebrates (olfactory bulbs) by their principal morphological organization into so called olfactory glomeruli, but also a number of basic physiological properties with respect to information processing (Hildebrand and Shepherd 1997). Glomeruli represent functional units for odour processing containing thousands of synapses between OSNs from the olfactory epithelium/antenna and neurons of the olfactory bulbs/ALs. Each glomerulus receives input from OSNs expressing particular ORs (Vosshall 2000; Korsching 2002; Jefferis & Hummel 2006; Mombaerts 2006). Odours are finally encoded by activation patterns of defined sets of glomeruli, resulting in a spatial odour map and a chemotopic representation of odour information in the brain (Galizia et al. 1999, Leon & Johnson 2003, Vosshall & Stocker 2007). Owing to these similarities, the antennal lobes of several neopteran insects serve as important models to further understand olfactory information processing, development, and adult plasticity of the first odour integration center. From the ALs, information is conveyed to the calyces of the mushroom bodies (Mbs) and to the lateral protocerebrum by means of antenno cerebral tracts (reviewed in Anton & Homberg 1999; Hansson & Anton 2000).

Owing to different live styles of larva and adult, the larval olfactory system in holometabolous insects differs from the adult olfactory system. Depending on the holometabolous group, the differences are more or less pronounced. Typically,

the antennal appendages are smaller; they contain less olfactory sensilla, less OSNs and less ORs. *Drosophila* larvae have 21 OSNs located in a single sensillum (Singh and Singh 1984) each expressing a single specific OR and each OSN projects to one of 21 glomeruli in the larval AL. In contrast, adult *Drosophila* have 1300 OSNs, distributed in about 600 individual sensilla and project to about 50 glomeruli in the adult AL (reviewed in Vosshall & Stocker 2007). Most if not all OSNs project to individual glomeruli in the larval antennal lobe (LAL). In contrast to larva, each glomerulus receives not only one, but a certain number of OSN axons typically carrying the same specific OR. In the red flour beetle *Tribolium castaneum*, the difference between larva and adult seems much less pronounced as in *Drosophila*. The LAL contains about 50 glomeruli and neuroanatomical stainings suggest that more than one OSN is entering a single glomerulus (Götz et al. 2007; J. Schachtner, personal communication). OR numbers in *Tribolium* are under debate but it seems as if the number of functional ORs may exceed the number of glomeruli (Engsontia et al. 2008). Adult *Tribolium* ALs contain about 70 glomeruli and backfills revealed many OSN axons per glomerulus suggesting a similar situation as described for adult *Drosophila* (Goetz et al. 2007; J. Schachtner, personal communication). Neuroanatomical studies on the hymenopteran and the lepidopteran olfactory system underline a similar organization of the olfactory system in all adult insects (reviewed in Schachtner et al. 2005). In contrast to the larval olfactory system of *Drosophila* and *Tribolium*, the larval olfactory system in the honey bee and in the moth *Manduca sexta* is only rudimentary developed with no olfactory glomeruli (Kent & Hildebrand 1987; Schröter & Malun 2000).

1.4 Above- and Belowground Constitutive and Induced Defense Strategies

Plants in nature often are attacked by herbivorous insects (*e.g.* biting-chewing feeders, cell-piercing feeders, phloem and xylem feeders), and different pathogenes (several bacteria, fungi, or viruses). Terpenes and monoterpenes are released by plants in response to herbivory (*e.g.* Takabayashi et al. 1994). It is not clear yet, if the biosynthesis of the volatiles emitted by the infested plants is induced by herbivore feeding or if they are stored in plant cells and released at time of insect attack (Paré & Tumlinson 1996). Plants kept in the greenhouse of course interact with insects and pathogenes which are different from those faced by plants living in the wild.

Plant defence strategies exist in constitutive (*e.g.* spines, hairs, enzymes, secondary metabolites, which are present and produced irrespective of herbivore attack) and induced defense mechanisms (expressed only as a reaction on herbivorous attacks). The latter can be divided again in directly induced (through accumulating secondary metabolites) and indirectly induced defense (through emitted VOC, which attract predators and parasitoids, Dicke & van Loon 2000 and references therein, Fatouros et al. 2008), but not all of the autors do distinguish between directly and indirectly induced defense.

There are many studies dealing with induced defence in plants. The wound hormone jasmonic acid and its ester methyljasmonate play an important role in the signal pathway leading to the induction of secondary metabolites which could act against herbivores and microorganisms by promoting resistance to them (Baldwin 1998, Steppuhn et al. 2004, Zayed and Wink 2004, Howe & Jander 2008). 2002 Gange et al. mentioned that insect herbivores can affect the mycorrhizal colonization of plants in a complex way. Relating to herbivory, plants can benefit from mycorrhizal colonisation or it can have detrimental effects. Foliar herbivory impaires arbuscular mycorrhization of roots, probably because of a reduced carbon allocation to the roots. Also the reverse interaction has been documented: mycorrhizal fungi deter herbivores and interact with fungal endophytes to influence herbivory (Gehring & Whitham 1994).

Plants are able to respond to different types of wounding (herbivory, mechanical damage) through the emission of different chemical volatiles, which may also depend on the herbivorous species attacking the plant (Gosset et al. 2009). Differences in the volatile emissions caused by different herbivorous insects can be perceived by several organisms (Loughrin et al. 1996, Takabayashi & Dicke 1996, Röse et al. 1998, Dicke 1999, van Tol et al. 2002/2004, Kessler & Baldwin 2004, Rasmann et al. 2005). Turlings et al. showed 1990 that even if a caterpillar regurgitant is applied to a mechanically damaged plant part, the volatile emission by the plant is the same as the volatile emission of a plant damaged by feeding of a caterpillar.

Simultaneous feeding on a host plant by multiple herbivores with diverse feeding strategies is very common in nature, but little investigation is done in this field (Shiojiri et al. 2001, Strauss 1991). Additionally, the different volatiles from neighbouring plants, simultaneously infested with different herbivorous insects, influence the foraging success of carnivorous arthropods (Dicke et al. 2003).

The volatile emission of carnivore-attracting volatiles takes place not only on the damaged plant part, but also on other parts. So, local herbivore-infestation mostly leads to systemic effects in other parts of the same plant (*e.g.* Turlings & Tumlinson 1992, Baldwin 1998). However, the emitted volatiles, differing qualitatively and also quantitatively from those of intact plants, can act as attractants for the natural enemies (carnivores such as parasitoids or predators, Kalberer et al. 2001, Bolter et al. 1997, Turlings et al. 1995, Tollrian & Harvell 1999, Van den Boom et al. 2004) or as repellents (Dicke 1986, De Moraes et al. 2001). Larval root feeding by *Agriotes lineatus* induces an increased production of aboveground foliar extra-floral nectaries, which aboveground attract carnivorous insects (Wäckers & Bezemer 2003).

Volatile emission can also be induced by oviposition on the plants (Meiners & Hilker 2000). Many of the oviposition-induced plant volatiles are similar to those induced by herbivory (Hilker & Meiners 2002) and can attract egg parasitoids (Colazza et al. 2004).

Herbivory and/or mechanical damage induce not only extensively modified volatile emissions but also modified gene expression in plants (Reymond et al. 2000).

Especially in this context one cannot only focus on above- or belowground aspects, because the possibilities of direct and indirect interactions are very diverse and complex, and it is not possible to separate them. „In fact, the division between above and belowground interaction is highly artificial and results from methodological rather than scientific arguments. Increasing the effort to make connections between the two will be a major and rewarding challenge in the coming year“ (Schoonhoven et al. 2005). Increasing numbers of studies investigating belowground behaviour of root-feeding insects become aware of the insect-plant interactions. Recent studies have shown that soil dwelling organisms, such as root-feeding insects, arbuscular mycorrhiza, and nematodes, can influence aboveground plant-herbivore-parasitoid-hyperparasitoid interactions via changes in plant quality (Bezemer et al. 2005, Soler et al. 2005, Rasmann & Turlings 2007). Some of the defense mechanisms known from aboveground also occur belowground in a similar manner, even if the physiochemical conditions (*e.g.* adsorption and desorption processes) between above- and belowground differ (van der Putten et al. 2001, Blossey & Hunt-Joshi 2003 and references therein, Wardle et al. 2004 and references therein).

So far each study, highlights only a small part of the full context in insect-plant interactions. Considering all the single contributions, a better understanding of this highly fascinating and complex topic appears still to be desirable and, at the same time, a challenge for further research. The present study hopes somewhat to narrow the gap.

1.5 Cockchafer(larvae) as Pests in Forest and Agriculture

As major damage to crops and forests is being done by the two types of cockchafer beetles (see chapter 1.1), and, as especially in the life cycle of the beetles the larval stage is considered to be the most damaging, we will, in this study, concentrate on the larvae as pests (figure 1.4).

Figure 1.4: 3rd larval stage of *Melolontha hippocastani*. White bar: 4 mm. Wolfgang Tambour.

The larvae of *Melolontha* sp. feed approximately three years on roots without provoking any visible damage on the upper parts of the plants. They are very polyphagous, in meadows they attack the roots of several wild grasses and weeds. Host plants are: *Rumex*, *Chenopodium*, *Stellaria*, *Achillea*, *Daucus*,

Solanum, *Festuca* and *Cirsium*; *Taraxacum* and *Plantago* are highly preferred. In experiments it was shown that the roots of *Taraxacum officinale* are the best source of nutrition and that the beetles are capable to select these weeds in the field for oviposition (Hauss & Schütte 1978, Horber 1961). In stony soils, even if they are covered with *Taraxacum*, less larvae are present compared with sandy soils (Hauss & Schütte 1978). A laboratory study shows that Leguminosae are preferred over Graminaceae (Schwenke 1974), except during the first weeks of the first larval stage, where the mortality is significantly lower, if the larvae being fed with Graminaceae like *Festuca rubra*, *F. pratensis*, *Agrostis tenuis* and *Cynosurus cristatus* and not with *Taraxacum officinale* (Hasler 1986, Hauss & Schütte 1976). *Taraxacum officinale* is the best diet for the larvae of all the three larval stages (Hauss 1975). It was proved that the females of *M. melolontha* prefer areas covered with *T. officinale* for the oviposition as well as the roots of *T. officinale* as the favoured host plant material (Hauss 1975, Hauss & Schütte 1978). In some countries such as France, *Melolontha* have become rare and cause almost no damage. This is most probably due to the common insecticide applications in former times, the widespread use of mechanical cultivation (*e.g.*intensive tillage, which kills the very fragile larvae) and herbicide application. The occurrence of *T. officinale* and *M. melolontha* in Europe over the past 30 years showed that the conditions for propagation of the two organisms have been changing. During the 1990's, in some regions the abundance of *Taraxacum* has increased in relation to decreasing herbicide usage. In an experiment, this weed was reduced to 12% of its abundance by spraying herbicides; by that, the abundance of the larvae was reduced to 55%. Today, many meadows and pastures are partially covered by *Taraxacum*. These conditions are favourable for mass occurrence of *Melolontha* (Schütte 1996). But also the type of soil may play an important role in the dispersion of the larvae. Therefore, in Germany very few organisms are found in the northern part, whereas in the central part and in the south the adults, and mainly the larvae, cause severe damage.

1.6 Orientation Behaviour of the Adults of *M. hippocastani* and *M. melolontha*

M. hippocastani occurs in central and northern Europe, whereas *M. melolontha* is distributed in the whole of Europe, except the most northern and southern parts. In the south of Germany *M. hippocastani* and *M. melolontha* are sympatric. Cross breeding was never observed but could theoretically happen (Niklas 1970).

Figure 1.5: Male (left side) and female (rigth side) of *M. hippocastani*: The antennae have 10 segments, male antennae show seven big lamellas whereas female antennae have six smaller ones, black bar: 1 cm.

After the maturation feeding, the females of the two *Melolontha* species stay in the trees, feed on the leaves and emit sex pheromones. The feeding causes emission of green leaf volatiles (GLVs). This attracts swarming male beetles, which then also start to feed. The damage caused by defoliation mostly can be compensated by the “lammas shot“ in june. GLVs emerge by enzymatic oxidation of unsaturated fatty acids and are released by all damaged green plants (Visser 1986). GLVs smell characteristically similar to freshly cut grass and include isomers of hexenol, hexenal and hexenyl acetate (Hatanaka 1993). Electrophysiological experiments with *Phyllopertha diversa* W. showed that these beetles are equipped with highly sensitive and specific olfactory sensory neurons (OSN) for detecting GLVs (Hansson et al. 1999). Among the green leaf volatiles only (Z)-3-hexen-1-ol attracts males of *M. hippocastani* (Ruther et al. 2002a),

while in *M. melolontha* also 1-hexanol and (E)-2-hexenol act as attractive volatiles for the males (Reinecke et al. 2002a). Male beetles of the forest cockchafer M. *hippocastani* are attracted by green leaf volatiles (GLV) and 1,4-benzoquinone as the species-specific sex-pheromone (Ruther et al. 2000). In contrast, toluquinone is identified as the species-specific sex-pheromone of *M. melolontha* (Reinecke et al. 2002b). The sex-pheromones are not attractive alone but they synergize the male response towards green leaf volatiles. In this case plant volatiles play a key role in mate location by acting as primary sex attractants. Therefore, they can be defined as sexual kairomones (Ruther et al. 2002a). Both benzoquinones are identified in whole body extracts from females and males, and are well known as defence compounds *e.g.* in the Blattodea species *Diploptera punctata* (Eisner 1958) and special beetles like Brachinidae (Schildknecht & Holoubek 1961), Tenebrionidae (Tschinkel 1975) and Staphylinidae (Steidle & Dettner 1993). Phenol as an attractant for *M. hippocastani* and *M. melolontha* is described by Ruther et al. (2002b).

In electroantennographic experiments, Reinecke (2005) mentioned that female and male antennae of *M. melolontha* showed responses to almost the same compounds. The antennal responses of males to special green leaf volatiles were stronger.

In an experiment by Reinecke et al. (2002b) it was shown that volatiles from damaged leaves of *Fagus sylvatica* were significantly more attractive for *M. melolontha* males than leaf volatiles from *Carpinus betulus* and *Quercus robur*. Leaf voaltiles from healthy *F. sylvatica* were not attractive at all. For females none of the tested volatile sources were attractive.

The following overview (table 1.1) shows some host tree preferences of the adults of *M. melolontha* (Huiting et al. 2006).

However, these results have to be handled with care, since the results of experiments highly depend on the choice situation and maybe on other factors as well, like physiological status and development of the larvae.

Table 1.1: Host and non-host tree preferences of adults of *M. melolontha*.

Highly preferred trees	**Rare feeding on**	**No feeding on**
Quercus	Castanea	Tilia
Acer	Aesculus	Robinia
Carpinus	Salix	Fraxinus
Fagus	Populus	Ulmus
Prunus	Betula	Several Conifers
Larix	Corylus	
Different Shrubs		

1.7 Orientation of the Larvae of Melolontha in the Soil

Larvae and adults of holometabolous insects are morphologically different, reflecting their different lifestyles and habitats. Abiotic factors like light can affect larval behaviour (Dethier 1943, de Wilde 1958, Tanton 1977a/1977b, Gilbert 1994, Busto et al. 1999). However, volatile compounds emitted by plant roots, are supposed to have a very strong effect on larval orientation behaviour (Nordenhem & Nordlander 1994). CO_2 is a key component of host odours and is a well known attractant to several terrestrial living organisms (Bernklau & Bjostad 1998a, Bernklau & Bjostad 1998b, Bernklau 2003, Bernklau et al. 2004, Bernklau et al. 2005, Gaugler et al. 1980, Prot 1980, Robinson 1995, Sage 2002, Johnson & Gregory 2006 and references therein) including also wood decaying larvae (White et al 1974) and the larvae of *Melolontha* species (Klingler 1957/1958/1959/1966, Hasler 1986, Reinecke et al. 2008).

However, little is known about other aspects of the orientation behaviour of *Melolontha* larvae. Several aboveground living insects are attracted by CO_2 as well (Agrell 2000, Stange 1999, Stange & Stowe 1999, Stange et al. 1995). Most probably the attractive range of CO_2 to an insect is related to the CO_2-concentration of the insect environment (Doane et al. 1975). 1917 Hamilton

pointed out that a CO_2-value of 4% was the best concentration to attract the larvae of the soil dwelling carabid species *Evarthrus* (*Cyclotrachelus*) *sodalis*. In *Atta cephalotes, sensilla ampullacea* are responsible for the perception of CO_2. The tested CO_2-concentrations ranged from 0,05 to 4% (Kleineidam & Tautz 1996). The exposure to CO_2 as a fumigant in high concentrations had a toxic effect to the termites *Cryptotermes brevis* (> 50% for 5 days, higher concentrations caused mortality in shorter periods, Delate et al. 1995). In mosquitoes, however, *sensilla chaetica* (both long and short), *sensilla coeloconica* and *sensilla ampullacea* are classified as non-olfactory sensilla (Boo & McIver 1995).

Acetone is another highly volatile trace component acting as attractant or repellent, depending on the concentration. It is an attractant for many, but not all, biting flies, especially if it is associated with other substances, for example like carbon dioxide or 1-octen-3-ol. The simultaneous presence of associated components could have synergistic effects on the attractance of insects (Krčmar et al. 2005). Dependent on the concentration, acetone is mentioned also to be a repellent (Opoku 2008). Several beetle-larvae are able to detect differences in humidity (Klingler 1957).

The repellent effect of attractive compounds occuring in high concentrations is also known from aboveground living insects (*e.g.* Wallbank & Wheatley 1979). From the sources cited above it is known that the attractant or repellent effect of all chemicals may depend upon the concentration.

Since the influence of VOCs (not CO_2) on the behaviour of belowground living insects was only investigated in few cases so far, this study tries to highlight the VOC-affected orientation process exemplified by *Melolontha* larvae in the soil.

1.8 Sampling, Analysis and Quantification of Volatiles

Because of the complex mixtures of organic compounds in the atmosphere and because of the low concentrations of some compounds, sensitive and selective methods are required for analysis and several techniques have been developed for pre-concentration of VOCs from the atmosphere.

In this study, active sampling methods (figure 1.6 a and 1.6 b) as well as passive sampling methods (figure 1.6 c) were employed.

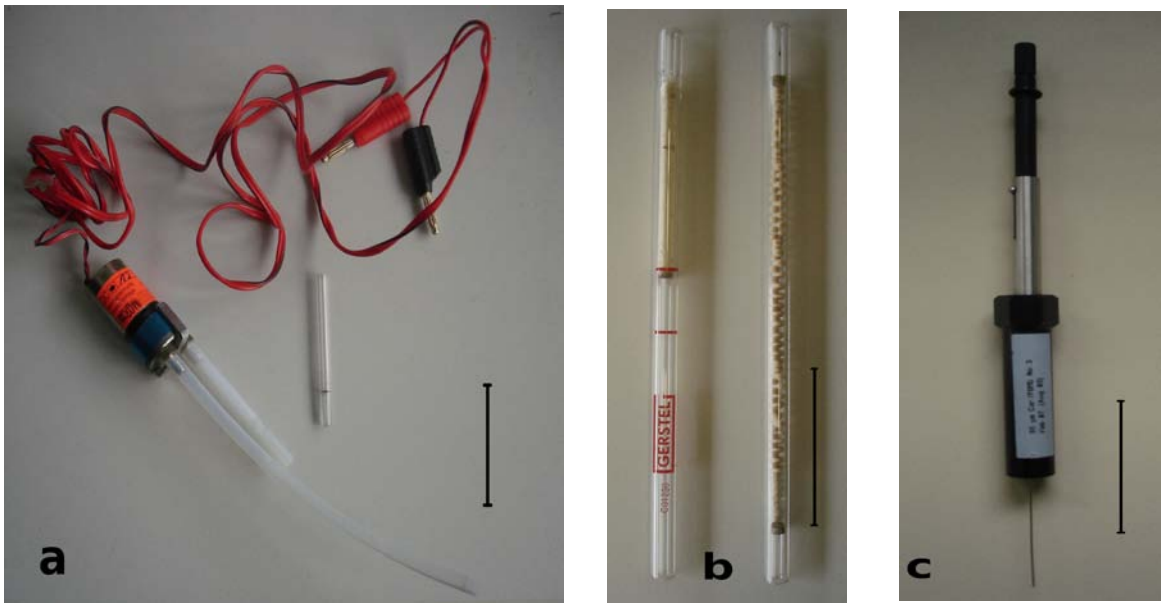

Figure1.6 a) Miniature pump (Fürgut, Tannheim, Germany) and an adsorbent trap (Daumazan sur Arice, France, 6 cm long) with a layer of activated charcoal; b) TDS-tube (termodesorption, Gerstel, Mühlheim an der Ruhr, Germany) with adsorbent polymer matrix TENAX® TA on the left side and molecular sieve, filled with filter pearls (OD of the pearls 1.6 to 2.5 mm, made of metal-aluminosilicate, 0.3 nm, Carl Roth GmbH + Co.KG, Karlsruhe, Germany) on the right side (both ID 4 mm, 17.8 cm long each); c) equipment/fixture for solid phase microextraction (SPME, Supelco, Sigma-Aldrich, Canada, USA, 20.5 cm long); black bar: 5 cm.

1.8.1 Sampling methods

1.8.1.1 Active Sampling Methods

A constant air volume passed via miniature pumps (type DC12/16NK, Fürgut, Tannheim, Germany) through adsorbent materials in sampling tubes where the VOCs are accumulated and trapped (figure 1.5 a and 1.5 b). The pumps were powered by an adjustable DC power supply (6 to 8 V), which could be used to adjust the air flow rate. To avoid mismatches in the accumulation rate, the flow rate was checked prior to every experiment. There were two different adsorbent materials used in the active sampling:

1. Volatiles were trapped on a thin activated charcoal layer (1.5 mg charcoal), and were eluted afterwards with organic solvents (75 µl of a 2+1 mixture of methylene chloride and methanol, both solvents Suprasolv-quality, Merck/VWR, Darmstadt, Germany). After elution the samples could be stored for several months at -73°C to -76°C with the possibility of multiple injections into the gas chromatograph.
2. Sampling was based on the absorbent polymer matrix TENAX® TA (Gerstel, Mühlheim an der Ruhr, Germany), combined with a successive thermodesorption, followed by gas chromatographic separation. With this method only one injection is possible.

The choice of adsorbent material highly influences the sensitivity and selectivity of the sampling process of air volatiles (Dettmer & Engewald 2002).

Two different experimental setups were used for volatile collection. In the first method, applied 2004 and 2005, volatiles from shoots and roots were sampled via closed loop stripping analysis (CLSA, Boland et al. 1984): The air was sucked out of the sampling space, where shoots and roots were enclosed by an oven bag made of polyethylene-terephthalate (PET, Toppits, Cofresco Frischhalteprodukte, Minden, Germany), which is free of plasticizer. The air was circulated in a closed system through stainless steel tubes and the adsorbent trap with activated charcoal (Daumazan sur Arice, France, figure 1.7 left), through miniature pumps, and back into the sampling space. The sampling time was one hour for the shoots and three hours for the roots. Via the permanent air flow

through the pumps, compounds from the samples were accumulated in the pumps. In subsequent measurements, compounds stored in the pumps may be transported into the sampling space, and therefore lead to subsequent contamination of the charcoal traps. Therefore, a second method was established, modifying the first method without guiding the air from the pumps back into the sampling space, but releasing it into the laboratory air space (figure 1.7 right). Additionally, the charcoal trap was combined with a TENAX® trap, which was able to sample supplementary compounds, especially those with high vapour pressure like aldehydes. Charcoal trap and TENAX® trap were connected via a polytetrafluorethylen (PTFE) tube. For the TENAX® traps, sampling time for the shoots was 20 minutes, for the roots it was 40 minutes. For the traps loaded with charcoal, the sampling time for shoots was 1 hour, for roots it was 3 hours, as in the method described above. The sampling time was depending on the adsorbent traps used in the experiments, because of the different sensitivity and storage capacity of the traps. Molecular sieves were used to filter the air entering the enclosed sampling space inside the oven bag.

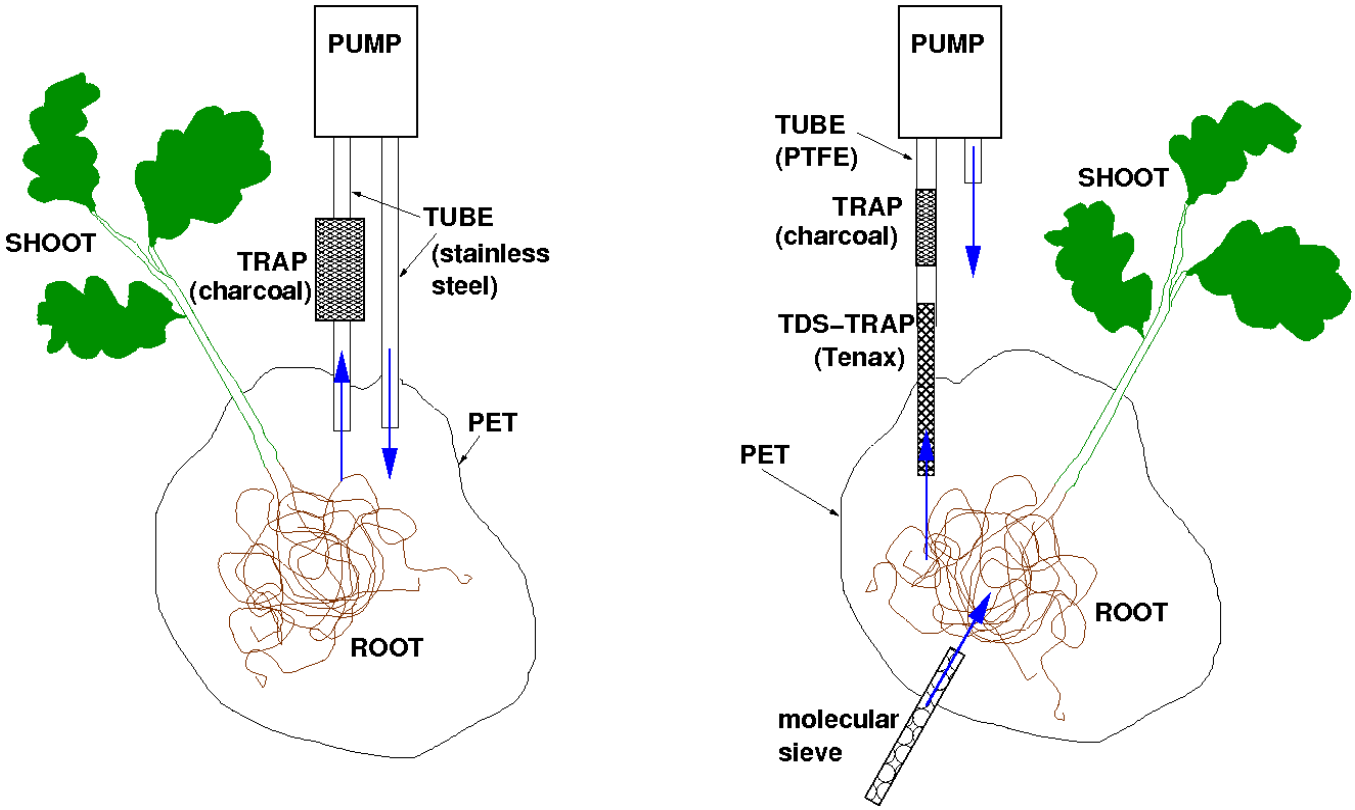

Figure 1.7: The two different experimental measurement setups used in the experiments. Left: root volatile sampling in the closed loop stripping analysis method (CLSA), using a charcoal trap. The tube with activated charcoal layer is enclosed in a stainless steel

holder. Right: root volatile sampling with combined charcoal and TENAX® trap. Both traps are connected with PTFE tubes. Air flow is indicated by blue arrows.

If measurements were done with combined charcoal traps and TENAX®-traps for the first 20 minutes in shoots and 40 minutes in roots, pumps were operated on a 6 to 7 V DC power supply, which corresponds to an air flow rate on the order of 1 l/min. During sampling with charcoal traps, pumps were operated on a 8 V DC power supply. The higher the voltage, the higher the air volume pumped through the adsorbent material, which is highly desired using the less sensitive charcoal traps.

1.8.1.2 Passive Sampling Methods

Passive sampling methods (figure 1.5 c) are widely used in case of high VOC-concentrations. The volatiles are adsorbed on special materials without any air circulation. In solid phase microextraction (SPME), a fused silica fiber coated with a stationary phase on the surface is exposed to the headspace of the sample. Headspace sampling under equilibrium conditions in a static system is called static headspace analysis (Ettre 2002). During the passive sampling process the volatiles stick on the surface of the chosen adsorbent, and accumulate by gradient-driven diffusion.

Soil and root volatiles were sampled using a Polydimethylsiloxane (PDMS) fiber, which was sterilized before each sampling process by exposing it to the GC injection port at 250°C for 10 minutes. The sampling time varied between 8 to 36 hours.

Generally, passive sampling methods are less sensitive than active sampling methods. However, they require less technical equipment and less effort in preparation and operation.

1.8.2 Volatile Analysis

The most widely used methods of analysis are gas chromatography (GC) coupled with mass spectrometry (MS) or with flame ionization detection (FID) (Hutte et al. 1984).

The GC-MS analysis is performed by separating the VOC compounds after injecting a sample into the GC system. Retention time (RT) and mass spectra, allow a qualitative analysis by GC/MS.

Preliminary compound identification is done semi-automatically in a computer aided work flow using the NIST Mass Spectral Database (National Institute of Standard an Technology, Gaithersburg, Maryland, USA) and the Wiley Registry of Mass Spectral Data (Wiley Intersicence, New York, USA, containing more than 390,000 spectra). Final VOC identification (qualitative analysis) is obtained by comparing the mass spectra and the RTs with those of commercially available, authentic standards.

1.8.3 Quantification of Volatiles

Semi-quantitative analysis with the GC-MS can be obtained by evaluating the total peak area using the total ion current mode (TIC). A more precise method to quantify with the GC-MS can be based on the evaluation of the total peak area in the more sensitive selected ion monitoring mode (SIM). In addition, a calibration with an external standard is required, to account for column properties and detector properties (De Oliveira 2010).

Because of the lack of any structural information given by the GC-FID (gas chromatograph- flame ionisation detector), the compound identification is possible only via retention time. The quantification is based on calculating combustible carbon and relating it to the peak area.

Holm (1999) characterizes the system of FID, which does not provide the selectivity to identify compounds. This method coupled with MS for identification depends on the formation of ions and would be an appropriate way to identify and quantify volatile compounds (Zielinska et al. 1995, Jurvelin et al. 2001).

1.9 Electrophysiology to Record Sensory Reaction

Sensory reaction to pure chemical compounds or blends can be recorded by different electrophysiological techniques (Frazier & Hanson 1986). Not only the olfactory, but also the gustatory sensilla respond to chemical volatiles (Städler & Hanson 1975).

The electroantennogram (EAG) signal is the „summed changes in potential of the chemoreceptor neurons in an antenna in response to an odour and is a relative measure of the number of receptors stimulated by the odour molecules“ (Howse 1998). Also the response from the mechanoreceptors is included in the reported signal. This EAG signal is related only to the antenna and provides a screening of the entire antennal receptor population. It does not give any information about the behavioural significance of the activity (Ômura et al. 2000). It detects only the firing of receptor cells if different odours are perceived. „In some instances, however, a false negative may be obtained where key responses to particular chemicals are controlled by only a few neurons, and too few sensilla respond to generate measurable changes in potential on an EAG“ (Lofstedt et al. 1982). The physiological proof of odour perception is a compound specific dose-dependent electrophysiological response of the organ.

EAG recordings usually are performed on isolated appendages like antennae or legs, but also alive, immobilised insects can be used. In the first case the background noise is smaller because of the steady mounting of the antenna in the special holder made of acrylic glass (Färbert et al. 1997). In the second case the antennal signals can be investigated for longer periods. Background noise can be of different origin. It can depend on the antennal preparation, muscle activity in or close to antennal structures, on the EAG-set up like the amplifier, or external sources interfering with the input circuit. Figure 1.8 shows in an electroantennogram the antennal response of a 2nd larval instar of *M. hippocastani* to several stimuli:

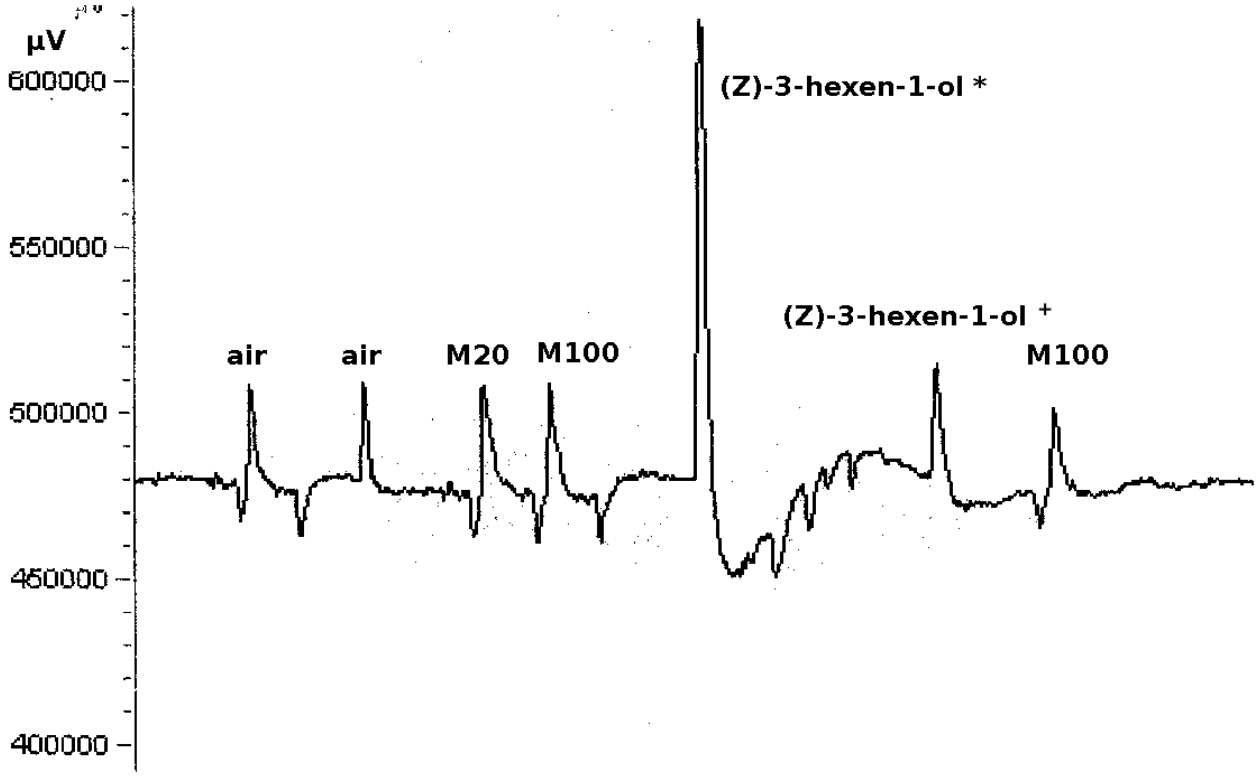

Figure 1.8: Electroantennogram antennal response of a 2nd larval instar of *M. hippocastani* to:
air (drought out of the EAG-system),
silicone oil in two different viscosities (M20 is more fluid than M100), and
(Z)-3-hexen-1-ol:
+ : sampled fresh from a silicone oil dilution of 10^{-3} ,
* : several days old, in a paraffin oil dilution 10^{-3}.

The rather small action potentials from extracellulary recording have to be amplified. The EAG signal increases with higher concentration of the stimulus (injected chemical) until a saturaion level is reached. In addition, the intensity of the signal depends on the quantity of sensitive receptors (Bernays & Chapman 1994). Large bumblebees showed higher antennal response to given odour concentrations than smaller individuals, because of a higher number of olfactory sensilla on the antennae (Spaethe et al. 2007). In EAG measurements insect antennal responses are species-specific.

In the experiments with antennae of *M. hippocastani* larvae, chemicals diluted in silicon oil were tested. Silicon oil was chosen, because only a very small antennal response could be observed (except those of the mechanoreceptors), in contrast to paraffin oil. The reaction of the mechanoreceptors was checked by puffing "clean air" (of the EAG system) with a glass syringe over the antenna. Additionally the response to silicon oil was recorded. Approximately 30 µl of the dilution were enwrapped in a piece of aluminium foil and given into the glass syringe. The obtained value was subtracted as a control from the EAG signal to each puff from the dilution series. Between the puffs (about 5 ml each), the antennal receptors needed about two minutes to recover. The antennal responses were electronically amplified by a factor of 100 and a high pass filter suppressed the drift of the antennal signal. The amplified and filtered signal was digitized and recorded by the GC ChemStation software. The antennae could typically be used for several days before the EAG signals became to weak.

Besides the EAG, the SCR (single-cell recording, also called single-sensillum recording) is developed as an electrophysiological technique to study the specificity and sensitivity of the olfactory system in insects. In this method the spike activity from an individual sensilla, innerved by different neurons, is recorded. This technique directly shows the responsiveness of the OSNs (olfactory sensory neurons). Computer programs analyse the measured complex spike pattern. The obtained results differ, depending if EAG or SCR is used as investigation-method (Wibe 2004).

Another method in electrophysiology is a gas chromatograph coupled with an electro-antennograph, which allows to receive a direct response of insect antennae on different chemical compounds by an equal splitting of the substance between the mass spectrometer (MS) and the electroantennographic detector (EAD) (figure 1.9). The MS works under vacuum, the EAD under atmospheric pressure. This coupled system was described in detail by Weißbecker et al. 2004. It allows to identify volatiles in complex blends and the simultaneous determination of the biological activity of single chemicals. In this method insect antennae act as detectors:

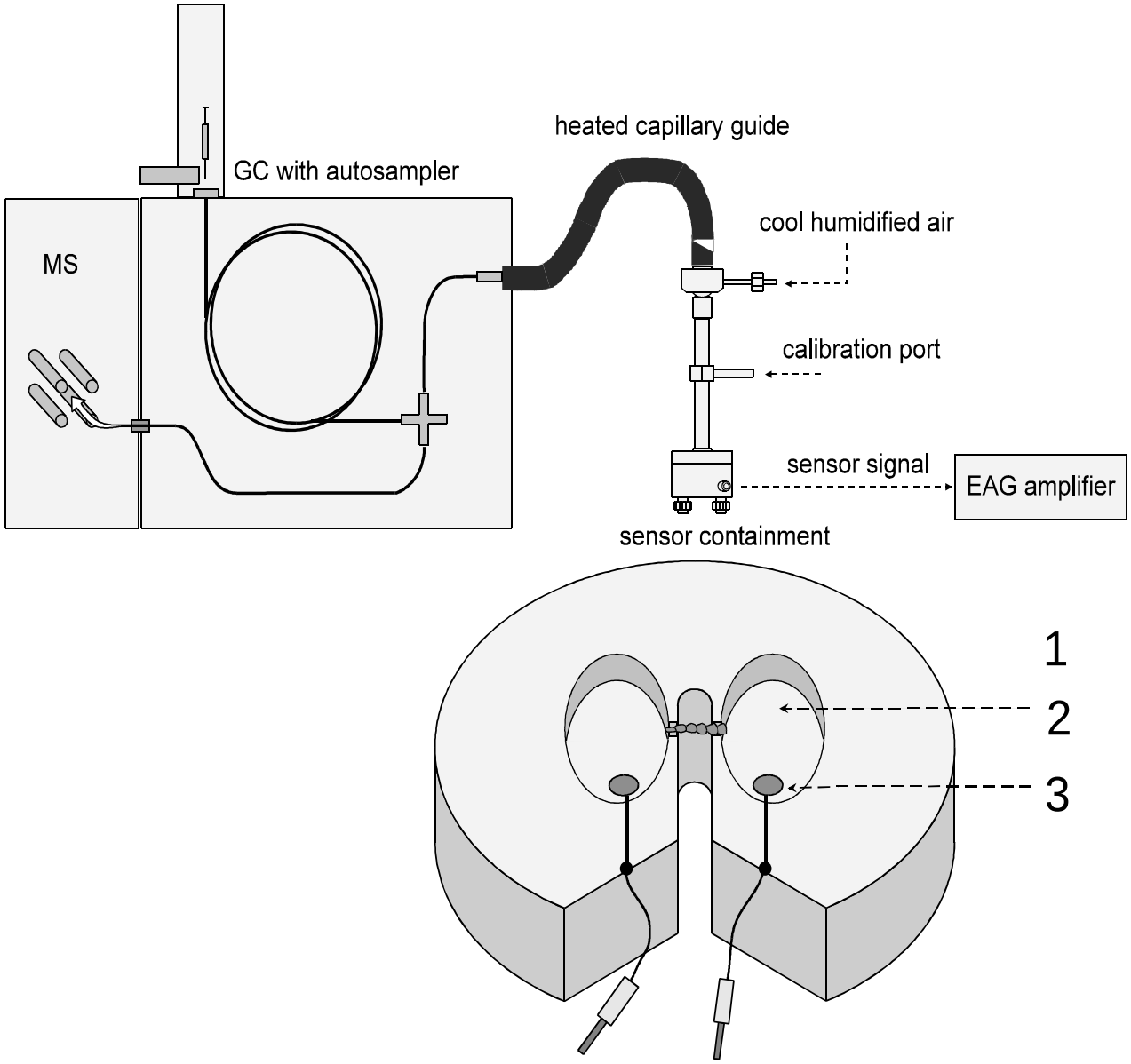

Figure 1.9: Coupled gas chromatograph with electroantennograph and antenna holder made of acrylic glass with fixed antenna (1), inserted into a PTFE support, electrolyte reservoir (2) and Ag/AgCl-electrode (3).

1.10 Behavioural Tests

Behavioural experiments in soil areas are necessary to understand subterranean living organisms. So far, little is known about the interactions between soil volatiles and the belowground biota, but more and more research is done in this field. Because of the difficulty to observe organisms living in opaque substrate, different adopted techniques are used in the experiments (*e.g.* Tanton 1977b, Murray and Clements 1992, Jewett & Bjostad 1996, Mankin et al 2001, van Tol et al. 2001, Johnson et al 2004, Rasmann et al. 2005, Kepler & Bruck 2006, Reinecke et al. 2008, Thomas et al. 2008).

In this study, experimental tests (preference and choice tests) were carried out to highlight the behaviour of selected larvae of *M. hippocastani* in the soil. In a first approach the preference of larve was tested between biologic-organic cultivated carrots (*Daucus carota* ssp. *sativus*) and about 5 year old oaks (*Quercus* sp.) in spring 2004. The experiment was carried out in the greenhouse. The larvae were kept individually in black plastic buckets together with the two plants, the larva was placed in between. After one week, the position of the larva was recorded, additionally the roots were inspected for feeding damage traces. For those larvae, who did not show any decision, the experiment was prolonged for another week.

In a second experimental design, in summer 2004, larvae (3rd instar larvae of *M. hippocastani* and larvae of *Agriotes sp.)* were kept in black plastic buckets together with two plants: *Daucus carota* ssp. s*ativus* and *Solanum tuberosum. M. hippocastani* larvae were kept individually, larvae of Agriotes sp. were kept five each in one bucket. Every week the position of the larvae in the buckets was checked, and the roots were visually inspected for signs of feeding. If a decision of the larva could be observed for the roots of one or the other plant, the experiment ended. After three weeks, the entire experiment was terminated.

A third type of experiments were carried out in autumn/winter 2007/2008, summer 2008, and autumn/winter 2008/2009, using pure chemicals diluted in silicon oil in the concentration 10^{-2}. In addition, some experiments were carried out with a concentration of 10^{-4}. One experimental run was carried out with 15 or 30 units.

1.11 Objectives of this Study

This study investigated the orientation process of cockchafer larvae *M. hippocastani* towards forage sources. The aim was to more thoroughly understand the incentives which influence the larvaes' behaviour. Therefore, particular attention was given to the following key aspects:

- Analysis of different host plant volatile patterns;
  Do the following volatile patterns differ:
  - Healthy plant roots
  - Mechanically damaged plant roots
  - Roots damaged by feeding of *M. hippocastani* larvae?
- How differentiated can the antennae of the larvae perceive the single components of the volatile pattern?
- Which influences have the single components on the orientation behaviour of the *M. hippocastani* larvae?
- Which influences have the different investigated host plants on the orientation behaviour of the larvae?

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CHAPTER 2

Is Differentiated Host Plant Preference of *Agriotes* sp. and *Melolontha hippocastani* Mediated by Root Volatiles?

Weissteiner S. & Schütz S.

c.p. less advanced: Weissteiner S. & Schütz S. (2005): Is differentiated host plant preference of *Agriotes* sp. and *Melolontha* sp. mediated by root volatiles? IOBC/wprs Bulletin 28: 175-178.

2.1 Abstract

Effects of different root volatiles on the behaviour of belowground living larvae were investigated. Choice tests were performed with larvae of *Melolontha* and *Agriotes* in order to determine the role of different volatiles on the orientation of the underground moving larvae. The investigated organisms had to choose between carrots (*Daucus carota* ssp. *sativus*) and potatoes (tubers and roots, *Solanum tuberosum*). In this experiment the organisms show a clear preference for carrots. GC-MS (Gas Chromatography-Mass Spectrometry) analysis of volatile compounds released by undamaged and damaged roots shows different feeding induced volatile pattern if chewed by *Melolontha* or *Agriotes* larvae.

Key words: belowground living beetle larvae, choice test, root volatiles

2.2 Introduction

Plants and insects live and function in a complex multitrophic environment. Most multitrophic studies, however, almost exclusively focussed on aboveground interactions (Dicke 1994, Schütz & Hummel 1997, Schütz et al. 1997, Apel et al. 1999, Schütz et al. 1999, Turlings & Fritzsche 1999, Dicke & Bruin 2001a, Dicke & Bruin 2001b). There are a lot of speculations about belowground living insects and their way of living, but until now there was very little experimental investigation (Horber 1954, Hauss & Schütte 1976/1978, Hasler 1986). A rather unknown topic is the orientation behaviour of soil living organisms. One of the current hypotheses indicates that the orientation of belowground living insects is partly guided by a CO_2-gradient (Hasler 1986) which is caused by plant root respiration. This means that CO_2 for soil inhabiting polyphagous larvae could function as a non specific lure to find their potential host plants. In addition, volatile secondary plant substances released by the roots might be utilized by the larvae as an important additional clue

for their orientation toward host plants. Furthermore, no volatile secondary plant substances which are released by roots as root exsudates, can act as feeding stimulants.
Odourant compounds were identified which are released by plant roots and which may be able to attract or repel belowground living insects.

2.3 Material and Methods

2.3.1 Insect Provenience and Growth Conditions

Organically cultivated carrots (*Daucus carota* ssp. *sativus*) and potatoes (tubers and roots, *Solanum tuberosum*) were used for the study. Larvae of *Melolontha hippocastani* were collected in a forest near Darmstadt, larvae of *Agriotes* sp. originate from outdoor experiments carried out near Mainz and Braunschweig.

2.3.2 Experimental Design

During the experiment *Melolontha* larvae were kept individually in black 10 l- plastic buckets together with carrots and potato plants whereas *Agriotes* larvae were kept in groups of five larvae per bucket.

2.3.3 Belowground Feeding Experiment

After one week the roots were visually inspected for signs of feeding damage. The experiment was prolonged for those larvae who did not show any clear decision for one of the plants. After three weeks all but one of the larvae had fed at least on one type of the two kinds of roots available.

2.3.4 Sampling of Root-Volatiles

At the end of each experiment, samples for GC-MS analysis were collected from the bare roots for two hours using the cloosed-loop-stripping-analysis (CLSA) method (Boland et al. 1984).

2.4 Results and Discussion

2.4.1 Choice Test

The results show clear feeding preferences: both *Melolontha* and *Agriotes* highly favoured carrots, if they had the opportunity to decide between carrots (c) and potatoes (p).

Table 2.1: Percentage rates of damaged carrots (c) and potatoes (p) caused by larvae of *Melolontha* and *Agriotes*. In the test with the larvae of *Agriotes* we found feeding signs on carrots and potatoes in 30% of the buckets.

M. hippocastani **(N = 10, χ^2 p < 0.001)**		***Agriotes*** **sp. (N = 12, χ^2 p < 0.05)**	
c	**p**	**c**	**p**
90%	**10%**	**75%**	**25%**

2.4.2 Gas-Chromatography/Mass-spectrometry(GC-MS) of Carrots and Potatoes

Volatile pattern of carrots and potatoes are quite different. Moreover, feeding damage on carrots caused by the different insect species led to different damage induced root-volatile pattern (figure 2.1 a-c).

First electrophysiological experiments demonstrate that antennae of *Melolontha* larvae are able to detect some of these compounds (Weissteiner & Schütz 2004). So, considering 1) the differences in volatile pattern from roots of different plant species, 2) the possibility of damage induced compounds specific to the insect species feeding on the plant root, and 3) the fact that antennae of *Melolontha* larvae are able to detect at least a part of these compounds, it seems highly probable that these insects use volatile organic compounds released by plant roots to perform their root choice demonstrated in behavioural assays. Moreover, volatile emissions by plant roots specific to the feeding insects might be the basis for aggregation behaviour or density regulation of larvae causing these emissions.

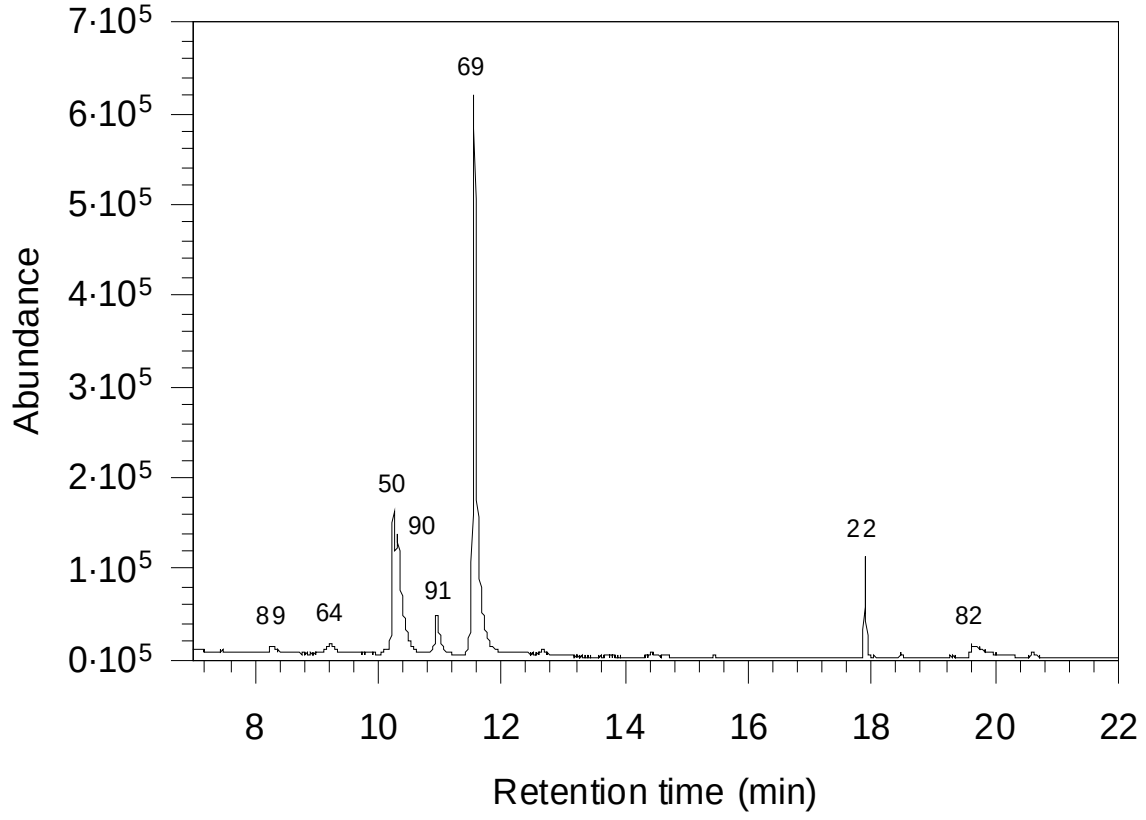

Figure 2.1 a: root volatiles of *Daucus carota* ssp. *sativus*, undamaged plant (N=5)

22→ β-caryophyllene (5/5)
50→ cymol (2/5)
64→ β-pinene (5/5)
69→ terpinolene (5/5)
82→ β-farnesene (2/5)
89→ α-pinene (4/5)
90→ limonene (3/5)
91→ τ-terpinene (5/5)

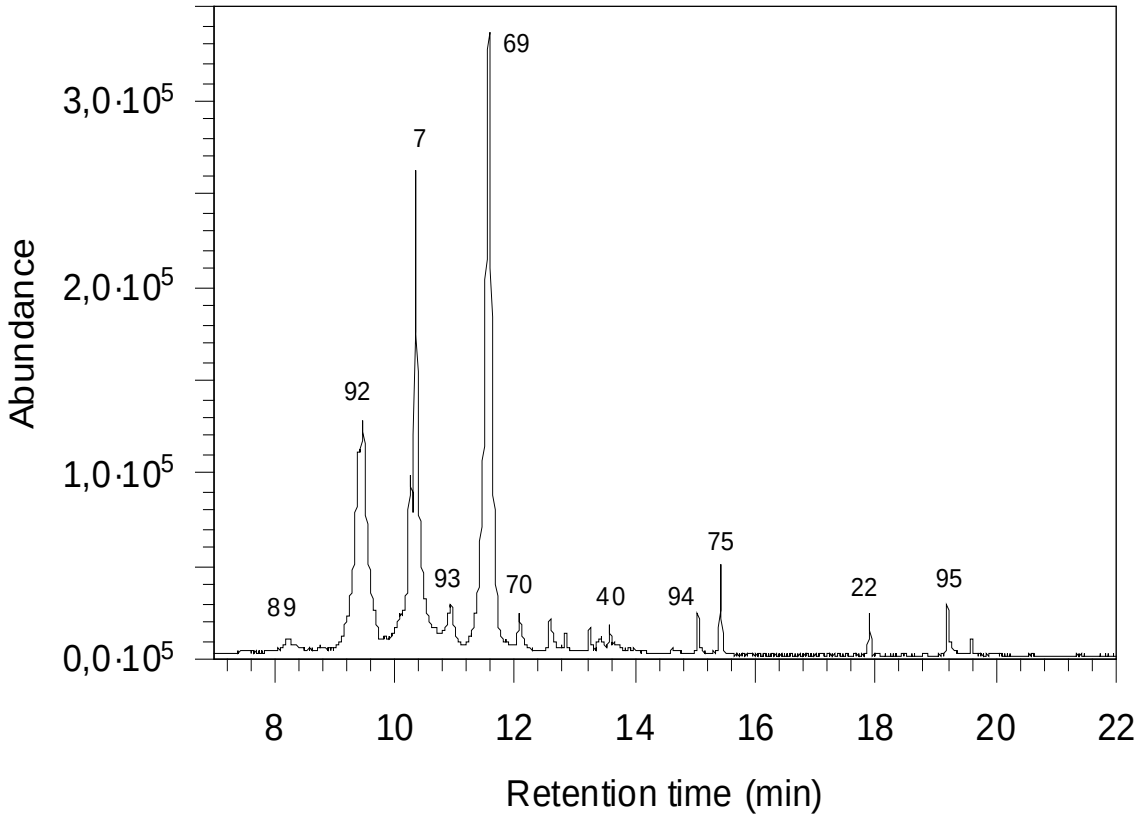

Figure 2.1 b: root volatiles of *Daucus carota* ssp. *sativus*, damaged by feeding of *Agriotes* sp. (N=6)

7→ 2-ethyl-1-hexanol (5/6)
22→ β-caryophyllene (6/6)
40→ borneol (4/6)
69→ terpinolene (6/6)
70→ 1,3,8-p-menthatriene 5/6)
75→ bornyl acetate (6/6)
89→ α-pinene (4/6)
92→ β-myrcene (6/6)
93→ 1,4-p-menthadien-7-ol (3/6)
94→ 1,2-dimethyl-3-vinyl-1,4-cyclohexadiene (2/6)
95→ 2,4-di-t-butylphenol (3/6)

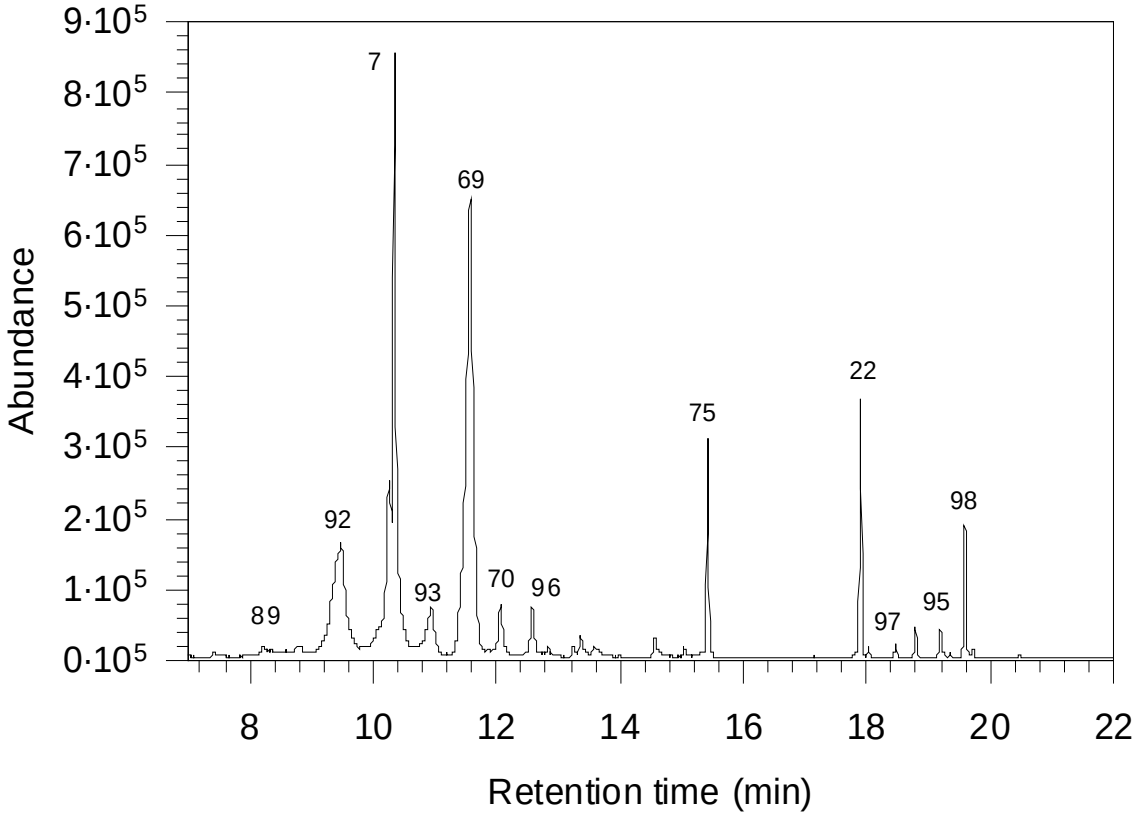

Figure 2.1 c: root volatiles of *Daucus carota* ssp. *sativus*, damaged by feeding of *Melolontha hippocastani* (N=5)

7→ 2-ethyl-1-hexanol (5/5)
22→ β-caryophyllene (5/5)
69→ terpinolene (5/5)
70→ 1,3,8-p-menthatriene (4/5)
75→ bornyl acetate (5/5)
89→ α-pinene (3/5)
92→ β-myrcene (5/5)
93→ 1,4-p-menthadien-7-ol (4/5)
95→ 2,4-di-t-butyl phenol (2/5)
96→ p-vinyl anisol (4/5)
97→ α-curcumene (4/5)
98→ unidentified compound (3/5)

Acknowledgements

We thank Stefan Rath and Jörg Berger for their help in the field, Katrin Katzur who supplied the larvae of *Agriotes* sp. and Matthias Schulz for helpful discussions.

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CHAPTER 3

On the Host Plant Choice by Belowground Living Insects, Influenced by Root Volatiles

Weissteiner S. & Schütz S.

c.p. less advanced: Weissteiner S. & Schütz S. (2006): Are different volatile pattern influencing host plant choice of belowground living insects? Proceeding of German Society for General and Applied Entomology (DGaaE), 54.

3.1 Abstract

Beeinflussen verschiedene Volatilenmuster die Wirtspflanzenwahl unterirdisch lebender Insekten?
Zum besseren Verständnis der Orientierung und Fraßpräferenz von Maikäferlarven *Melolontha hippocastani* Fabr. (Coleoptera: Scarabaeidae) im Boden wurden Wahltests durchgeführt. Den Larven von *M. hippocastani* wurden Karotten (*Daucus carota* ssp*. sativus*) und Eichenwurzeln (*Quercus* sp.) zur Auswahl angeboten. Die Duftstoffe von Karotte und Eichenwurzel wurden auf Aktivkohle gesammelt und mit Gaschromatographie – Massenspektroskopie untersucht (GC-MS). Unverletzte Karotten sowie Eichenwurzeln unterschieden sich in ihren Volatilenmustern deutlich voneinander. Darüber hinaus konnten Unterschiede im Volatilenmuster unverletzter, mechanisch verletzter sowie angefressener Wurzeln nachgewiesen werden.

Key words: choice test, GC-MS, *Melolontha hippocastani*, root volatiles, Scarabaeidae

3.2 Introduction

Cockchafers of the genus *Melolontha* (Coleoptera: Scarabaeidae) can be severe pests in forestry, agriculture and horticulture. Gradation of the two most important species, the forest cockchafer *M. hippocastani* Fabr. and the European cockchafer *M. melolontha* L., currently occurs in several parts of central Europe.
Orientation behaviour of the adult beetles has been the focus of recent studies (Reinecke et al. 2002 a, b, 2005). Most dreaded, however, is the root-damage caused by larvae feeding belowground, as it is not directly to be detected.
There are some speculations about preferential feeding behaviour of belowground living insects, but, up to now, only few experimetal investigations.
There are some publications discussing orientation behaviour of belowground living insects (Horber 1954, Hauss & Schütte 1976, Hasler 1986, Hibbard et al. 1994,

Jewett & Bjostad 1996, Bernklau & Bjostad 1998a, Bernklau & Bjostad 1998b, Bernklau et al. 2005) but more detailed experimental investigations seem to be inevitable. The present study hopes to help filling the gap.

3.3 Material and Methods

In order to understand behavioural patterns of belowground living insects in selecting plants for feeding I isolated and selected organically cultivated carrots (*Daucus carota* ssp. *sativus*) and about 5 year old oak trees (*Quercus* sp.). These I offered as feed to cockchafer larvae. The plants investigated were from a forest near Göttingen (Germany). The larvae (L3) of *M. hippocastani* were collected in a forest near Darmstadt (Germany).

For measuring root volatiles in different stages I only slightly damaged the capillary roots and external bark (undamaged roots), others I damaged massively by cutting roots by knife (mechanical damage), some I – prior to the experiment – exposed for feeding by larvae (insect damage).

For collecting the root volatiles (N=5-10) I used bags of PTFE foil. At the beginning and at the end of each behavioural experiment (according to the three stages of the setup of our experiments) root volatiles were collected from the bare roots using the closed-loop-stripping-analysis (CLSA) method (Boland et al. 1984). Within these bags the air was circulated through a charcoal filter with a flow of 1 l/min for a sampling time of 3 hours for oak roots. Carrots were sampled only for 1 hour in order to avoid overloading charcoal trap and GC column. Volatiles were eluted from the charcoal with a 2+1 mixture of methylene chloride and methanol. Odour samples were analysed by coupled gas chromatography-mass spectrometry (Weissbecker et al. 2004). The GC (model 6890N, Agilent, Palo Alto, USA) employed the temperature program: start: 50°C, hold for 1.5 min, ramp 6°C/min to 200°C, hold for 5 min. It was equipped with a split/splitless-injector operated at 250°C in the pulsed-splitless-mode and two GC-columns were employed for identification: HP-5MS column (length 30m, ID 0.25 mm, film thickness 0.25 µm, Agilent) and HP-Innovax column (length 30m, ID 0.25 mm, film thickness 0.25 µm,

Agilent). Helium was used as carrier gas at a constant flow of 1 ml/min. The odour compounds were identified by comparison of retention time and mass spectra with the NIST library and the MASS FINDER library.

During the dual choice tests the larvae of *M. hippocastani* (N=20) were kept individually in 20 cm high black 10 l-plastic buckets with a diameter of 28 cm together with carrots and oak trees. The distance between the two plants was about 15 cm. The larva was placed halfway from carrot and oak and about 10 cm below soil surface. Humous, sandy and clayey soil was used as a substrate. The experiment was performed in June 2004 in the glasshouse under controlled conditions (photoperiod 16 hours, 10 kLux, 19-25°C, 40-50% relative humidity). Position of the larvae in relation to the roots and feeding traces of larvae at the roots were assessed in order to evaluate the decision of the larvae.

After one week the roots were visually inspected for signs of feeding damage. The experiment was prolonged for those larvae that did not show any clear decision for one of the plants.

3.4 Results

Undamaged oak roots predominantly release fatty acid derivatives whereas damaged oak roots release phenols and monoterpenoids (figure 3.2 a-c). Both undamaged and mechanically damage roots caused volatile patterns distinctive from the volatile pattern caused by larval feeding (figure 3.1 a-c, figure 3.2 a-c)

In dual choice tests feeding preferences of larvae of *M. hippocastani* were observed: carrots were clearly favoured if the larvae had the opportunity to decide between carrots and oak roots. After 3 weeks, 4 larvae had died during the experiment. Four of the remaining 16 larvae (=25%) caused feeding traces on both of the roots (by considering only the main roots of the oak trees and carrots), 1 larvae (=6%) showed no decision for one of the plants. 11 larvae (=69%) fed on the roots of carrots exclusively.

According to the null hypothesis the roots of carrots and oaks would be accepted to the same extent. The validity of the null hypothesis was checked by the sign test with correction for continuity. The test statistic u was 2,581989 and thus lead to the

rejection of the null hypothesis for α = 0.05. Therefore the acceptance of the carrots by the larvae was significantly higher than the acceptance of the oak roots.
Volatile pattern released by carrot roots and oak roots differ both qualitatively and quantitatively. Undamaged carrot roots release predominantly monoterpenoids whereas damaged carrot roots release sesquiterpenoids (figure 3.1).

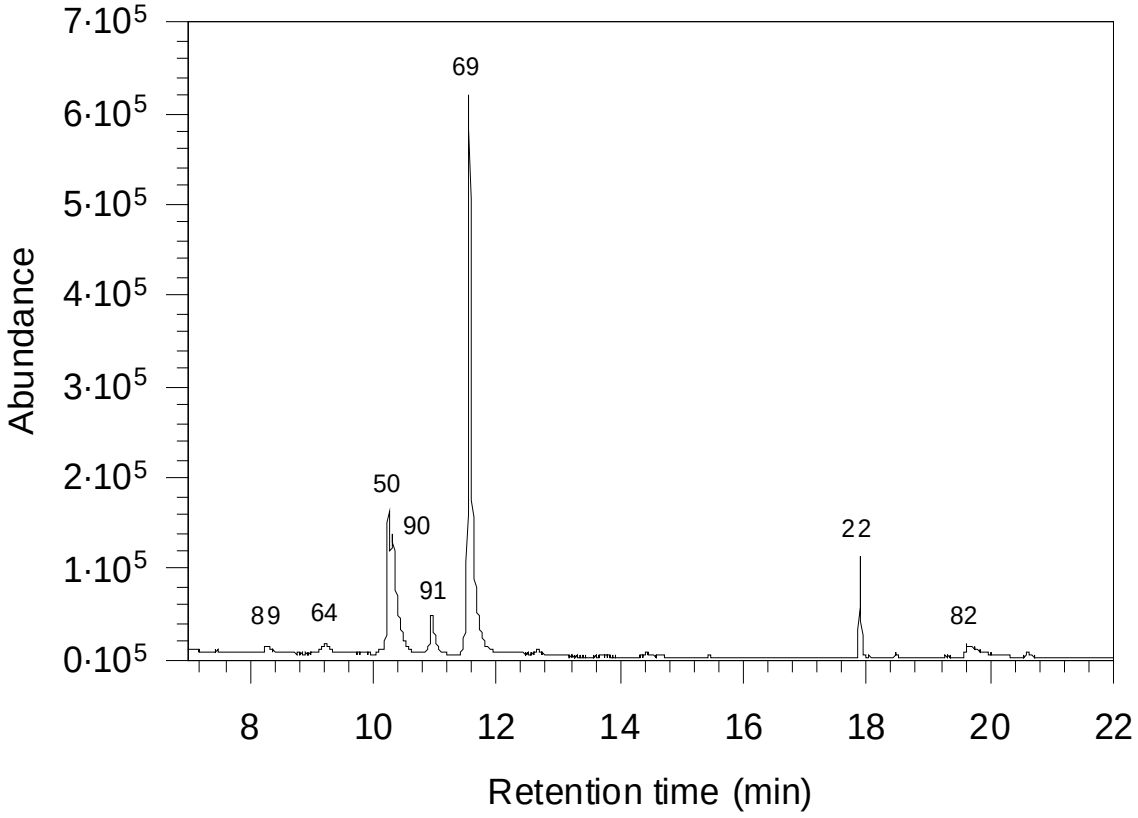

Figure 3.1.a: root volatiles of *Daucus carota* ssp. *sativus*, undamaged plant (N=5)

22→ β-caryophyllene (5/5)
50→ cymol (2/5)
64→ β-pinene (5/5)
69→ terpinolene (5/5)
82→ β-farnesene (2/5)
89→ α-pinene 4/5)
90→ limonene (3/5)
91→ τ-terpinene (5/5)

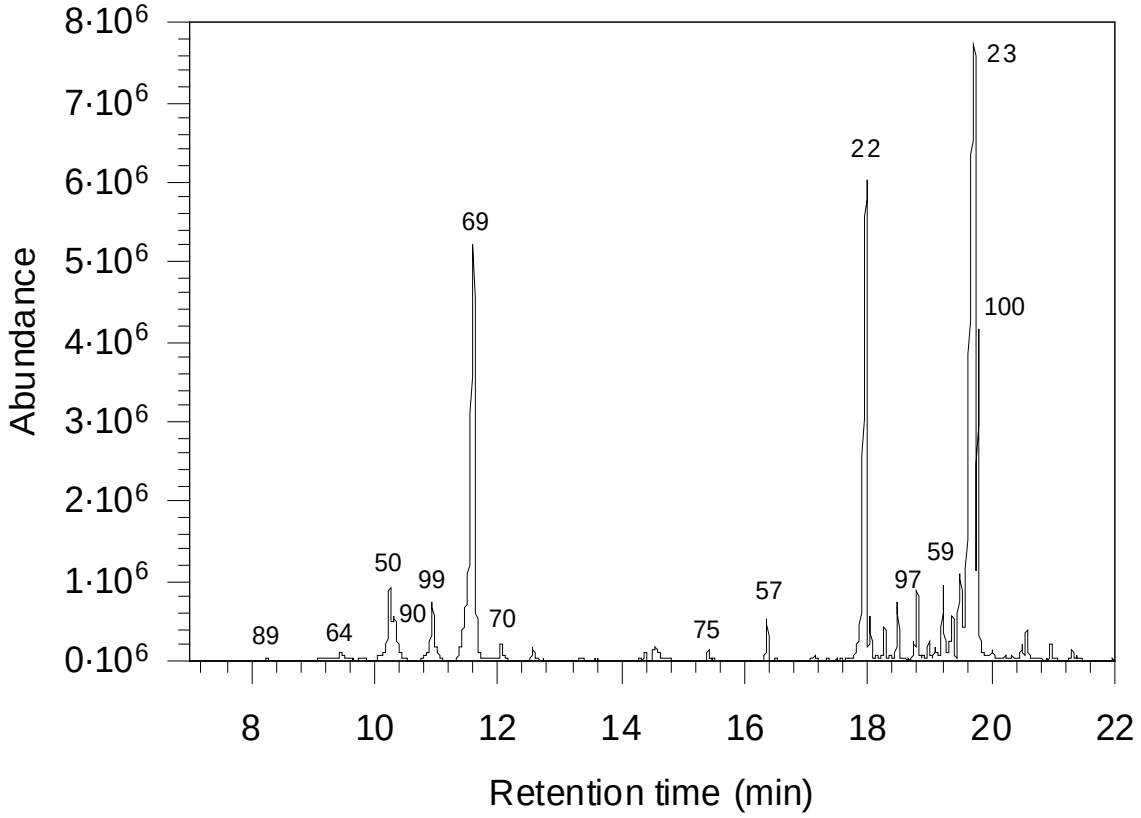

Figure 3.1.b: root volatiles of *Daucus carota* ssp. *sativus*, mechanically damaged (N=4)

22→ β-caryophyllene (4/4)
23→ α-farnesene(4/4)
50→ cymol (3/4)
57→ δ-elemene (3/4)
59→ β-bisabolene (2/4)
64→ β-pinene (4/4)
69→ terpinolene (4/4)
70→ 1,3,8-p-menthatriene (4/4)
75→ bornyl acetate (4/4)
89→ α-pinene (3/4)
90→ limonene (4/4)
97→ α-curcumene (4/4)
99→ 1,4-p-menthadiene (3/4)
100→ unidentified compound (3/4)

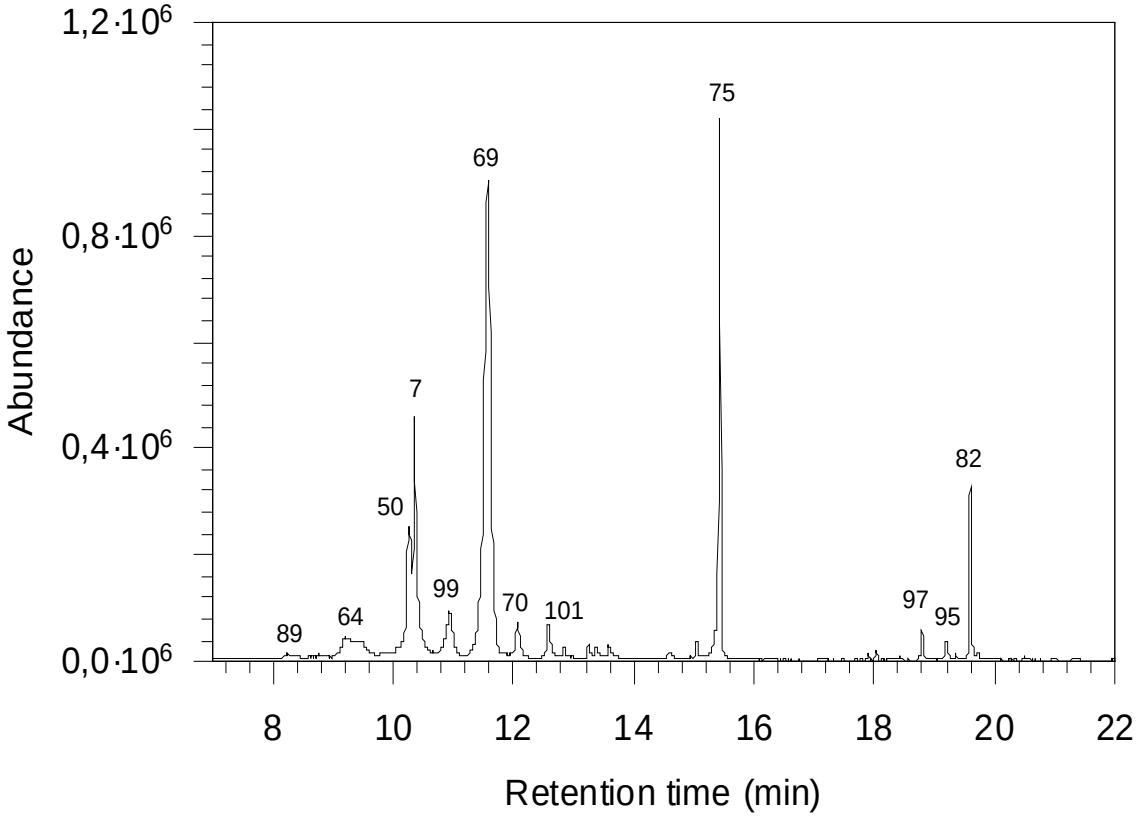

Figure 3.1.c: root volatiles of *Daucus carota* ssp. *sativus*, damaged by feeding of *M. hippocastani* (N=4)

7→ 2-ethyl-1-hexanol 4/4)
50→ cymol (2/4)
64→ β-pinene (4/4)
69→ terpinolene (4/4)
70→ 1,3,8-p-menthatriene (4/4)
75→ bornyl acetate (4/4)
82→ β-farnesene
89→ α-pinene (3/4)
95→ 2,4-di-t-butyl phenol (2/4)
97→ α-curcumene (3/4)
99→ 1,4-p-menthadiene (3/4)
101→ 1-ethyl-3,5-dimethyl benzene (2/4)

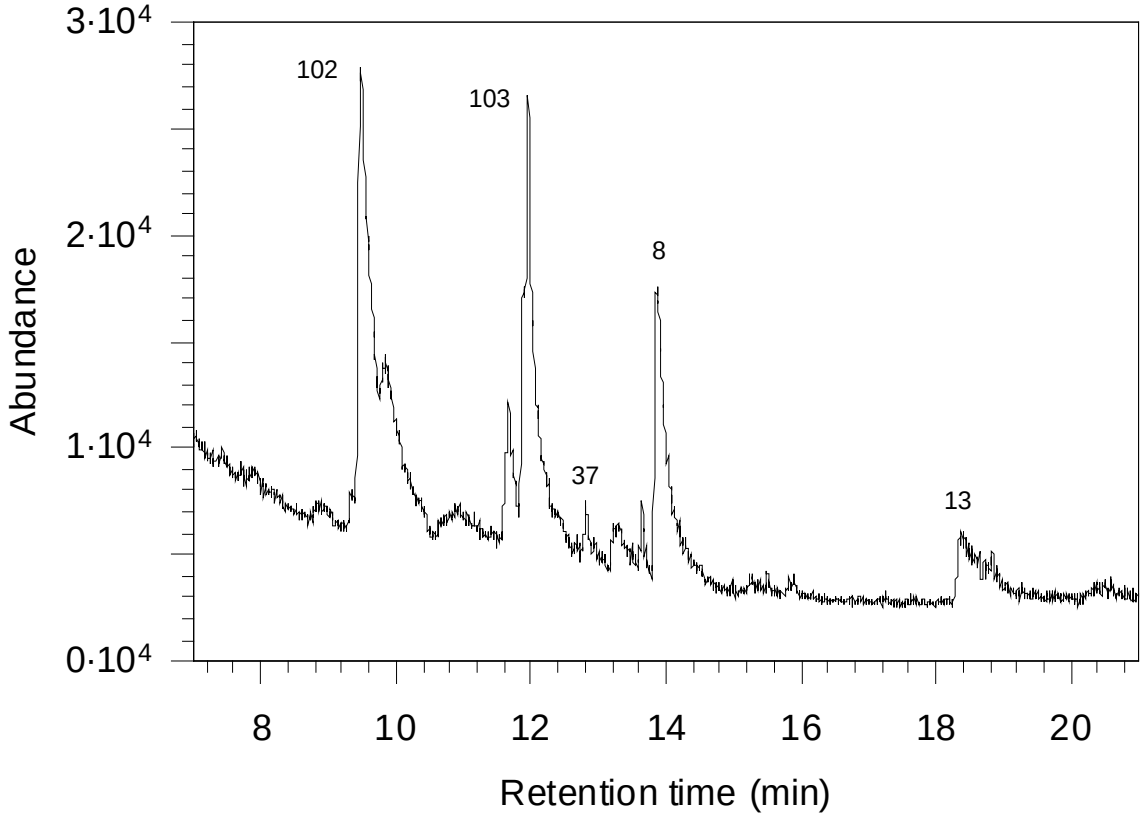

Figure 3.2.a: root volatiles of *Quercus* sp., undamaged plant (N=5)

8→ decanal (4/5)
13→ geranyl acetone (5/5)
37→ camphor (2/5)

102→ methyl heptenone (3/5)
103→ 3-methyl-3-cyclohexen-1-one (2/5)

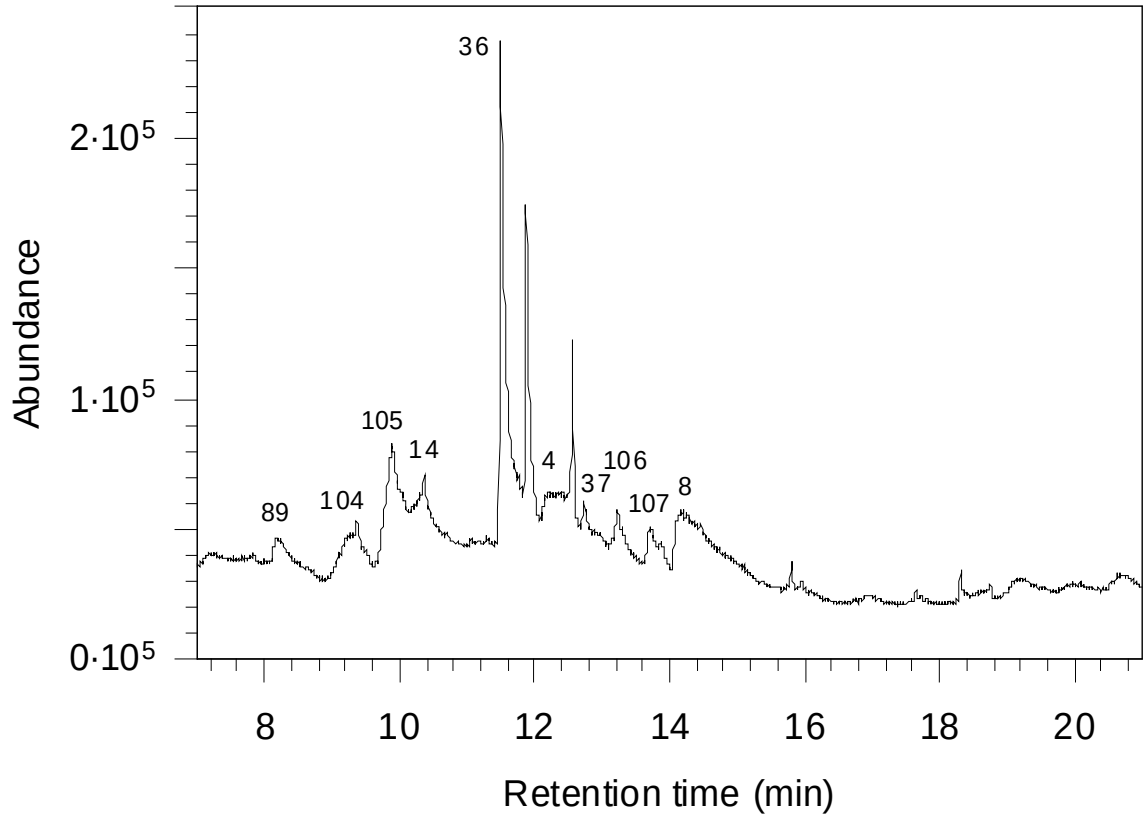

Figure 3.2.b: root volatiles of *Quercus* sp., mechanically damaged (N=4)

4→ nonanal (4/4)
8→ decanal (4/4)
14→ 1,8-cineol (3/4)
36→ geranyl acetone (4/4)
37→ camphor (3/4)

89→ methyl heptenone (3/4)
104→ ethyl toluene (1/4)
105→ 3-carene (3/4)
106→ epoxy linalool (2/4)
107→ dodecane (2/4)

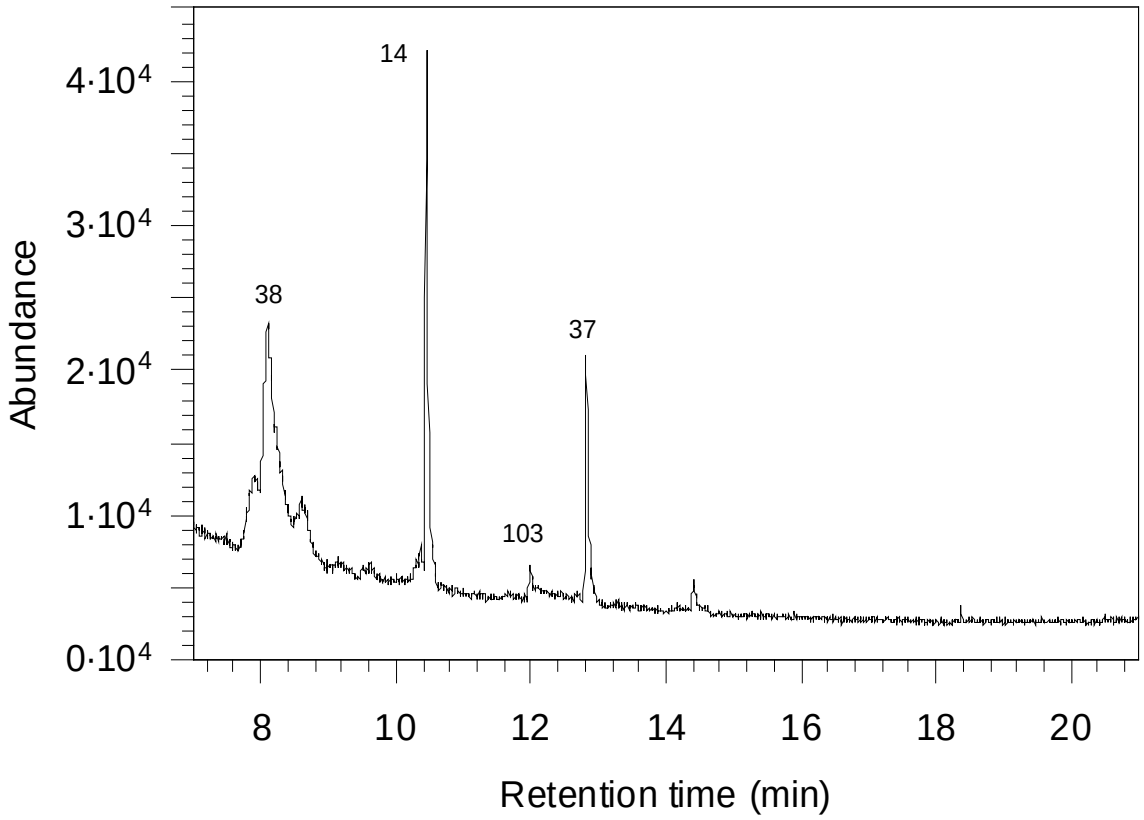

Figure 3.2.c: root volatiles of *Quercus* sp., damaged by feeding of *M. hippocastani* larva (N=3)

14→ 1,8-cineol (3/3)
37→ camphor (2/3)
38→ anisol (3/3)

103→ 3-methyl-3-cyclohexen-1-one (1/3)

3.5 Discussion

The common hypothesis about the behaviour of in soil living organisms suggest that orientation towards host plant roots of *Melolontha*-larvae is principally guided by a CO_2-gradient (Horber 1954, Hauss & Schütte 1976, Hasler 1986) which is caused by plant root respiration. This means that CO_2 for soil inhabiting polyphagous larvae could function as a non specific lure to find their potential host plants. In addition, volatile secondary plant substances released by the roots might be utilized by the larvae as an important additional cue for orientation and choice of host plants. However, it is important to consider that the composition of the root volatiles is not only influenced by the species but also by the physiological status of the plants (mechanical damage, feeding damage, colonisation by microorganisms). Moreover, the rhizosphere is inhabited by numerous microorganisms modifying plant root volatiles. Additionally plant volatiles might be transformed by these microorganisms, which, in turn, release their own volatile metabolites. Thus, it is a demanding task for the larvae to find the proper food source belowground.

Ene (1942) mentioned that the orientation of *M. melolontha* larvae is depending more on the quality of the root than on the plant species. Thiem observed some years later (1949) that not only the root tissue quality is important for the behaviour, because in his experiments the larvae preferred clearly carrots over potatoes (*Solanum tuberosum*). However, Hoffmeister (1957) performed experiments with larvae of *M. melolontha* and found out that the level of lignification is an important factor of the choice by the larvae. It is unlikely that these discriminations can be performed on the basis of CO_2-gradient only. Thus, differentiation of plant species by *Melolontha*-larvae needs a contribution of secondary plant metabolites. Moreover it was shown that volatiles released by damaged roots have an impact on orientation behaviour of belowground invertebrates (Rasmann et al. 2005). Insect pathogenic nematodes are attracted by damage induced root volatiles. This suggests that similar mechanisms of volatile guided orientation might be used by invertebrates belowground in a similar way to mechanisms known from insects aboveground. Larvae of *M. melolontha* might serve as a first potential example.

Acknowledgements

We are grateful for the financial support of S. Weissteiner by the CUSANUSWERK. We thank the RF Griesheim for supplying the larvae of *M. hippocastani* and the RF Ebergötzen for supplying the trees. Moreover we want to thank Jörg Berger and Stefan Rath for their assistance.

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CHAPTER 4

Volatile Emissions of Oak Trees Modified Through Shoot and Root Damage by Arthropod Feeding

4.1 Abstract

Below ground feeding of 3rd larval instar of *Melolontha hippocastani* on the roots of *Quercus* sp. was studied with respect to the volatile emissions of these plants. Shoot and root volatiles were trapped on active charcoal and analysed by GC-MS (Gas Chromatography-Mass Spectrometry). The volatile patterns differ clearly between shoots and roots. Moreover, different volatile compounds were emitted by plants above- and belowground, if the roots were damaged by feeding of *M. hippocastani*.

Key words: shoot volatiles, root volatiles, *Melolontha hippocastani*

4.2 Introduction

The emission of volatile organic compounds (VOCs) is very common in plants and occurs during all ages of plant life (Arimura et al. 2000). VOCs such as isoprene and monoterpenes are synthesized in plant plastids (Croteau 1987, Kleinig 1989, Chappell 1995) and are emitted through the stomata into the atmosphere (Sharkey 1991, Fall & Monson 1992, Loreto et al. 1996). Volatile emissions of herbivore infested plants are complex blends, often composed of more than 100 different compounds (Dicke & Vet 1999). Belowground damage like root herbivory by *Melolontha* larvae is responsible for reduced plant growth and increased plant mortality (Wildbolz 1982, Pötsch et al. 1997). Plants frequently are damaged simultaneously by above- and belowground feeding organisms (Muller-Scharer & Brown 1995) giving rise to all kinds of interactions (Masters et al. 1993, Masters & Brown 1997).

Several studies have shown that root herbivory can influence aboveground plant emissions (*e.g.* Wäckers & Bezemer 2003). In trees, to my knowledge, this phenomenon is not investigated yet. Herbivory is able to induce a stress response within the host plant, which can lead to a re-allocation of plant resources like carbohydrates and soluble nitrogen between root and shoot tissue. The

concentration of these compounds is known to directly affect insect growth parameters (Masters & Brown 1997). Root herbivory may enhance aboveground herbivores by accumulating carbohydrates and soluble nitrogen in the shoot as a stress response to root damage, which may be an advantage for herbivores living aboveground. Thus, perception of volatiles induced by root herbivory might be important for aboveground herbivorous insects.

The effect of aboveground damaging processes on belowground plant emissions is not extensively investigated yet (Bezemer & van Dam and references therein 2005). Feeding by folivorous insects may have a negative impact on root feeders (Masters et al. 2001 and references therein). Thus, perception of volatiles induced by shoot herbivory might be important for belowground herbivorous insects.

The effect of root feeding by larvae of *M. hippocastani* on aboveground and belowground volatile emissions in *Quercus* sp. is not known yet and, to my knowledge, is investigated in this study for the first time.

4.3 Material and Methods

4.3.1 Insects and Plants

The larvae of *M. hippocastani* were collected in the urban forest of Griesheim, near Darmstadt (Germany). They were kept individually in plastic pots (8 cm x 13 cm x 7 cm, filled with sieved native soil from Griesheim) at 20°C and fed once a week with carrot slices. The humidity of the substrate was checked once a week as well.

The plants used for the experiments were oak trees (*Quercus petraea* and *Q. robur,* about 7 years old), from a forest near Göttingen. The trees were kept individually in 10 l plastic buckets (20 cm high x 28 cm diameter) in a greenhouse under controlled conditions (photo period 16 hours, 10 kLux, 19-25°C, 40-50% relative humidity). The substrate was sandy, clayey soil. All trees were visually inspected for Tetranychidae, Thripidae and Aphididae as well as for mildew (*Microsphaera alphitoides*). The roots of *Quercus* sp. were visually inspected for ectomycorrhiza.

4.3.2 Experimental Conditions

4.3.2.1 Treatments

About 120 trees of *Quercus* sp. were dug out in february 2004 in the forest. They were planted in single plastic buckets in mother soil from the forest. For acclimatization the trees were placed for three months in the greenhouse, before they were planted in a sandy, clayey soil in single plastic buckets. During the winters the oaks were kept outside, with buckets buried in the soil.

Undamaged Above- and Belowground

The first volatile sampling in early july of 2006 aimed to record volatile patterns of the shoots and roots of 10 undamaged *Quercus* sp. trees. However, slight mechanical damage due to handling and preparation, as well as an infestation of about 15% of the leaves by Tetranychidae, Thripidae and Aphididae has to be accounted for as a general condition of all samples. Additionally, about 15% of the leaves were infested with mildew. Ectomycorrhiza could be found in all samples. These trees were indicated as “undamaged” and, after being subjected to the sampling process, they were not used for further experiments.

Piercing-Sucking Damage Aboveground by Arthropods

Volatile patterns of 10 shoots of *Quercus* sp. indicated as “aboveground damaged” were obtained about one week later. About 70% of the leaves of Quercus sp. trees were colonised by Tetranychidae, Thripidae, and Aphididae. About 30% of the leaves were infested with mildew.

Feeding Damage Belowground by Beetle Larvae

During the volatile sampling of undamaged shoots and roots, larvae of *M. hippocastani* of the 3rd larval stage were kept inside the plastic buckets of trees, for about one week. This allowed them to damage the roots of 15 Quercus sp. by feeding. The position of the larvae as well as feeding signs on the main roots were

recorded. Volatile patterns of the roots were taken the same day as the volatiles of the damaged shoots.

4.3.3 Sampling

For volatile sampling the trees were transferred to the laboratory at 21°C. The aboveground and belowground parts of the trees were enclosed in PET foil (Toppits, Cofresco Frischhalteprodukte, Minden, Germany). In a first step, volatiles of the shoots were collected and trapped with charcoal traps. In a second step, roots and larvae were dug out carefully, the position of the larvae as well as feeding signs on the main roots were recorded. Only plants with significant feeding traces on the main roots were regarded as “damaged by root feeding”. Roots were carefully washed with tap water before the sampling process of the roots could start.

Volatiles were obtained by circulating the air through adsorbent traps loaded with 1.5 mg charcoal (Daumazan sur Arize, France), by miniature pumps (Fürgut, Tannheim, Germany). The sampling time for the shoots was 1 hour with a flow rate of 60 l/h, for the roots it was 2 hours. The volatiles were eluted from the charcoal with 75 µl of a 2+1 mixture of methylene chloride and methanol (both solvents Suprasolv-quality, Merck/VWR, Darmstadt, Germany). After elution, the samples were immediately analysed by using a gas chromatograph coupled to a mass spectrometer (6890N and 5973, Agilent, Palo Alto, USA, technical information see Weissbecker et al. 2004) or stored at -76 °C in an ultra low temperature freezer for later analysis.

4.3.4 Analytical Procedure

1µl of the eluate was injected into the S/SL injector operated at the pulsed splitless mode (pulse pressure 150 kPa until 1.5 min), at a temperature of 250°C. For chemical identification, a polar column was used (HP-INNOWAX, length 30 m, ID 0.25 mm, film thickness 0.25 µm, Agilent). The GC (6890N, Agilent, Paolo Alto, USA) was operating in the following temperature program: start: 50 °C, hold for 1.5

min, ramp 7.5 °C/min to 200 °C, hold for 5 min. Helium (purity 99.999%) was used as carrier gas with a flow rate of 1 ml/min. The temperature at the transfer-line was 280°C. The mass spectrometer (5973, Agilent, Paolo Alto, USA) operated in the scan mode with a mass range from 35 to 300 atomic mass units.
The volatile compounds were identified using the Mass Spectral Search library of the National Institute of Standards and Technology (NIST, Gaithersburg, USA) and the database of MassFinder 3.0 software in conjunction with the library “Terpenoids and Related Constituents of Essential Oils” (Hochmuth, König, Joulain, Hamburg, Germany). Selected compounds were identified by direct comparison of retention time and mass spectra recorded from authentic standards.

4.4 Results

In a qualitative analysis, most of the root volatiles differ from the shoot volatiles. The volatile emissions of the roots are changing if larvae of *M. hippocastani* are feeding on it. Moreover, also the shoot volatiles are changing, if larvae are damaging the roots by feeding.
Still, statements to the appearance of chemicals, which were not identified by comparing retention time and mass spectra with those of authentic standards but solely relied on matches with mass spectra and retention order of the database, have to be handled with care.
The chromatograms of the different treatments are shown in the appendix.

6-methyl-5-hepten-2-one was emitted in almost all samples, as well as nonanal, 2-ethyl-1-hexanol, hexadecane, isopropyl laurate and geranyl acetone.
Benzyl alcohol, 2-pentanol, trimethyl benzene and acetic acid were emitted by shoots as well as by roots without any clear distribution pattern.
The green leaf volatiles (GLV) (Z)-3-hexenyl acetate and (Z)-3-hexen-1-ol were observed as shoot specific in all shoot samples.
Benzaldehyde, 3-octanone, linalool oxide and camphor were emitted by roots of *Quercus* sp. damaged through larval feeding and by some undamaged.

Table 4.1 shows the compounds occurring only in one of the five different treatments. Numbers given behind compounds show how often the compound was present in all samples of the treatment above detection threshold. These compounds occurred as so-called “marker-compounds” for the particular treatment.

Table 4.1: SDA Shoot measured, colonised aboveground by arthropods and infestation of mildew; SUA Shoot measured, plant undamaged; SDB Shoot measured, colonised aboveground by arthropods with additionally infestation of mildew as well as root feeding of *M. hippocastani;* RDA Root measured, plant undamaged; RDB Root measured, shoot damaged aboveground by arthropods and infestation of mildew with additionally root feeding of *M. hippocastani.*

SDA
β-ocimene 6/7
hexyl acetate 7/7
β-caryophyllene 1/7
α-farnesene 6/7

SUA + SDA
2,6-dimethyl-1,3,5,7-octatetraene 2/7 + 7/7
germacrene 1/7 + 2/7
2,6-dimethyl-3,5,7-octatriene-2-ol 3/7 + 6/7

SDB
2-butoxy ethanol 7/9
β-bourbonene 4/9
methyl salicylate 7/9

RDA
heptanal 4/9
3-ethyl toluene 8/9
sabina ketone 5/9
diethoxy methane 9/9

RDB
anisole 10/11
methyl benzyl ether 4/11
borneol 8/11

Table 4.2 gives an overview of all detected chemicals in *Quercus* sp. They are sorted by their occurrence presented in the results.

Table 4.2: Compounds emitted by *Quercus* sp. in the five different treatments. Compounds, appearing in less than 50% of the single measurements, are marked with **O**, those, appearing in more than 50% of the single measurements are marked with **X**. Nr...Numbers referring to the identification/indication in the chromatograms, RT...Retention time, SUA...Shoot measured, plant undamaged, SDA...Shoot measured, colonised aboveground by arthropods and infestation of mildew, SDB...Shoot measured, colonised aboveground by arthropods with additionally infestation of mildew as well as root feeding of *M. hippocastani*, RDA...Root measured, plant undamaged, RDB... Root measured, shoot damaged aboveground by arthropods and infestation of mildew with additionally root feeding of *M. hippocastani.*

	ABOVEGROUND	**BELOWGROUND**
***Quercus* sp.**	2,6-dimethyl-3,5,7-octatriene-2-ol 2-butoxy ethanol	3-ethyl toluene anisol
Aesculus hippocastanum	3-hexenyl isovalerate δ-cadinene 2-hexenyl acetate 2-hexen-1-ol 1-penten-3-ol 2-hexenal cubebene	curcumene cymol octanal p-methyl anisol thymol methyl ether

4.5 Discussion

As shown in this study and mentioned in earlier own studies (Weissteiner & Schütz 2006), plants vary in the volatile composition above- and belowground. Plant shoots and roots are attacked by several herbivorous organisms and thus plants emit special volatiles above- and belowground, which in turn are able to attract or repel other organisms (Dicke et al. 2009). The emission of so-called herbivore induced plant volatiles (HIPV) is dependent on abiotic factors, of which light is the most important (Gouinguene & Turlings 2002). It occurs locally as well as systemically (Turlings & Tumlinson 1992). The systemic emission is mediated by internal

signals, which may be transported through the vascular tissue (Dicke et al. 1993, Jones et al 1993). Isoprene and monoterpene emission rate is strongly affected by temperature and light (Rasmussen & Jones 1973, Tingey et al. 1979, Monson & Fall 1989, Loreto & Sharkey 1990, Staudt & Seufert 1995, Staudt & Bertin 1998) as well as by CO_2 (Loreto et al. 1996/1998). The aboveground volatile emissions of *Quercus sp.* were studied by several groups, such as Kesselmeier et al. 1996/1997/1998, Staudt et al. 1993/2001, Fischbach et al. 2000, Niinemets et al. 2002, Loreto et al. 2009. “Large differences in emissions from species within the same genus have been described. For example, *Q. ilex* is known to be a strong emitter of monoterpenes (Bertin et al. 1997, Street et al. 1997) whereas other *Quercus* species, e.g. *Q. virginiana*, are isoprene emitters (Tingey et al. 1981)” (Owen et al. 1997).

In the experiments described in the present study, the fatty acid derivative (Z)-3-hexenyl acetate and the alcohol (Z)-3-hexen-1-ol are typical shoot volatiles appearing in all shoot samples, whereas benzaldehyde, 3-octanone, and camphor appear as typical root volatiles in almost all root samples.

Arimura et al. (2008) mentioned that in lima bean (*Phaseolus lunatus* L.), the emission of terpenoids like β-ocimene is dependent on photosynthetic fixation of CO_2, whereas the emission of fatty acid derivatives like (Z)-3-hexenyl acetate is mediated by constitutively expressed enzymes as well as phytohormone induced biosynthesis.

(Z)-3-hexenyl acetate is emitted by aboveground parts of damaged plants a few hours after herbivore feeding or mechanical damage (Röse & Tumlinson 2004).

As stated by Dicke et al. (1990), Lima bean leaves infested by *Tetranychus urtica* emit the kairomone terpenoids β-ocimene, (E)-4,8-dimethyl-1,3,7-nonatriene and linalool and the phenolic compound methyl salicylate (MeSA, the methyl ester of salicylic acid). This odour complex attracts predatory mites and a special predatory beetle (*Oligota kashmirica benefica* N., Shimoda & Takabayashi 2001). These compounds are known to be produced by plants but not by animals, and they were not emitted by undamaged or mechanically damaged Lima bean leaves.

In the experiments with *Quercus* sp., β-ocimene was emitted only in case of heavy infestation of the shoots by arthropods as well as with mildew. It was not emitted in detectable abundances in undamaged shoots of the investigated *Quercus* sp. trees,

although they were all slightly mechanically damaged during the preparation process, and all subjected to a slight arthropod as well as mildew infestation. (Z)-3-hexenylacetate and β-ocimene were observed also in Lima bean leaves during feeding of *Spodoptora littoralis* B. larvae (Kunert et al. 2002). Whereas *Spodoptera littoralis* feeds in a biting-chewing way, Tetranychidae, Thripidae and Aphididae are more piercing-sucking organisms.

MeSA and several other terpenoids are discussed as useful chemicals for enhancing the effectiveness of carnivorous natural enemies of spider mites (Dicke et al. 1990, Shimoda et al. 2002). MeSA was described as the most abundant compound emitted by infested plants (Dicke et al. 1999). In *Quercus* sp. it was emitted only by shoots of plants which were infested aboveground with Tetranychidae, Thripidae and Aphididae as well as with mildew and simultaneously damaged belowground by larval feeding of *M. hippocastani*. Cardoza et al (2002) mentioned that MeSA, (Z)-3-hexenyl acetate and linalool significantly inhibited fungal growth on solid culture media.

As a typical indicator for fungal growth, 3-octanone (Combet et al. 2006) was emitted by the roots of undamaged and larval damaged *Quercus* sp. This C_8-compound may be released upon mycorrhiza fungi colonization of the undamaged and damaged roots. Mutualistic arbuscular mycorrhizal (AM) fungi affect even parasitoids and pollinators by colonising the roots, depending on the fungal species (Gange & Smith 2005). Some fungal combinations showed increasing effect on parasitism, some showed a decreasing one, whereas others had no effect (Gange et al. 2003). *Quercus* sp. shoots infested with the fungus *Microsphaera alphitoides* did emit the terpenoid 1,8-cineol, in few of the heavily infested shoots, as well as by almost all roots damaged by larval feeding.

In the experiment, anisol, methyl benzyl ether, and borneol were emitted only by roots, which were damaged by larval feeding. In another study (Weissteiner et al., in prep.), borneol was emitted also by undamaged and mechanically damaged roots of *Quercus* sp., whereas anisol was emitted only by roots, which were damaged by feeding of *M. hippocastani* larvae. Methyl benzyl ether may be defined as a marker compound for insect infestation. It can as well be emitted by green walnuts infested with the codling moth *Cydia pomonella* L. (Buttery et al. 2000). However, the volatile emission belowground has so far not been studied as intensively as aboveground. Rasmann et al (2005) as well as Köllner et al (2008) investigated the volatile

emission of maize plants attacked by corn rootworm larvae *Diabrotica virgifera virgifera* L. The roots emitted (E)-β-caryophyllene, which attracted the entomopathogenic nematode *Heterorhabditis megidis* P., which in turn attacked and killed the rootworm larvae. The simultaneous infestation of maize plants *Zea mays* L. with *D. v. virgifera* and the foliar herbivore *Spodoptera littoralis* reduced significantly the attraction of the entomopathogenic nematodes by a lower emission of (E)-β-caryophyllene (Rasmann & Turlings 2007).

Volatile emission in plants is a highly complex process, affected by many factors. Belowground insect damage, which was investigated in this study, is focussing one aspect. The additional foliar damage, in the experiment by arthropod feeding of Tetranychidae, Thripidae and Aphididae as well as additional mildew infestation, is enhancing the complexity of the system. It might be responsible for altering volatile patterns and may affect as well plant fitness directly by reducing the photosyntetic area (Strauss 1991). Very few studies have investigated the effect of multiple stresses to volatile emissions, which is not simply an addition of single stress factors (Mittler 2006). Knowing single attacker systems does not allow to predict in general the responses in multiple attacker systems (de Vos et al. 2006, Moayeri et al. 2007, de Boer et al. 2008). However, knowledge of multiple stress effects is highly relevant to practical issues, since in nature plants are rarely exposed to single stress factors (Mittler 2006).

Thus, the results of this study should be complemented by further investigations in order to specify possible interaction effects of different kinds of stress inflicted to different organs of the trees.

Acknowledgements

I'm grateful for the financial support by the CUSANUSWERK. I thank the forestry district Griesheim for supplying the larvae of *M. hippocastani* and the forestry district Ebergötzen for supplying the trees. Moreover, I thank Jörg Berger, Reinhold Dankworth and Stefan Rath for their assistance.

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CHAPTER 5

Antennal and Behavioural Responses of *Melolontha hippocastani* larvae to Different VOCs of *Quercus* sp. Root Respiration Based on Morphological Findings

in preparation for journal submission

5.1 Abstract

The larvae of *Melolontha hippocastani* F. (Coleoptera: Scarabaeidae) are known as pest organisms in agriculture and forestry. Roots of *Quercus* sp. are a preferred forage. In the present study the root volatiles of *Quercus* sp. (undamaged, mechanically damaged, and damaged by feeding) were investigated and analysed by using gas chromatography – mass spectrometry (GC-MS). Furthermore, via electroantennogragraphy (EAG) we proved the physiological impact of typical volatiles found in damaged roots on the antennae of *M. hippocastani* larvae, and we investigated the behavioural responses of the larvae to these volatiles, namely: anisol, 1,8-cineol, 3-octanone, camphor, and furanoid trans-linalooloxide. Here we show that oak roots release different volatile compounds upon different type and state of damage and that cockchafer larva antennae are able to detect some of these volatile compounds in electrophysiological tests. Moreover, we demonstrate that some compounds detected in roots damaged by larval feeding lead to significant responses in larval orientation behaviour and that these antennal structures send this message into the glomeruli of the larva, a brain structure attributed to the first processing of olfactory signals, eliciting behavioural responses as attraction or avoidance to the perceived odours. Thus, cockchafer larvae are employing mechanisms for belowground host plant finding that are similar to those employed by the adult beetles flying aboveground, despite drastically different physicochemical conditions in the soil.

Key words: root VOC, *Qercus* sp., *Melolontha hippocastani*, GC-MS, EAG

5.2 Introduction

Larvae of the cockchafer *M. hippocastani* F. (Coleoptera: Scarabaeidae) cause conspicuous root damage to a broad range of horticulturally and silviculturally important plants, especially to young oaks on reforestation sites.

While the adult beetles migrate kilometres and defoliate several tree species, the grubs show a pronounced preference to a variety of tree roots and are able to move towards their targets as far as several meters through the soil (Ene 1942, Hasler 1986). The larvae face the situation that belowground orientation cannot rely on vision and that every movement in the wrong direction is quite energy consuming. Carbon dioxide gradients were proven to attract cockchafer larvae (Hasler 1986) but it seems unlikely that host plant discrimination is mediated by carbon dioxide only. Already behavioural studies exist indicating that volatile organic compounds might play a role in belowground host plant finding of coleopteran larvae (Nordenhem & Norlander 1994, Thomas et al. 2008).
The blend of compounds emitted by damaged plants depend on the animal, causing the damage by specific feeding habits (Delphia et al. 2007, Gosset et al. 2009) and on the type of damage.

5.3 Materials and Methods

5.3.1 Insects

Second instar larvae of *M. hippocastani* were collected in late may of 2007 in a forest near Darmstadt (Germany). The larvae were kept for several months at 20°C in the dark. To prevent cannibalism, larvae were kept individually in small boxes (250 ml), filled with sieved native soil from Darmstadt. Once to twice a week the humidity of the substrate was checked and the larvae were fed with fresh slices of carrot. Old carrot slices were removed. Only actively feeding insects were used for the experiments.

5.3.2 Plants

120 young oak trees (*Quercus petraea* and *Q. robur*) from a forest near Göttingen (Germany) were kept from 2004 – 2007 in a greenhouse under

controlled conditions (photoperiod 16 hours, 10 kLux, 19-25 °C, 40-50% relative humidity, individual buckets). During the winters the oaks were kept outside, with buckets buried in the soil.

5.3.3 Sampling of Volatiles

One month before starting the measurements, larvae of *M. hippocastani* were placed each in one pot in the root zone of a fraction of the *Quercus* sp. trees, to enable feeding on the roots. Prior to the measurements, roots were rinsed with tap water and the soil was carefully removed from the roots. According to the morphological state of the roots, they were defined as undamaged roots (UR) and as roots with feeding damage (FR). A subset of the undamaged, washed roots was mechanically damaged by cutting the roots into pieces using a pair of scissors (mechanical damage, MR). Samples of root volatiles were obtained using dynamic headspace sampling of humid roots enclosed into bags of PET-foil (Toppits, Cofresco Frischhalteprodukte, Minden, Germany). The air was circulated by miniature pumps (Fürgut, Tannheim, Germany) through adsorbent traps loaded with 1.5 mg charcoal (Daumazan sur Arize, France) connected by flexible tubes of teflon (5 mm ID) to the PET bags. The sampling time for the roots was 3 hours with a flow rate of 1 l/min. Volatiles were eluted from the charcoal with 75 µl of a 2+1 mixture of methylene chloride and methanol (both solvents Suprasolv-quality, Merck/VWR, Darmstadt, Germany). After elution, the samples were stored at -76 °C in an ultra low temperature freezer until analysis.

5.3.4 Gas Chromatography-Mass Spectrometry and Analytical Conditions

The root volatiles were analysed using a gas chromatograph coupled to a quadrupole mass spectrometer (6890N and 5973, Agilent, Paolo Alto, USA, technical details: see Weißbecker et al. 2004). For compound identification an apolar column (HP-5MS, length 30 m, ID 0.25 mm, and film thickness 0.25 µm,

Agilent) and a polar column (INNOWAX, same conditions as the apolar column)were used.

1 µl of the eluate was injected into the GC-injector in the pulsed splitless mode (pulse pressure 150 kPa until 1.5 min) at a temperature of 250 °C. With the apolar column, the GC was operating in the following temperature program: start: 40 °C, hold for 2.5 min, ramp 6.2 °C/min to 250 °C, hold for 10 min. With the polar column the following parameters were used: start: 50 °C, hold for 2.5 min, ramp 7.5 °C/min to 250 °C, hold for 5 min. Helium (purity 99.999%) was used as carrier gas after passing through a combined adsorbent trap for removal of traces of water, oxygen and hydrocarbons ("Big Universal Trap", Agilent). The carrier gas flow was set to 1 ml/min resulting in a gas vector of 24 cm/s. The GC-MS interface was operated at a temperature of 280°C.

The mass spectrometer used electron ionisation (EI) at 70 eV and was used in the scan mode with a mass range from 35-300 atomic mass units at a scan speed of 2.78 scans per second.

The preliminary peak identification of the odour compounds was carried out by using the Mass Spectral Search library of the National Institute of Standards and Technology (NIST, Gaithersburg, USA) and the database of MassFinder 3.0 software together with the library "Terpenoids and Related Constituents of Essential Oils" (Hochmuth, König, Joulain, Hamburg, Germany). The identification of the compounds was confirmed by comparing mass spectra and retention times to those of authentic standards.

5.3.5 Electroantennogram (EAG) Dilution Series

Dilution series from 10^{-7} to 10^{-2} of methoxybenzene, 1,8-cineol, 3-octanone, (1R) -camphor and the furanoid form of trans-linalooloxide were prepared in silicon oil (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Responses from at least three antennae from different individuals were recorded per each tested compound. The cutted antenna was placed in an antenna holder (Prof. Koch, Kaiserslautern, Germany) out of acrylic glass (Färbert et al. 1997). The ends of the antennae were immersed in a electrolyte solution (Kaissling & Thorson 1980). Volatile solutions (~30 µl) were applied on pieces of aluminium foil (9 cm^2), folded and put

in a 10 ml glass syringe (Poulten & Graf GmbH, Wertheim, Germany). The dilution series were measured by manual injection of these odour standards onto the antenna of 2nd instar larvae of *M. hippocastani* contacted to the EAG-setup: inside the air volume of the syringe, the odourant accumulates at a concentration proportional to the concentration of the compound in the solution and its vapour pressure according to Henry's law. By puffing five ml of air over the antenna a reproducible stimulus could be provided (Schütz et al. 1999). Humidified air out of the GC-system (23°C, 80% RH) was used as a negative control. Antennal responses were electronically amplified by a factor of 100. They were subtracted to the silicon oil response, which was used as control. Additionally antennal responses to anisol at the dilution 10^{-3} were measured as positive control to check the fitness of the antenna.

5.3.6 Behavioural Tests

A dual choice bioassay was used for the behavioural part of the experiment. Each experimental set up consisted of a Petri dish (14 cm ID, 2 cm deep) with the corresponding lid, two smaller Petri dishes without lid (5 to 6 cm ID), and a small cage made of steel gauze (2.5 cm x 2.5 cm x 1.5 cm). The lid of the Petri dish had two holes at opposite locations of the lid. The larva was placed in the centre of the 14 cm Petri dish inside the steel cage and surrounded by sieved, native soil. After at least 15 hours of acclimatisation, the cage was removed and the Petri dish was turned up side down. The smaller Petri dishes were placed below the holes, with the diluted test compound (10^{-2}) in one dish and pure silicon oil as the control in the other dish (~30 µl each). The Petri dishes were distributed and oriented randomly to avoid position effects. The experiments were performed in a dark room at 20°C.

In one experimental cycle the choice of 15 larvae was recorded. They could choose between the test compound and the control, or stay in the central part (a central segment of 3 x 3 cm), or move in the central band (a central bar of 3 x 14 cm, orthogonal to the connection line of the holes). The position of the larvae was recorded each 10-15 minutes, because it was not clear how quickly beetle larvae move through the soil. First choice position in relation to the stimulus compound,

the control or the neutral area is recorded. The entire experiment was terminated after one hour.

An activity index A_cI was defined as the number of active animals (sum of attracted and repelled larvae) divided by the total numbers of larvae. The attraction index A_tI is the number of larvae in the area of the test compound divided by the number of active larvae. The significance of the results was statistically evaluated by chi^2 tests.

5.3.7 Morphological Examination of the Larval Antenna of *M. hippocastani*

5.3.7.1 Scanning Electron Microscopy (SEM)

Ten *M. hippocastani* larvae were used for the observations. Insects were anaesthetized using CO_2 and kept at -18 °C until death. Then, individuals were dissected removing the antennae from the head capsule. Specimens were dehydrated in a series of graded ethanol, from 50% to 99% (10 minutes each) After dehydration, the specimens were treated with HMDS (Hexamethyldisilazane, Sigma®) and gold-sputtered using a “Balzers Union® SCD 040” unit. On each aluminium stub 5 specimens were mounted, taking care to place them with different orientations in order to obtain a clear view of the ventral, dorsal and both lateral sides. The observations have been carried out using a scanning electron microscope Philips® XL 30.

5.3.7.2 Transmission Electron Microscopy (TEM)

Ten *M. hippocastani* larvae were anaesthetized with CO_2 and immediately immersed in a solution of glutaraldehyde and paraformaldehyde 2.5% in cacodylate buffer + 5% sucrose, pH 7.2 - 7.3. In order to achieve optimal fixation, the last antennomere was detached from the rest of the antenna to help fixative penetration, and left at 4°C for 2 hours. After rinsing overnight in cacodylate

buffer, the specimens were post fixed in 1% osmium tetroxide for 1 h and rinsed in the same buffer. Dehydration in a graded ethanol series was followed by embedding in Epon-Araldite with propylene oxide as bridging solvent. Semi-thin and thin sections were taken with a diamond knife on a LKB® "Nova" ultramicrotome, and mounted on formvar coated 50 mesh grids. Finally, the sections were investigated with a Philips® EM 208, after staining with uranyl acetate (20 min, room temperature) and lead citrate (5 min, room temperature). Digital pictures (1376x1032 pixels, 8 bit, uncompressed grey scale Tiff files) were obtained using a high resolution digital camera MegaViewIII (SIS®) connected to the TEM.

5.3.8 Immunocytochemistry and Antennal Backfills

To selectively label neuropil structures in 3rd larval instars of *M. hippocastani* including olfactory glomeruli we used a monoclonal antiserum from mouse against the ubiquitous synaptic vesicle protein synapsin I (SYNORF1, kindly provided by Dr. E. Buchner, University of Würzburg, Germany; Klagges et al. 1996). For whole-mount staining we adapted the staining protocol described by el Jundi et al. (2009). Whole brains were dissected under cold phosphate buffered saline (PBS 0.01 M, pH 7.4) and fixed subsequently at 4°C overnight in a solution composed of one part formaldehyde (37%, Roth, Karlsruhe, Germany), one part methanol, and eight parts PBS 0.01 M. These brains were then rinsed in 0.01 M PBS for 1 hour at room temperature followed by preincubation overnight at 4°C in 5% normal goat serum (NGS; Jackson ImmunoResearch, Westgrove, PA) in 0.01 M PBS containing 0.3% Triton X-100 (PBST). The synapsin I antibody was diluted 1:100 in PBST containing 1% NGS: in this solution the brains were incubated for 5 to 6 days at 4°C. Subsequently the brains were rinsed six times in 2 hours with PBST before they were incubated with the secondary goat anti mouse antibody conjugated to Cy2 (1:300, Jackson ImmunoResearch) in PBST and 1% NGS for 4 days at 4°C. After this time the brains were rinsed again with PBST six times in 2 hours. Thereafter the brains were dehydrated in an ascending alcohol series (50% to 100%, 10 minutes each) and then cleared in

methyl salicylate (Merck, Gernsheim, Germany) for about 40 minutes. Finally, the brains were mounted in Permount (Fisher Scientific, Pittsburgh, PA) between two coverslips using three spacers (Zweckform, Oberlaindern, Germany) to prevent compression of brains.

Antennal backfills were performed according to the method described in Schachtner et al. (2004). Crystals of biotinylated dextran (lysine-fixable, molecular mass 3000 Da; Molecular Probes, Eugene, OR, USA) were placed on the cut ends of one antenna of immobilized L3 larva. The antennal stump was sealed with vaseline. The animal was kept in a humid chamber overnight at 4°C to allow the dextran to diffuse through the antennal nerve into its target area in the brain. The next day animals were dissected, and the brains were processed for immunocytochemistry as described above. Dextran was visualized using Cy3-coupled streptavidin (1:300, Jackson Immuno Research), which was applied for 1·h at room temperature.

Fluorescence was analyzed using a confocal laserscan microscope (Leica TCS sp2). The wholemount preparations at 512 x 512 pixel resolution by using a 20x oil immersion objective (HC PL APO 20x/0.70 lmm Corr CS; Leica, Bensheim, Germany). All brains were scanned with a voxel size of 0.73 x 0.73 x 1 µm, a speed of 200 Hz, a pinhole of 1 Airy unit and a line average of 2 to 4.

5.4 Results

5.4.1 Volatile Compounds of *Quercus* sp.-Roots

A total of 12 volatile chemical compounds were characterised with GC-MS of undamaged roots of *Quercus* sp., mechanically damaged roots and roots damaged by larval feeding of *M. hippocastani.* For identification an apolar and a polar column were used. Table 5.1 shows the identified compounds in the different treatments. Seven different volatile organic compounds could be detected in undamaged roots of *Quercus* sp. (UR) and 8 compounds in mechanically damaged roots (MR) as well as in roots damaged by larval feeding (FR).

Table 5.1: LRI (Linear Retention Indices) of the present Volatile Organic Compounds of three different root-treatments of *Quercus* sp. identified with GC-MS. All compounds were verified by comparing the mass spectra and retention indices of authentic standards using the apolar column (* for anisol the polar column was used: INNOWAX, length 30 m, ID 0.25 mm, and film thickness 0.25 µm, Agilent)

UR Volatiles found in undamaged roots

MR Volatiles found in mechanically damaged roots of *Quercus* sp.

FR Volatiles found in damaged roots by feeding of larvae of *M. hippocastani*

COMPOUNDS	UR	MR	FR
1-octen-3-ol			985
3-octanone			990
6-methyl-5-hepten-2-one	991	989	
2-ethyl-1-hexanol		1030	1032
1,8-cineol			1034
furanoid trans-linalooloxide	1091	1089	1091
nonanal	1105	1104	1105
(1R)-camphor	1148	1147	1148
borneol	1168	1168	1169
decanal	1205	1205	
anisol			1323 *
geranyl acetone	1454	1454	

The alcohols 1-octen-3-ol and anisol, the keton 3-octanone and the monoterpen 1,8-cineol are found only in the samples damaged by larval feeding, whereas 6-methyl-5-hepten-2-on, decanal and geranyl acetone are found in the healthy and mechanically damaged roots. 2-ethyl-1-hexanol appears in mechanically damaged oak-roots and in those who are damaged by feeding of the larvae. Furanoid trans-linalooloxide, nonanal, (1R)-camphor and borneol could be found in all of the samples.

5.4.2 Electrophysiological Response of *M. hippocastani* to Some Root-VOCs of *Quercus* sp. Damaged by Larval Feeding

The results from measurements with a gas chromatograph coupled to an electro-antennograph (EAD) were the basis for selecting the compounds for the electrophysiological experiments (see chapter 6.2.1).

In the electrophysiological investigations the five prominent “key-compounds” anisol, 1,8-cineol, 3-octanone, (1R)-camphor and furanoid trans-linalooloxide were tested. They are released particularly by oak-roots damaged by larval feeding of *M. hippocastani* larvae. Stimuli were generated as a puff of air in equilibrium with a dilution of the stimulus compound in silicon oil. The highest response to a puff in the dilution 10^{-2} was observed for the alcohol anisol and the monoterpene 1,8-cineol, it was for anisol 58 mV (±16 mV) on average, for 1,8-cineol about 34 mV (±13 mV). The mean response for the two ketones 3-octanone and camphor was 23 mV (±7 mV) and 17 mV (±4 mV) respectively and for the furanoid form of trans-linalooloxide it was 16 mV (±2 mV). For camphor we tested the two enantiomers and we could not observe any different reaction from the antennae. The detection limit was given in the dilution 10^{-6}.

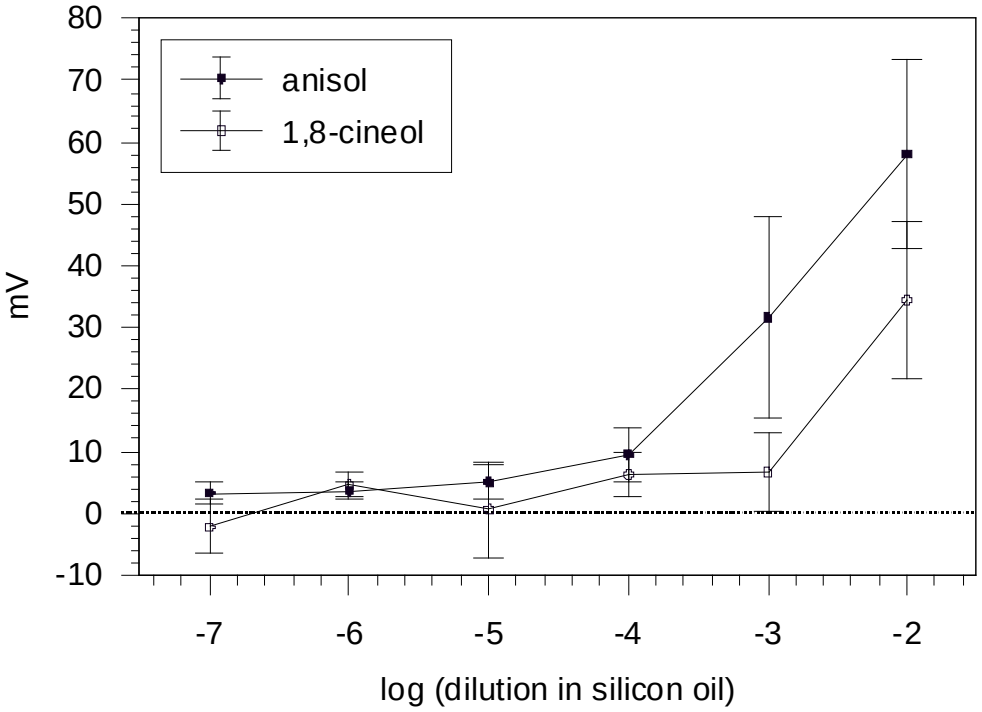

Figure 5.1 a: Dose-response curves of 2nd instar larvae of *M. hippocastani* to anisol (N = 8) and 1,8-cineol (N = 4) released by oak-roots damaged by feeding of the larvae. SE of the mean is indicated by error bars.

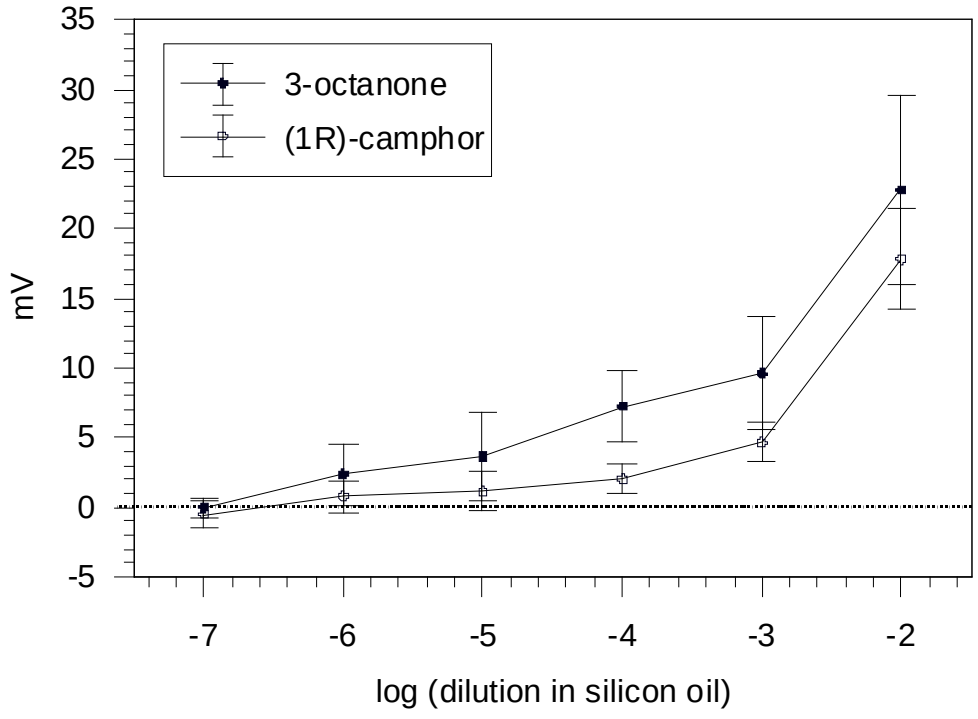

Figure 5.1 b: Dose-response curves of 2nd instar larvae of *M. hippocastani* to 3-octanone (N = 6) and (1R)-camphor (N = 8) released by oak-roots damaged by feeding of the larvae. SE of the mean is indicated by error bars.

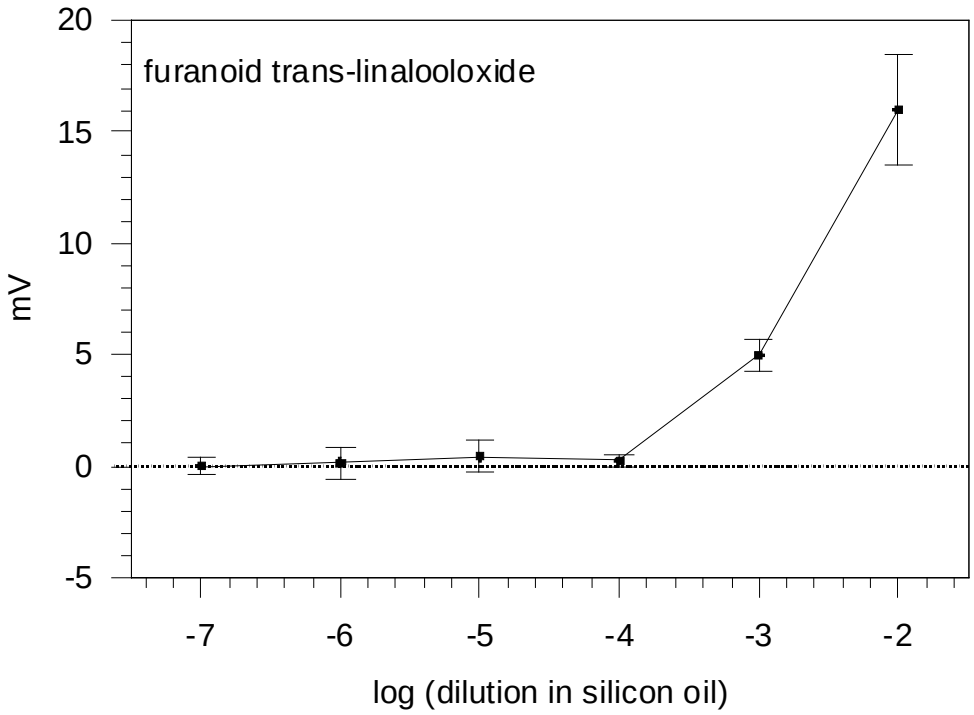

Figure 5.1 c: Dose-response curves of 2nd instar larvae of *M. hippocastani* to furanoid trans-linalooloxide (N = 3) released by oak-roots damaged by feeding of the larvae. SE of the mean is indicated by error bars.

5.4.3 Functional Anatomy of the Antennal Olfactory Sensilla of *M. hippocastani* Larva

The antennae of *M. hippocastani* larvae consist of 4 antennomeres. The apical antennomere is shorter than the sub-apical one, and has a typical triangular shape (when observed from one of the external sides) (figure 5.2 A). Apically, the antennomere presents a specialised, truncated area housing 10 pegs of various shapes (figure 5.2 C). Preliminary observations carried out on these sensilla strongly indicate that they are not involved in olfaction. External observations of

the long (dorsal) and short (ventral) side show the presence of three smooth, slightly depressed areas (figure 5.2 B-D). The dorsal area is more rectangular in shape (figure 5.2 E), while the two ventral areas are sub-elliptical (figure 5.2 D). The average total surface area occupied by the three sections is about 900 μm^2. SEM high magnification pictures show the presence of numerous scattered minute pores evenly distributed on the whole surface (figure 5.2 F). Light and TEM serial cross section revealed that these three areas are large multiporous olfactory sensilla resembling the pore-plate sensilla (figure 5.2 A-B). The porous cuticle is considerably thinner that the cuticle of the antennal wall, and is crossed by pore canals connecting the external pores with the lumen of the sensillum. Below the porous cuticle, an impressive number of dendritic projections completely fill the sensillar lumen (figure 5.2 C-D). At the level of the pore canals openings, pore tubules can be found (figure 5.2 F). The multiporous olfactory sensilla are innervated by an undefined number of sensory neurons, typically grouped in bundles of 4 (figure 5.2 E).

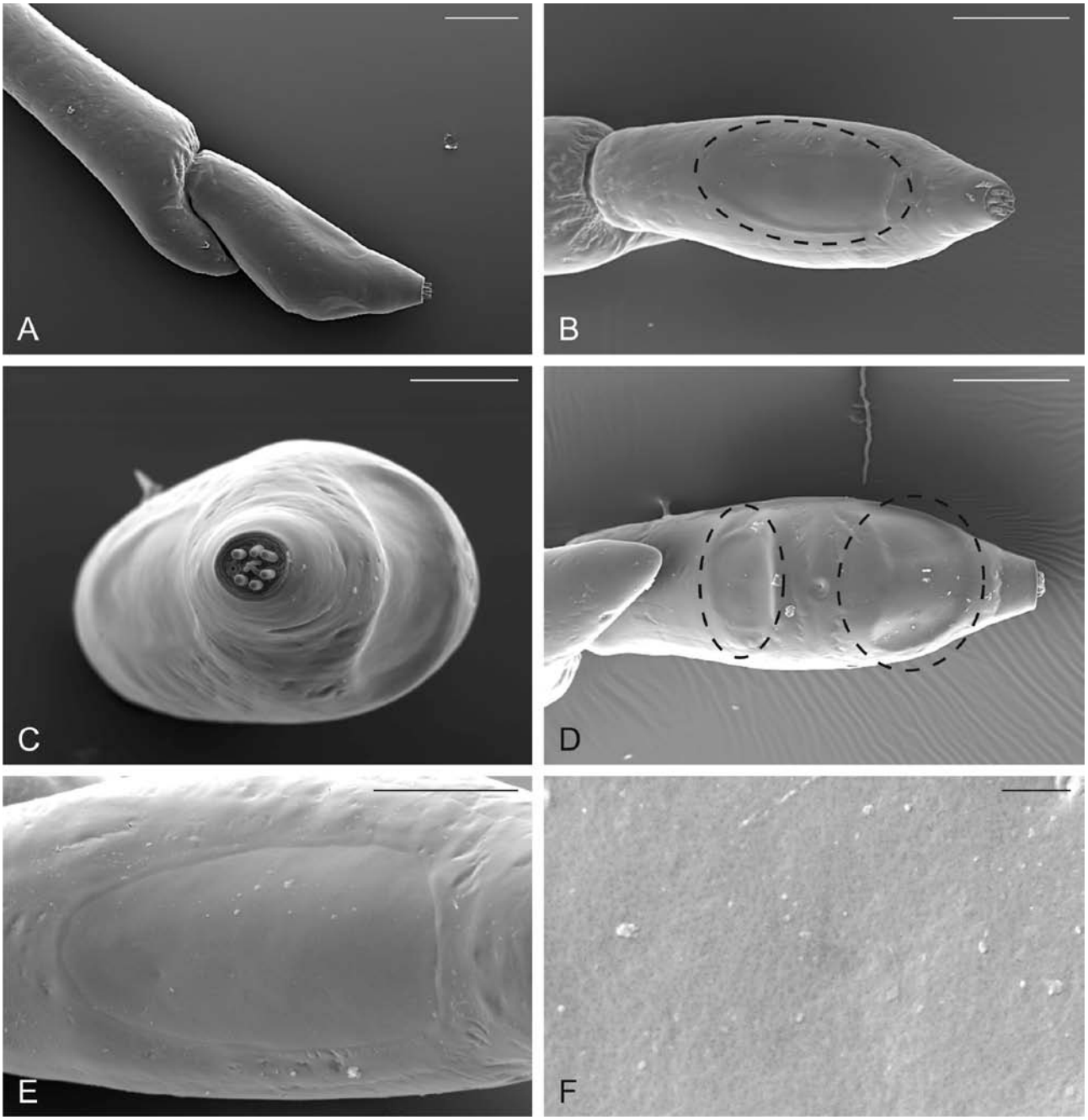

Figure 5.2: SEM pictures of *M. hippocastani* apical antennomere. A) Lateral view of the apical and sub-apical antennomere. B-D) Dorsal, apical and ventral view of the apical antennomere, respectively. In B and D the multiporous olfactory sensilla (MOS) can easily be observed. In C the apical part of the antennomere is shown, with the dorsal (left) and ventral (right) MOS. E) Detail of the dorsal MOS. F) Close up view of the MOS surface, pierced by numerous tiny cuticular pores. Bar scale: A, B, D: 200 µm; C, E: 100 µm; F: 2 µm. Roberto Romani.

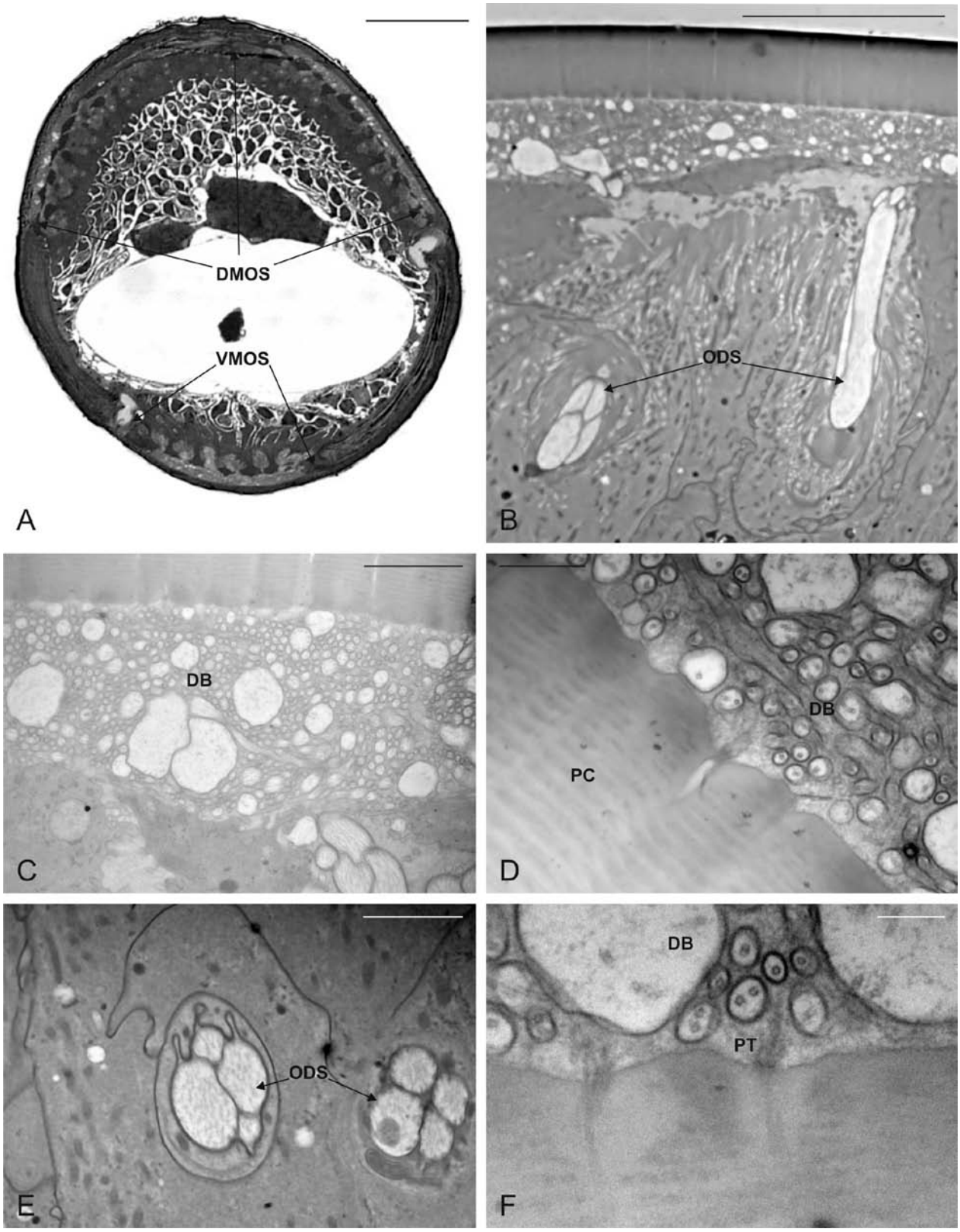

Figure 5.3: *M. hippocastani* apical antennomere. A) Light microscopy coss section showing the dorsal (DMOS) and ventral MOS (VMOS). B) TEM cross section at the level of the dorsal MOS, showing two bundles of outer dendritc segments (ODS). C, D, F) Details of the dendritic branches (DB) filling the space below the porous cuticle (PC), pore tubules (PT) can also be observed. E).

Two bundles of four dendrites taken at the level of the ODS. Bar scale: A 50 µm; B 10 µm; C E: 2 µm; D 500 nm; F 200 nm. Roberto Romani.

5.4.4 Neuroarchitecture of the L3 Antennal Lobes

Immunostaining against the ubiquitous synaptic vesicle protein synapsin and antennal backfills revealed a typical insect like glomerular organization of the antennal lobe of third instar *M. hippocastani* with about 70 olfactory glomeruli (Fig. 4.4). The backfills showed projections only in the ipsilateral AL and did not show any projections to the contralateral AL as it has been described for the majority of OSNs in *Drosophila* (reviewed in Stocker 2001). The antennal backfills additionally revealed two cell bodies lateral to the AL, very likely belonging to motorneurons innervating antennal muscles, and projections to the lateral protocerebrum and the subesophageal ganglion (SEG) (Fig. 4.4). Antenna are multimodal sensory appendages and house different sensilla with receptor neurons detecting different sensory modalities including mainly olfactory but also contact chemosensory, mechanosensory, temperature and humidity information (e.g. Altner et al. 1977; Staudacher et al. 2005). While OSNs typically project into the olfactory glomeruli of the AL, the mechanosensory axons typically project into a deutocerebral area posterior to the glomerular area called the antennal mechanosensory and motor center (AMMC) or dorsal lobe (reviewed in Staudacher et al. 2005). The axons of the contact chemoreceptors project into the AMMC but also to the SEG and even further to the thoracic ganglia (Kent & Hildebrand 1987; Nishino et al. 2005; Jørgensen et al. 2006). While the projections towards the SEG may thus belong to contact chemoreceptors, the source of the projections to the lateral protocerebrum remains unclear but are not very likely OSNs. OSNs in insects seem to exclusively project to the AL (for review see Schachtner et al. 2005).

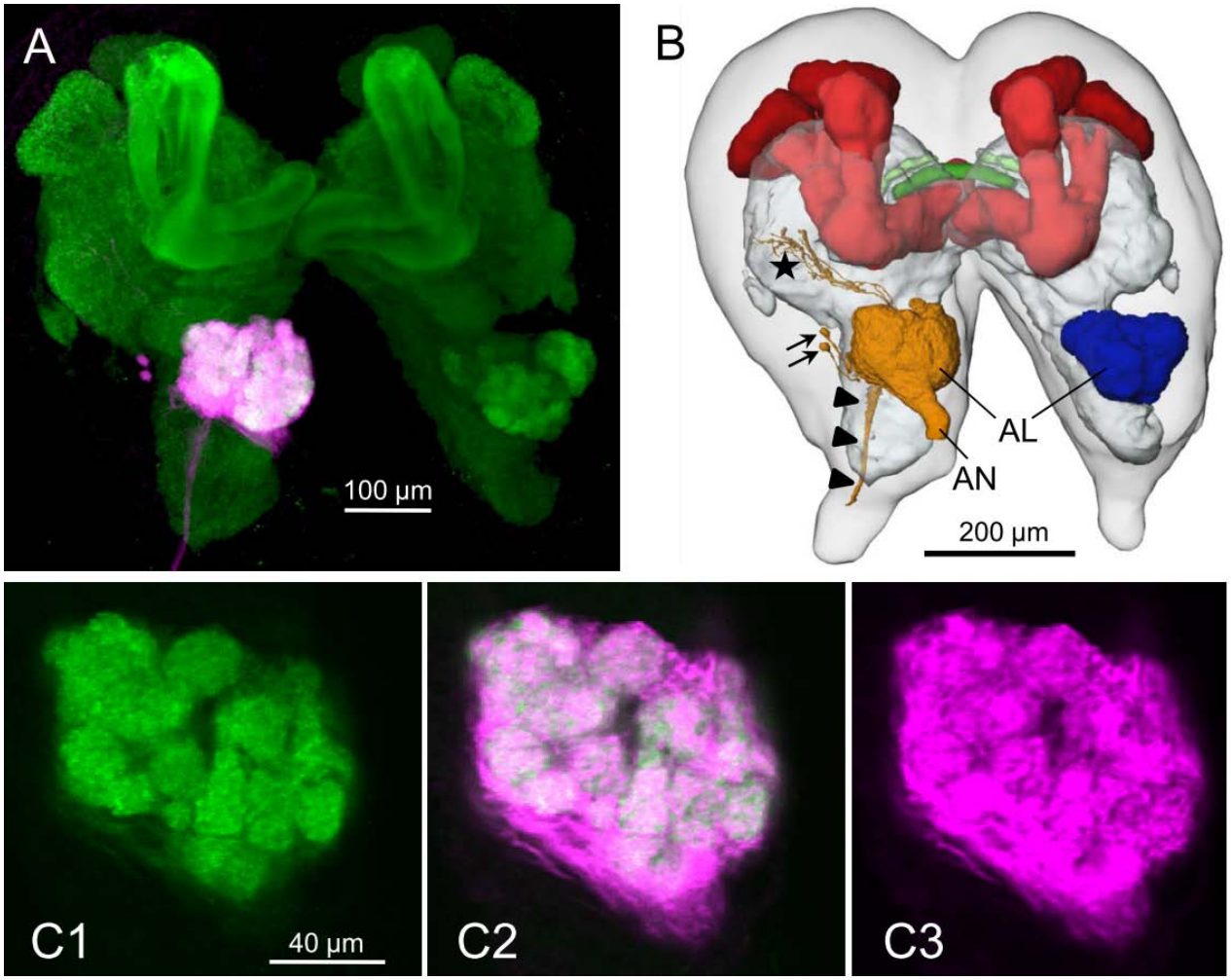

Figure 5.4: *M. hippocastani* brain including the antennal lobes (AL) of a 3rd instar larva, frontal views. A) Maximum projection of 229 serial confocal images: Green codes for anti synapsin immunostaining, magenta for a dye (dextran) backfill from the antenna. B) 3D-reconstruction of A showing the brain outline (light gray) and selected brain areas: yellow, reconstructed from the antenna backfill; the other brain areas, including the contralateral AL (blue), the mushroom bodies (red), the central complex (darker green), the protocerebral bridge (lighter green), and remaining neuropil (gray) are reconstructed from the anti synapsin immunostaining which can be used to label neuropil areas in insects (see *e.g.* Utz et al. 2008). Arrowheads, projection to the subesophageal ganglion; arrows, cell bodies of two antennal motoneurons; star, projections to the lateral protocerebrum; AN, antennal nerve. C) Single confocal images of the image stack of the left antennal lobe in A, clearly showing many spheroidal structures, the so called olfactory glomeruli in the larval *M.* AL - labeled by the synapsin (C1) and the backfill staining (C3). C2: Overlay of C1 and C3. Joachim Schachtner.

5.4.5 Behavioural Tests

The attraction of *M. hippocastani* larvae to dilutions of pure reference compounds released by roots of *Quercus* sp. was tested in dual choice tests. The reference compounds were diluted in silicon oil to the concentration 10^{-2} and were tested against the pure silicon oil. The experiments were carried out in autumn-winter of 2007/2008 and in autumn/winter of 2008/2009 with 2nd and 3rd instar larvae. The experiments with acetone were done in summer of 2008. In all experiments, we did not observe any differences in the behaviour of the two larval instars. Therefore the data were pooled.

During the preparation time the larvae were enclosed by the steel cage and placed in the middle of the big Petri dish, which was surrounded by soil from Griesheim (Germany, the larvaes place of origin). At least 15 hours later the steel cage was removed and the Petri dish turned up side down with the lids positioned over the smaller Petri dishes. One Petri dish contained the diluted compound in the respective concentration ("compound", see figure 5.5 below), the other one the silicon oil as the control ("control"). The central bar (14 x 1.5 cm) was defined as neutral area ("neutral"), including also the inactive area ("inactive", 2.5 x 1.5 cm) in the centre of the petri dish.

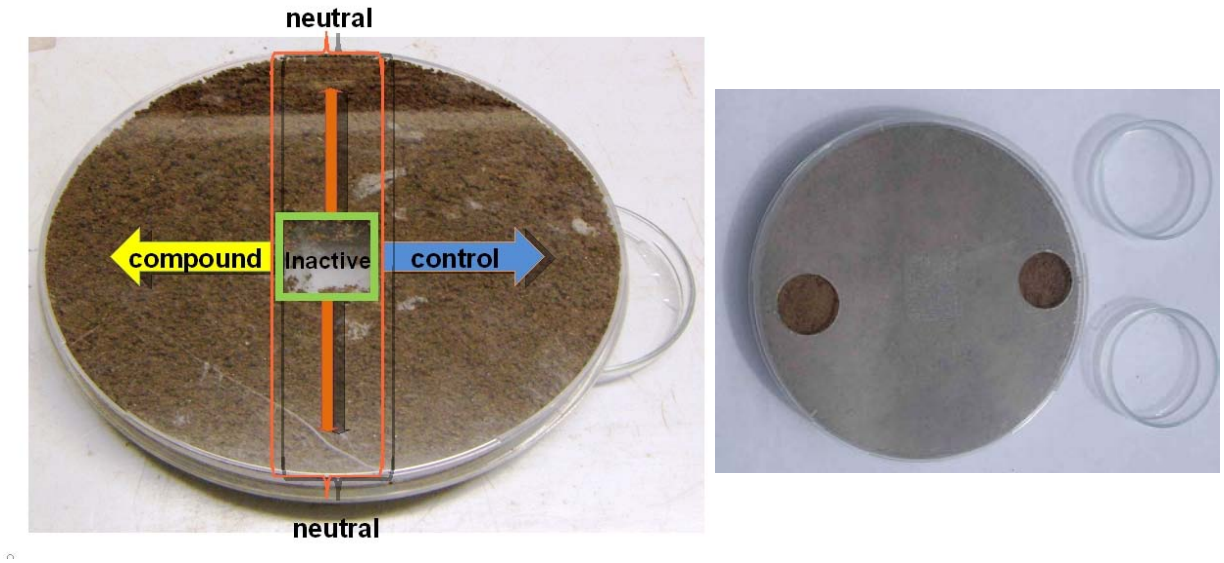

Figure 5.5: Design of one experimental unit of the dual choice bioassay (picture left, according to Henrik Ziegenhagen, 2009), consisting of one Petri dish (ID 14 cm) with two holes (diameter 24

mm each) in the lid, two Petri dishes (ID 5 to 6 cm) and a cage made of steel wire (2.5 cm x 1.5 cm) (foto right).

Figure 5.6 shows the results of the behavioural dual choice arena tests.

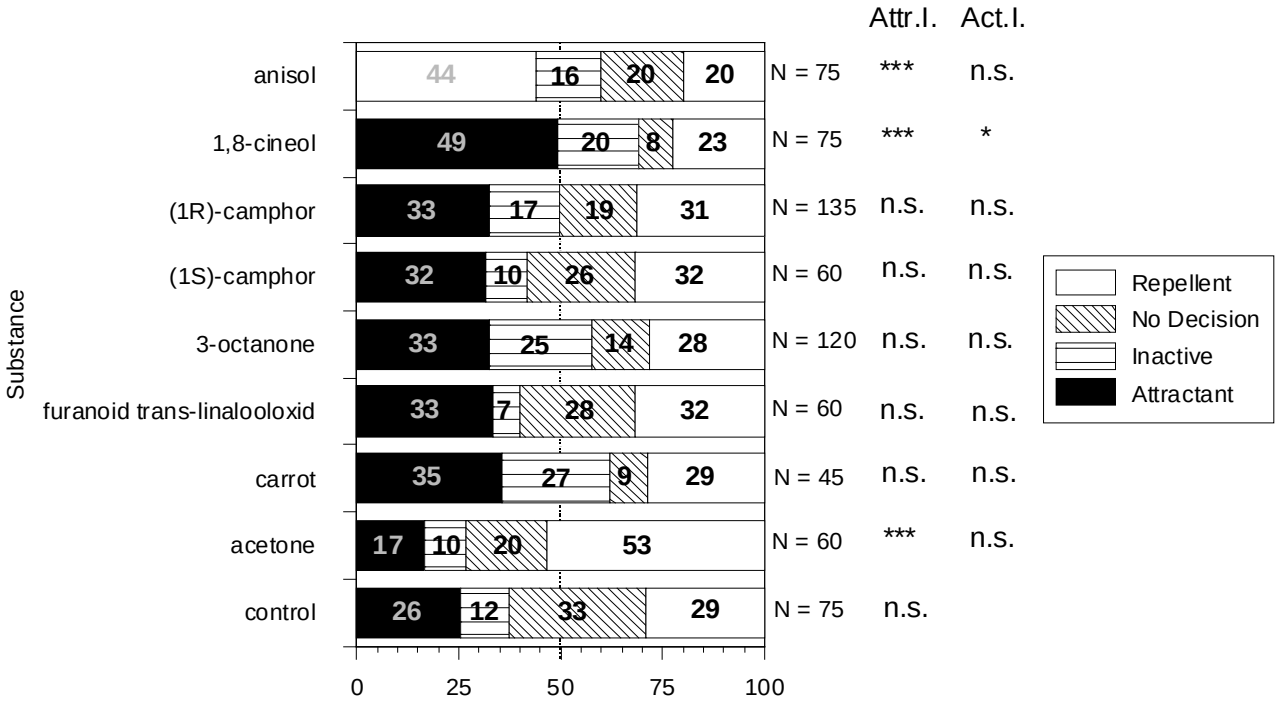

Figure 5.6: Behavioural data in percent of all 2nd and 3rd instar larvae of *M. hippocastani* in dual choice tests in soil. Numbers in the bars show the percentage, numbers next to the bars indicate the total number of individuals in the different experiments. Statistical analysis for the Attractivity Index (Attr. I.) was done excluding the inactive animals and those who showed no decision. The Activity Index (Act. I.) was calculated with all animals, confronting the attracted and repelled larvae against the inactive and those who showed no decision to the larvae of the control. Statistical significance is indicated by *** ($p < 0,001$), * ($p = 0,05 – 0,01$) and n.s. ($p > 0,05$, not significant, chi^2 test, $\alpha = 0,05$).

In the control run no preference for one of the two directions could be observed ($p > 0,05$, n.s., chi^2 test, $\alpha = 0,05$). Also with carrot slices, furanoid trans-linalooloxide, 3-octanone, (1S)-camphor and (1R)-camphor (in each case $p > 0,05$, chi^2 test) any clear attractant or repellent effect could be observed. In the

experiments with acetone this compound has a strong repellent effect ($p < 0,001$, chi^2 test), whereas anisol and cineol act as strong attractant compounds (each with $p < 0,001$, chi^2 test).
The Activity Index of 1,8-cineol was statistically different ($p = 0,05 – 0,01$) from those of the control.

5.5 Discussion

Volatile emissions of the aboveground parts of *Quercus* sp. are investigated by several groups (e.g. Vrkočová et al. 2000, Niinemets et al. 2002 and references therein). The adults of *M. hippocastani* were attracted by green leaf volatiles (GLV) and 1,4-benzoquinone as the species-specific sex-pheromone (Ruther et al. 2000, see also chapter 1.6). The authors performed also experiments with volatile compounds of different plants testing the attractiveness on the adults of *M. hippocastani.* The tested host plants were *Carpinus betulus* L. and *Quercus rubra* L., the non-host plant was *Prunus serotina* Ehrh.
Experiments performed by Reinecke et al (2008) showed that orientation behaviour of *M. melolontha* larvae was guided by CO_2 gradients (also shown by Hasler 1986), but it changed, if plant roots or root exudates were present. Root volatiles from *Taraxacum officinale* (attractive host plant) and *Trifolium pratense* (accepted host plant) did not attract *Melolontha* larvae if additionally CO_2 enriched air is provided. Thus, the authors supposed an interfering or “masking” effect of plant roots or root exudates of the attractive impact of CO_2. As a consequence, the authors mentioned that *M. melolontha* larvae need more chemical stimuli than CO_2 alone to localize their hosts.
In this study, the trees were manipulated as little as possible in order to maintain the natural character. Only the roots were treated carefully in the three different ways before sampling the volatiles. However, just removing the soil particles and washing the roots with tap water may influence the volatile pattern. Moreover the physiological status of the tree and the organisms living on the tree (on the roots and in the soil surrounding them as well as on the parts aboveground) may have

an additional impact on the volatile composition (*e.g.* Soler et al. 2005, Rasmann & Turlings 2007).

GC-EAD/MS-experiments are performed in chemical ecology to identify volatiles with a biological acivity for the insect. The biorhythm of the larvae of *M. hippocastani* may be influenced by several circadian and seasonal factors (see chapter 6.1.3), to obtain repeatable results was not possible. However, in certain moments a part of the tested antannae revealed compound specific response of the cockchafer larva antennae to some root volatiles. The compounds, selected on the basis of those GC-EAD/MS experiments yielded dose-dependent responses with detection limits down to 10^{-6} dilutions of stimulus compounds in silicon oil.

The behavioural experiments with the selected volatiles showed that root volatiles such as anisol and 1,8-cineol elicited a significant attractive response of *M. hippocastani* larvae, whereas acetone had a significant repellent effect. However, about 2 g of carrots (*Daucus carota ssp. sativus*) cut into pieces had no effect on the behaviour of the larvae, although CO_2 was released from the carrot pieces as well as a strong smelling mixture of carrot compounds (Weissteiner & Schütz 2006). In behavioural dual choice tests with the two highest abundant compounds terpinolene and β-caryophyllene, terpinolene had an attractive effect on the behaviour of *M. hippocastani* larvae, whereas β-caryophyllene had a repellent one (see chapter 6.2.2.1). Rasmann et al. (2005) mentioned an attractive effect of β-caryophyllene for the entomopathogenic nematodes *Heterorhabditis megidis* P. The compound was released by maize roots after feeding of *Diabrotica virgifera virgifera* L. larvae.

The typical adult olfactory pathway in insects consists of olfactory sensillae mainly on the antennae which house olfactory sensory neurons (OSNs). OSN axons project via the antennal nerve to the antennal lobes (AL), the first central processing unit for olfactory information processing in the insect brain. From the AL, odour information is then conveyed to higher integration centers including the mushroom bodies and the lateral protocerebrum (reviewed in Schachtner et al. 2005).

The neuroarchitecture of the olfactory pathway in 3rd instar larvae of *M. hippocastani* clearly resembles the anatomy of a typical adult insect olfactory

system. This compares to findings in the last larval instar of another beetle, *Tribolium castaneum*. The antennae in the 3rd instar larvae of *M. hippocastani* bear two large pore plate sensillae which house a large number of OSNs. The sensory neurons are grouped into bundles of 4 sensory neurons, each one ensheathed by its own dendrite sheath. This organization of the olfactory sensilla was reported also in other groups (Homoptera, Lewis & Marshall 1970), for which has been hypothesized an origin as merged, originary isolated sensilla basiconica (Bourgoin & Deiss 1994). The high number of sensory neurons, associated with the large antennal surface occupied by the pore plates suggest a key role played by the olfaction in these belowground larvae. The axons of the OSNs innervate via the antennal larval AL. Anti-synapsin immunostaining and antennal nerve backfills revealed in the 3rd instar of *M. hippocastani* ALs containing about 70 glomeruli. The glomeruli are regarded as the functional subunits of odour discrimination (Hildebrand & Shepherd 1997). The high number of glomeruli clearly indicates a highly developed odour discrimination ability of the cockchafer larvae.

Acknowledgements

Sonja Weissteiner is grateful for the financial support to the Cusanuswerk government scholarship. Moreover, the authors thank the forestry office Griesheim (Germany) for supplying the larvae of *M. hippocastani* and the forestry office Ebergötzen (Germany) for supplying the trees.

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CHAPTER 6

General Discussion

6.1 Discussion of the methods

6.1.1 Sampling, Analyzing and Storage Conditions of Volatile Organic Compounds (VOCs)

Volatile sampling with the combined system (TENAX® plus charcoal, see chapter 1.8.1.1) allows additional chemical detection of volatile compounds with high vapour pressure, like aldehydes or several acidic substances. These compounds can be detected and identified via the GC-MS when trapped with TENAX®. They cannot be detected, however, when trapped exclusively in a CLSA-charcoal trap. In the latter, the volatiles have to be eluted with a solvent. CLSA-charcoal traps detect principally volatile compounds with a lower vapour pressure. The peaks of the solvent may mask the peaks of volatiles detected in the first minutes, which are exactly those with a high vapour pressure. The TDS-measurement equipment has a higher storage capacity because of a higher mass of adsorbent material. This causes a higher resistance to air flow; therefore, the pumps were operated with lower voltage. Before each measurement run the pumps were checked with a mechanical gas flow meter on their respective capacities to guarantee equal conditions. The individual air flow of several pumps varied quite strongly from the typical average of 1l/min at the same voltage. The process for developing the combined system as it was used at the end took almost three years of sampling volatiles with different traps at different voltage conditions, differing as well in the time length.

VOCs should be collect and trapped in a reproducible time interval after preparing the sampling equipment. In the same way, the elution should shortly follow the sampling, to minimize volatilization of volatile components and changes in the volatile bouquet, and avoid compound degradation or oxidation of the samples.

The storage of the CLSA sample eluates at -73°C to -76°C will change the volatile pattern within weeks by solvent evaporation, oxidation, and reduction processes. These processes may be slow, but stored over months or even years the volatiles in the samples are found to change significantly. In the present

study, the ageing and the consequent change over time of the volatile composition in the samples led to problems in the quantification process. The amount of the single compounds varies because of the evaporating solvent and also because of unknown interactions of the compounds with each other as well as with the solvent.
The measurement equipment is not a static system. The column of the GC ages and the conditions will not be constant over time. So measuring the samples and verifying the single components by commercially available standards should be done at the same time because of ageing processes of the samples as well as of the column.

6.1.2 Sampling VOCs of Shoots and Roots

Accidental wounding processes, below- or aboveground evoke complex defence mechanisms in the plant, which affects the volatile emissions (see also chapter 1.4). “It is well known that within hours (and probably sooner) of excision leaves undergo biochemical degradation and changes in water relationships” (Bowers et al. 1991, Wolfson 1988 in Schoonhoven 2005).
In this study no plant parts were detached. Mainly oak trees *Quercus* sp. and *Aesculus hippocastanum* from the greenhouse were used. Against pest organisms (red spider mites, aphids, and thrips) and fungal growth (mildew) different insecticide and fungicide applications were necessary during their stay in the greenhouse from March to October. Only during the winter months the trees were kept outside by burying the buckets in the soil. Plants from the greenhouse generally differ from plants grown in the field (Hammond et al. 1979). Pesticides probably additionally influence the plant chemistry, not only directly after the application, but also later on by influencing the microbial community. Even if plants, growing in open fields, are enclosed in a cage, their nutritional value and therefore their attractivity for insects may change (Stamp & Bowers 1994). The experiments in the present study were carried out with plants potted in containers and transported for measurements in a laboratory room. These experimental

conditions could put stress on the plants and the volatile emissions may differ from those grown in the nature.

Loreto et al. (1998) mentioned that oak species differ in their volatile emissions regarding isoprene and monoterpenes. The authors investigated the aboveground emissions of several oak species. In this study volatile emissions of above- and belowground parts of several plants were sampled and analysed.

The sampling process of root volatiles is much more time consuming than sampling VOCs aboveground, *e.g.* from the stem or from leaves of plants. The soil has to be removed carefully, by washing the roots with tap water, before the VOCs sampling process can start. Anyway, this activity affects the VOC emissions by slightly damaging the root bark. Therefore, it is hardly possible to obtain a typical VOC-spectrum for undamaged or healthy roots. Different studies show (*e.g.* Turlings et al. 1990) that plants are nearly odourless before damage occurs, but after arthropod feeding or artificial damage, large quantities of volatile compounds can be emitted. These induced odours have been shown to be powerful attractants for *e.g.* parasitic Hymenoptera and predatory mites (Soler et al. 2007, Rasmann & Turlings 2007). However, plants in nature are never completely undamaged. Thus, the “undamaged plant” is more an intelectual reference than a natural state.

Even if we normally deal with natural sourced VOCs, we have to keep in mind that in our systems we have to calculate with „man-made emissions“ (Holzer et al. 1977). A zero-sample is done by sampling the substances surrounding the sampling-setup in the laboratory. This sample is taken to characterise the compounds present in the laboratory air, and to compare them with those present in root and shoot volatile samples.

Molecluar filters (see chapter 1.8, figure 1.6 b right) were used to filter the laboratory air, which was sucked into the measurement space of the combined setup. Experiments performed 2006 in our institute showed that the filter pearls were effective in filtering anthropogenic, aromatic compounds (like 2-butoxyethanole, toluene, p-xylene) out of the laboratory air. The compounds namend above probably originate from outside the building. The air inlet of the lab air conditioning system is located next to the smokers area, near the entrance to the building. Volatiles like *e.g.* nonanal and decanal mainly originate from damaged plants (*e.g.* laboratory preparation activities) and indicate degradation

processes. These volatiles were highly abundant in the laboratory air, and were also absorbed by the molecular filters. However, a high number of samples are necessary to minimize the noise-induced error because of the multi-factored design on the obtained results.

The physiological status of the trees and presence or abundance of organisms like fungi, bacteria, mycorrhiza and small arthropods may influence the volatile pattern by living, sucking, and feeding on above- and belowground parts of the plants. Ubiquitously occurring arbuscular mycorrhiza (AM) fungi are symbiotically associated with about 80% of all terrestrial plant roots and thus important in nutrient cycles in the soil (Smith & Read 1996). AM fungi are involved in phosphorus uptake (Jakobsen et al. 1992) and in simultaneous reduction of the total amount of nitrogen in the leaves (Wurst et al. 2004). Additionally, secondary metabolites (Gange & West 1994) and phytosterols (Dugassa-Gobena et al. 1996) are affected by AM fungi as well. “In fact, the underground transfer of information may be facilitated by root networks and by mycorrhizal connections that may transport nutrients” (Simard et al. 1997), “potentially also elicitors of defence over considerable distances” (Dicke & Bruin 2001). Colonisation of AM fungi of tomato plant roots is able to alter the volatile emission of the shoots, attracting aphid parasitoids *Aphidius ervi* even more than aphid infested tomato plant shoots (Guerrieri et al. 2004). Changes in the mycorrhizal composition may influence the biomass and the nutrient status of the plants as well as the structure of the plant community aboveground (Stampe & Daehler 2003). As a result, aboveground living herbivores can either benefit from the presence of AM fungi (Goverde et al. 2000) or the beneficial effect can be reduced (Vicari et al. 2002). Within ecosystems, AM fungi can function along a continuum from parasitism to mutualism (Klironomos 2003). However, it is not clear yet, in which way root colonisation by AM fungi could affect the volatile emissions of *Quercus* sp. and *A. hippocastanum*.

6.1.3 Electrophysiology

For preparation of the antenna and description of the method and the instruments see chapter 1.9.

The electrophysiological measurements were done with antennae of 2nd and 3rd instar larvae of *Melolontha hippocastani*. Predominantly, larvae of the 2nd instar were used. No conspicuous differences in the antennal responses could be observed between the 2nd and 3rd larval stage. The rather robust antennae of *M. hippocastani* allowed measurements for several days, which is much longer than typical measurement periods of the more fragile antennae of aboveground living insects (which could be on the order of minutes to hours).

Circadian and particularly seasonal rhythm seem to play an important role in the performance of electrophysiological experiments as well as in monoterpene emissions of *Quercus ilex* L. under natural conditions (Kesselmeier et al. 1996, Bertin et al. 1997). Faria et al. (1996) investigated the diurnal changes in photoprotective mechanisms in leaves of *Quercus suber* in summer.

6.1.3.1 Circadian Rhythms

The electrophysiological experiments were mainly performed during the night, because a more stable response of the receptors to volatile stimuli could be observed. In addition, the background signal was normally smaller during the night.

Concerning the circadian rhythm, Krishnan et al. (1999) observed even higher EAG responses of two chemically and behaviourally distinct compound classes in *Drosophila melanogaster*, if the experiments were performed in the middle of the night. The authors mentioned that in olfactory responses mainly peripheral oscillators are necessary for regulating circadian rhythms. Additionally, cryptochromes (CRY, proteins, acting as photoreceptors), contribute to oscillator function and physiological output rhythms in the antennae (Krishnan et al. 2001).

Page & Koelling (2003) observed a 10-fold change in sensitivity measuring EAGs in the cockroach *Leucophaea maderae*, as a function of the time of day when the

measurements were done. The authors supposed that circadian rhythms are under control of a single pacemaking system in the optic lobes and that the olfactory sensitivity in the antennae is modulated by the circadian system. This should be considered by the accomplishment of the experiments.

The composition of the emitted volatiles can vary strongly throughout the photoperiod, depending also on a herbivory attack (Johne et al. 2006a). For example, volatiles emitted periodically by flowers can be similar to the induced release of volatiles by damaged plants (Matile & Altenburger 1988, Loughrin et al. 1991, Loughrin et al. 1994). This should be considered by planning and performing experiments.

6.1.3.2 Seasonal Rhythms (Circannual Rhythms)

Seasonal factors might influence the antennal response as well.

In contrast to the mechanisms controlling circadian rhythms, those controlling seasonal rhythms are poorly understood yet. However, circadian and seasonal rhythms differ in many aspects and the purposes of circadian and seasonal timing are totally different (Danks 2005).

Experiments performed in spring, summer and autumn from 2004 to 2006 showed highly incoherent EAG responses to diluted compounds in silicone oil. The dose-response curves obtained in these periods were not reproducible at all. However, EAG experiments done in autumn-winter of 2006/2007, 2007/2008 and 2008/2009 showed clear dose-response curves dependent on the concentrations of the compounds diluted in silicone oil. This led to the assumption that seasonal factors may play an important role in the antennal response of *Melolontha* larvae. The compound with the most stable antennal response was anisol (also methoxybenzene), which also resulted in reproducible dose-response measurements in the autumn/winter seasons 2006/ 2007, 2007/2008 and 2008/2009.

Seasonal changes in host plant preferences could be observed in different insect species, *e.g.* in Homoptera (Drosopoulos 1977) and larvae of Lepidoptera (Klos 1901). In aphid species complicated host alternations are common (Dixon 1985).

The reasons for changing feeding site could be versatile: e.g nutritional factors through seasonal changes in plant quality and reduced predation risk by growing. Seasonal factors may be able to change the chemistry and/or nutritional value of potential host plants that the insect switches from one plant species to another. Also the native preferences of the insects may change (Schoonhoven et al. 2005). Thus, we hypothesize that observed changes in host plant preference might be the reason for the strong seasonal differences in electrophysiological responses by larvae of *M. hippocastani*.

6.1.3.3 Electroantennography (EAG)

The kind of odor stimulus provided and the stimulation protocol can significantly affect the outcome of electrophysiological experiments (Dickens 1984, Burguiere et al. 2001, Weißbecker et al. 2004, Altuzar et al. 2007, Spaethe et al. 2007).
Paraffin oil, which is often used in electrophysiological experiments (Visser 1979, Page & Koelling 2003, Weißbecker et al. 2004, Johne et al. 2006b/2007, Thakeow et al. 2008), evoked a strong response of the antennal receptors. Other established solvents used for diluting the chemicals in electrophysiological experiments are ethanol (Spaethe et al. 2007), diethyl phthalate (Burguiere et al. 2001), hexane (Mayer et al. 1984, Light et al. 1992, Ho & Millar 2002, Altuzar et al. 2007) or pentane (Dickens 1984). Silicone oil as a solvent was used by Koch et al. (2002) working with the pink bollworm moth, *Pectinophora gossypiella* S. Silicone oil was chosen as the solvent, because only a very small antennal response could be observed by puffing over the antenna (except those of the mechanoreceptors). In contrast to paraffin oil, which gets frowsty with the time, silicone oil is inert. However, in comparison of results and handling it has to be kept in mind that silicone oil has another polarity and a higher fluidity.
Aluminium foil was used instead of filter paper, because the antennal receptors responded to filter paper as well: The longer the filter paper soaked with paraffin oil was stored in the syringe, the higher was the antennal response to the solvent control.

A positive control (antennal response to anisol in the dilution 10^{-3}) as well as a negative control (antennal response to silicone oil) were measured prior and after each dilution series. All antennal responses were subtracted from the negative control. Humidified clean from the GC-EAD system was used for filling the stimulus syringes with air (0-calibration).

Wibe (2004) showed that the choice of method influenced the results of electrophysiological studies with insects. He compared single cell recording linked to a gas chromatograph (SCR-GC) and electroantennography linked to a gas chromatograph (EAG-GC). He mentioned that “the response strength was usually not the same relative to the strongest response recorded by each technique ... by using SCR-GC more information was obtained.” However, requirements in technical predispositions and preparation skills to obtain recordings for hours are much higher in the SCR method. Therefore, it could not establish as a routine method in screening for perceived odours in Chemical Ecology.

6.1.4 Behavioural Tests

The dual choice tests were carried out in dark rooms, because of the natural living conditions belowground in the dark. Light, of course, influences the insect behaviour. During the experiments, several short observations (switching on the light) were necessary, to record the position and the choice behaviour of the larvae of *M. hippocastani*. These short interruptions of the darkness might disturb the larvae and might alter their behaviour. The feeding preference tests were performed in black plastic pots so that the larvae were not disturbed by the daylight in the greenhouse.

The larvae used in the experiments were collected in the field (near Darmstadt), kept individually in plastic boxes (13.2 cm x 8.4 cm x 6.5 cm) and fed once to twice a week with fresh slices of carrot. During the behavioural experiments the larvae were positioned either in the black plastic pots during the feeding preference tests or in petri dishes during the dual choice tests.

It is known that insects, kept in the laboratory, can loose their ability to grow successfully on their original host plants (Guthrie & Carter 1972) or they can accept plant species totally outside their natural host range (Schoonhoven 1967). Moreover, insects collected in the field may be infested with pathogenes and/or parasitoids which may affect insect behaviour compared with non-infected individuals. Likewise, experience and learning factors may have an additional effect on the physiological and behavioural response of the single individual.

Plant odours consist of specific and general components (Visser 1983/1986). Thiery & Visser (1986) showed that odour of non-host plants was able to block the response of females of *Leptinotarsa decemlineata* to the odour of their host plants in a wind tunnel.

Such effects could cause false negative results in choice tests, e.g. if the host plants are close to non-host plants. Similarly, root exudates were able to mask the attractive effect of CO_2 in the behaviour of cockchafer larvae (Reinecke et al. 2008), if a combined stimulus of root extract and synthetic CO_2 was offered to larvae of *M. melolontha*.

Generally, large, more natural arenas or open-fields fit better with the life style of insects (Withers & Barton Browne 1998), but it is expected that masking of host plant odour occurs often in mixed cropping systems (Thiery & Visser 1986). At the same time the observation of the behaviour of the larvae is highly limited. However, many of the environmental factors named above can also influence open-field tests, which also vary depending on the experimental design.

The results can depend strongly on the density of occuring test plants (Briese 1999). Even the temperature of the food plant can have an influence on the choice behaviour of insects (Bongers 1970, Schalk et al. 1969). It is still unclear, if the chemical composition of plants change with temperature, thereby affecting the sensory impressions on the insect, and if the insect's behaviour is modified by temperature through changes in the central nervous system or the chemoreceptors.

Both kinds of tests are important and should be performed. In the laboratory, the observability is better, and the experiments can be performed under controlled conditions. These should then be complemented by open-field studies under natural environmental conditions.

Therefore, especially experiments in behaviour need a thorough consideration of methodology in the planning phase. Otherwise, an adequate statistical evaluation of the results may become impossible (Martin & Bateson 1986). Statistical evaluation of behavioural experiments dealing with food-choice preferences as well as olfactometer assays seem not to be easy and the results vary with the applied statistical tests employed. Several authors concentrate on this topic (*e.g.* Horton 1995, Manly 1995, Bernays & Weiss 1996, Lockwood 1998, Sakuma 1998).

Several innovations in methodological approaches had to be established in this study, because of the special way of living of the *M. hippocastani* larvae.

6.2 Discussion of the Results

6.2.1 Electroantennographic Detection (EAD)

The measurements with a gas chromatograph coupled to an electroantennograph were performed from 2004 to 2007 with VOC samples from experiments described in chapters 2 to 5, and additionally with single compounds in different dilutions. It was not possible to obtain clear and repeatable results throughout the year. However, in certain moments, which could last only hours, a small part of the tested antennae showed similar responses to several compounds. This was the basis for choosing the compounds for EAG-measurements (see chapter 5.4.2) and for the behavioural experiments (see chapter 5.4.5). Figure 6.1 shows the antennal response of a 3rd instar larva of *M. hippocastani* in summer of 2004.

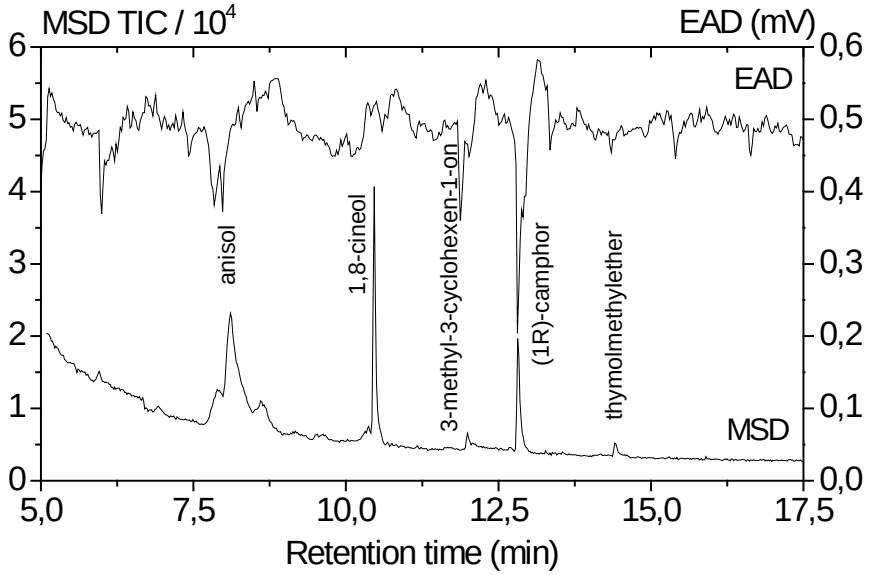

Figure 6.1: Chromatogram of a combined GC-MS/GC-EAD from the roots of *Quercus* sp. damaged by feeding of *M. hippocastani*. The response to (1R)-camphor was measured only once as an exception, maybe of special individual properties, maybe of technical origin. No response was found in any other GC-MS/GC-EAD-experiments to (1R)-or (1S)-camphor.

6.2.2 Behavioural Tests

Behavioural experiments can be performed in the laboratory or in the field. Semi-field systems (defined as enclosed environments, ideally situated within the natural ecosystem of the target insect and exposed to ambient environmental conditions, in which all features necessary for its life cycle completion are present) try to involve laboratory and open-field conditions and are often used for the environmental risk assessment of pesticides in soil (Schaeffer et al. 2010) as well as by medical entomologists (e.g. Ferguson et al. 2008). Ideally, behavioural tests are carried out first in the laboratory and afterwards in the open-field.

Probably, gases like CO_2, which is known to evoke an attraction of *Melolontha* larvae (Hasler 1986), and acetone are used by the larvae for the long range orientation, so the organisms can be attracted also by non-host plants, decaying

material or accumulations of organisms. For example, acetone had a repellent effect in the tested dilutions 10^{-2} and 10^{-4} in silicone oil (see figure 6.2). The experiments were carried out in July and August of 2008.

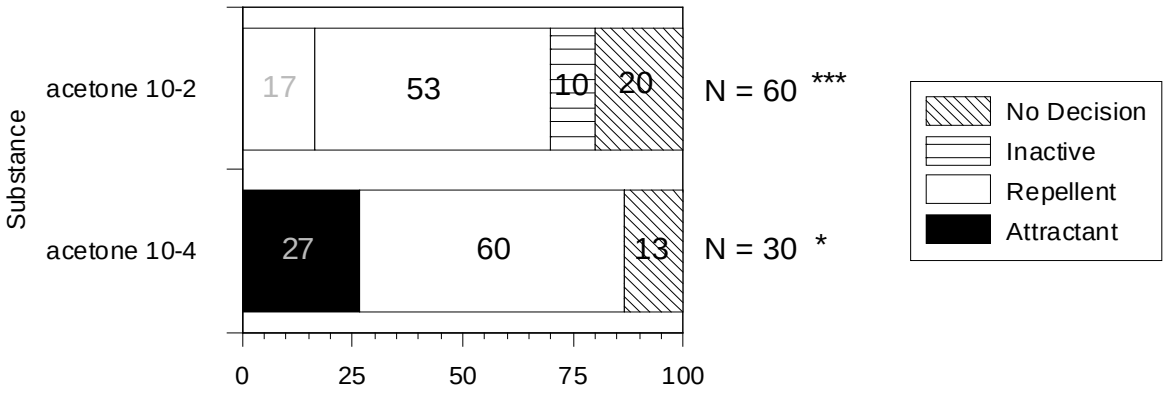

Figure 6.2: Behavioural data in percent of 2nd and 3rd instar larvae of *M. hippocastani* in dual choice tests of two different dilutions. Numbers in the bars show the percentage, numbers next to the bars indicate the total number of individuals in the two experiments. Statistical analysis was done excluding the inactive animals and those who show no decision. Statistical significance is indicated by * ($p < 0,05$) and *** ($p < 0,001$)

For the short range orientation, other substances obviously are responsible. The influence of nutrients and root exudates (which are dissolved in the soil water) on the behaviour, was not checked in this study. The diffusion of volatiles in soil medium is strongly dependent on the humidity of the substrate (Turlings, unpublished data) as well as on the physicochemical conditions in the soil. Volatile diffusion is, among others, decelerated by increasing humidity of the substrate.

6.2.2.1 Electrophysiological and Behavioural Responses in Dual Choice Arena Tests to Two Compounds of Carrot

On the basis of the results from measurements with a gas chromatograph coupled to an electro-antennograph (EAD), the two most abundant volatile compounds terpinolene and β-caryophyllene of *Daucus carota* ssp*. sativus* were selected for electrophysiological and dual choice arena tests.

The kind of behaviour of insects, if attracted or repelled by different volatile compounds, cannot be predicted via EAG tests (see chapter 1.9). The curves show only if the antennae receptors respond to volatile stimuli or not (see figure 6.4):

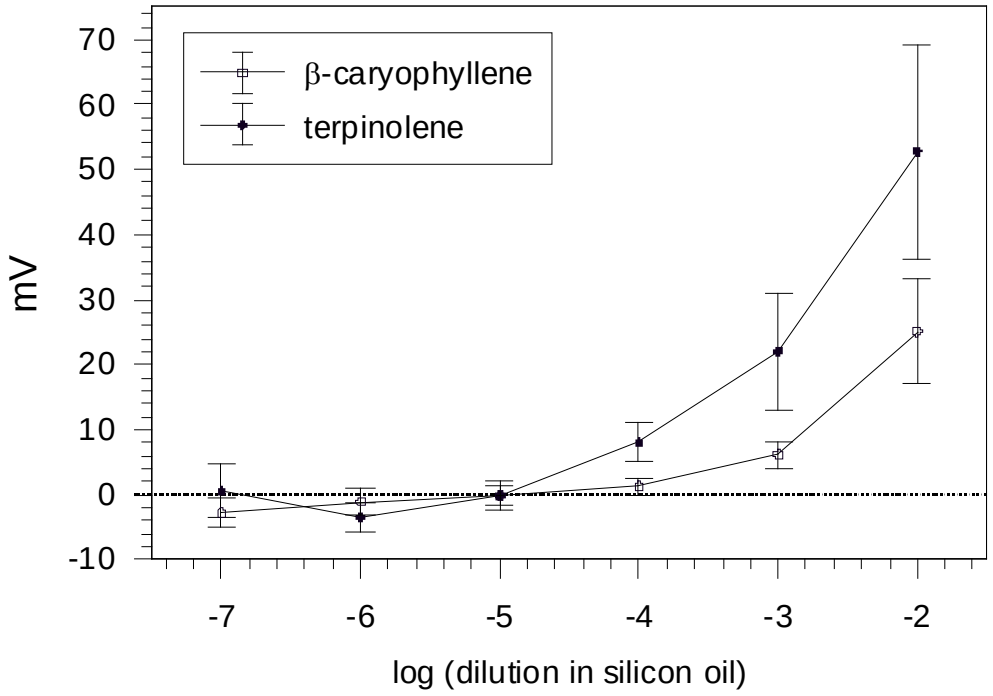

Figure 6.5: Dose-response curves of 2nd instar larvae of *M. hippocastani* to β-caryophyllene (N = 4) and terpinolene (N = 4) released by oak-roots damaged by feeding of the larvae. SE of the mean is indicated by error bars.

In the experiments concerning the behavioural responses of the larvae of *M. hippocastani* to volatile emissions of roots of *Quercus* sp. and *Daucus carota* ssp*. sativus*, the latter was clearly preferred by the larvae of *M. hippocastani* (see 3). This may be due to the volatile emissions, which qualitatively differ clearly between the two plant species (see chapter 3.4).

In dual choice arena tests, a clear attractive reaction could be observed to terpinolene at 10^{-2} dilution in silicone oil, whereas β-caryophyllene at 10^{-2} dilution in silicone oil had a clear repellent effect on 3rd instar larvae of *Melolontha* larvae (see figure 6.5):

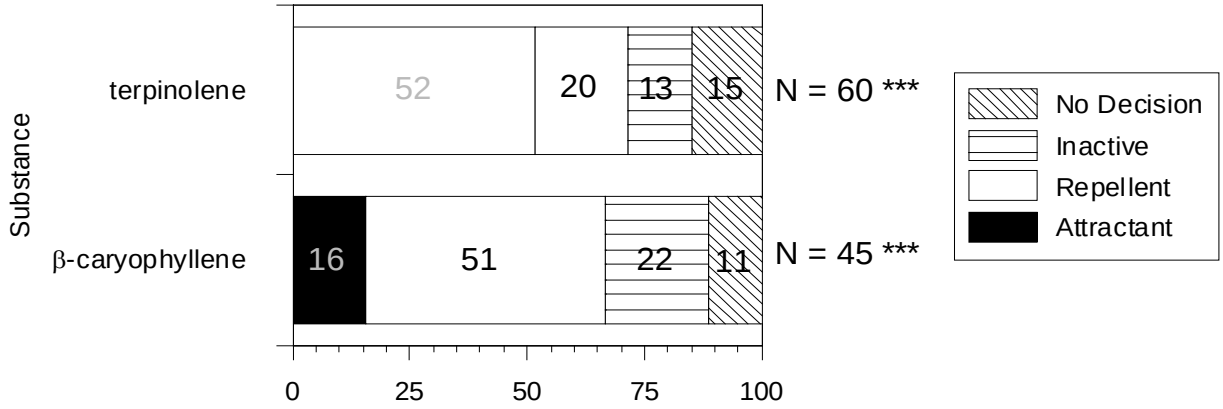

Figure 6.6: Behavioural data in percent of 2nd and 3rd instar larvae of *M. hippocastani* in dual choice tests of two main components in *Daucus carota* ssp*. sativus*. Numbers in the bars show the percentage, numbers next to the bars indicate the total number of individuals in the different experiments. Statistical analysis was done excluding the inactive animals and those who showed no decision. Statistical significance is indicated by *** ($p < 0{,}001$).

Although carrots were highly attractive in behavioral preference tests (see 3.4), β-caryophyllene as one of the highly abundant compounds of carrot had a clear repellent effect. This phenomenon is described by several other authors as well: certain compounds attract or repel certain species, whereas mixtures of compounds often attract or repel more selectively than single compounds (Dodson et al. 1969, Williams and Dodson 1972, Ackerman 1989, Schiestl & Roubik 2003). In *Cydia molesta* B., a pest of pomaceous and stone fruit, a 3-component mixture of peach shoot volatiles made of (Z)-3-hexen-1-yl acetate, (Z)-3-hexen-1-ol and benzaldehyde in a 4:1:1 ratio was attractive for the females,

whereas the components tested individuallly were not (Natale et al. 2003). These observations lead to the insight that correlations between single factors may be more important than the effect of single factors tested individually. This knowledge makes investigations much more complicated and urges the scientist to take a broader view.

The results of the electrophysiological and behavioural experiments suggest that the behaviour of the larvae of *M. hippocastani* is depending also on the seasonal and maybe also on the circadian rhythm. This may be because the food plant range changes during larval development (Schoonhoven et al. 2005). The earlier instars of the larvae of the garden tiger moth *Arctia caja* L. (Merz 1959) feed only on a few plants, whereas the later instars reject hardly any plant species. A similar feeding preference shows the larvae of *M. hippocastani* (Hauss & Schütte 1976). On the other hand, attack by early instars of root herbivores can result in completely different plant responses than feeding by mature larvae (Riedell & Evenson 1993).

Additionally, one has to take into account that each insect is an individual, with its own food preferences and aversions, which results in the deviation of behaviour. Thus, deviation from the mean, especially in behavioural tests, may be common and have to be regarded as normal. „When ignoring the extent of variation in behavioural or physiological parameters, as biologists often tend to do under the influence of Platonic philosophical traditions, essential information is lost. Such 'tyranny of the Golden Mean' disregards some basic principles of life (Bennett 1987 in Schoonhoven 2005).

But beside food, abiotic and biotic factors are important as well: The host plant is not merely something fed on, it is something lived on. Insects living on plants are faced with a lot of cohabitant e.g. natural enemies, competitors, host plant pathogenes and a specific microclimate (Kennedy 1953). Therefore, host plant preferences are governed not only by nutritional quality but also by environmental factors.

6.3 Conclusions

In this study, analysis of the volatile composition of root emissions, electrophysiological tests, and subsequently performed behavioural dual choice arena tests, aim to illuminate the behavioural orientation of *Melolontha* larvae.
Referring to the expected results (see 1.11), the effectively obtained results are listed here:

- *Quercus* sp. and *A. hippocastanum* differ in their volatile emissions above- and belowground.
- The volatile patterns of *Quercus* sp. differ in several compounds between healthy plant roots, mechanically damaged plant roots, and roots damaged by feeding of *M. hippocastani,* like 1-octen-3-ol, 3-otanone, 6-methyl-5-hepten-2-one, 2-ethyl-1-hexanol, 1,8-cineol, decanal, anisol, and geranyl acetone. Infestation by additional above- and belowground organisms had an eliciting effect on infestation specific volatile emissions of *Quercus* sp. and *A. hippocastanum* belowground.
- In electrophysiological tests with the antennae of *M. hippocastani*, dilutions down to 10^{-6} could be perceived in 3-octanone, camphor, and β-caryophyllene. Dilutions down to 10^{-4} could be perceived in anisol, 1,8-cineol, and terpinolene, whereas furanoid trans-linaloloxide was perceived in dilutions down to 10^{-3}.
- In dual choice tests it was shown that some volatiles emitted by damaged oak roots are able to elicit an attractive orientation and behavioural response in dual choice arena tests of cockchafer larvae (*M. hippocastani)*. These volatiles were anisol (in the dilution 10^{-2}) and 1,8-cineol (in the dilutions 10^{-2} and 10^{-4}). The volatile compound terpinolene, emitted by belowground parts of carrots (the wild form as well as the cultured form), was attractive in the dilution 10^{-2} as well. Repellent effects could be observed by testing acetone (in the dilutions 10^{-2} and 10^{-4}) and β-caryophyllene (in the dilution 10^{-2}). β-caryophyllene was emitted by belowground parts of carrots as well as by shoots of *A. hippocastanum,* but not in the roots of *A. hippocastanum.*

- *Daucus carota* ssp. *sativus* were highly attractive when tested in feeding choice tests against *Quercus* sp. and potatoes with larvae of *M. hippocastani*, and when tested against potatoes with larvae of *Agriotes* sp.

Larvae of *M. hippocastani* are able to distinguish between different volatiles and to respond with attractive or repellent behaviour.

6.4. Prospects and Applications

After discovering that larvae of *M. hippocastani* are able to perceive VOCs, more interesting questions result from the conclusions:

Single sensillum recording (SSR) could be used as a more suitable electrophysiological method to investigate in detail the olfactory system of *M. hippocastani*. Compounds, which were able to cause an antennal response, may be tested in electrophysiological tests systematically in different dilutions in dual choice arena tests.

Mixtures of electrophysiologically identified candidate compounds can be tested in different ratios. Natural volatile compositions could serve as basis for creating the mixtures.

This study focused on host plants of *M. hippocastani*. However no compounds were found, which elicited reproducible response in electrophysiological and behavioural experiments over an extended time of the year. It would be highly desirable to find such a compound. Therefore the present study could be complemented by a wider search, including non-host plants. There exist hints (Brückner 1999, Liu 1999), which indicate a lower preference of *M. hippocastani* larvae for plants such as the invasive plant *Prunus serotina* E. At

the same time these plants show a higher regeneration ability of the roots, if they were damaged by larval feeding of *M. hippocastani*, whereas *Quercus rubra* L. and *Pinus sylvestris* L. show a clearly lower tolerance against the grub. Ruther et al. (2000) indicate *Prunus serotina* as a non-host plant. Studying the recent literature, more potential non-host plants, or plants with a lower preference, may be found. However, it is important to keep in mind that also the dissolved root exudates may play an important role in forage choice and feeding behaviour of belowground living arthropods. This effect was not investigated in the present study.

A practical appliance in the open field based on this study could be the development of capsules enclosing a formulation of either attractive or repellent volatile compounds in order to establish control systems on the basis of a push-and-pull concept. The compounds could be provided either as single volatiles or as mixtures of several compounds. Prior to practical application volatile composition and the carrier substance have to be checked on the compatibility of floral and faunal organisms' activity (non-target organisms).

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APPENDIX A

A.1 Total Ion Chromatograms

A.1.1 *Quercus* sp.

The following total ion chromatograms show the volatile patterns of damaged shoots and roots of *Quercus* sp. (figures A.1 a-e) and *Aesculus hippocastanum* (figures A.2 a-e). *N* indicates the number of samples (i.e. trees) used in each treatment. Only one chromatogram of each treatment is shown as an example. Within the chromatograms, peaks of volatile compounds are labelled by arbitrarily chosen numbers. A list below each chromatogram translates these numbers to the compound names. Several compounds were not detected in every single sample of a treatment. Behind each name, the number of samples showing an abundance of this compound above detection threshold, is given versus the total number of samples measured.

Different y-axis scaling factors were used due to different total abundances of emitted volatiles.

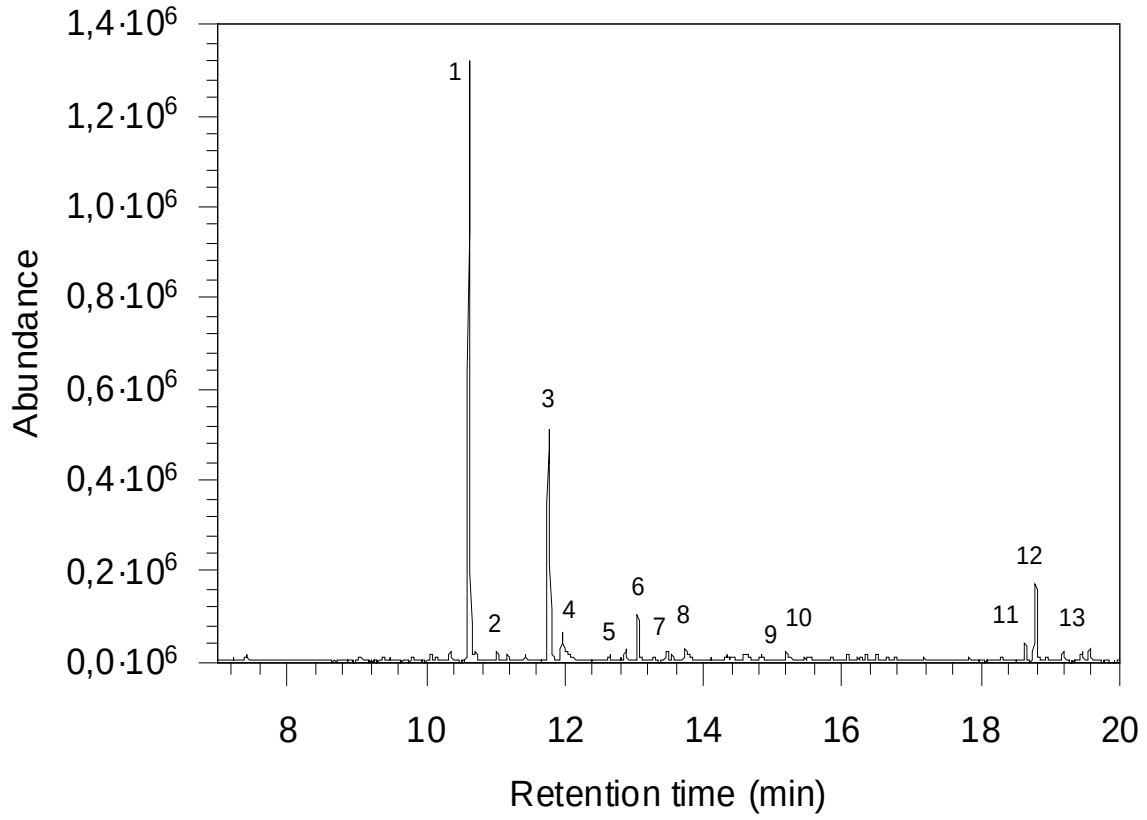

Figure A.1 a: shoot volatiles of *Quercus* sp., undamaged plants (N=7)

1→ (Z)-3-hexenyl acetate (7/7)
2→ 6-methyl-5-hepten-2-one (7/7)
3→ (Z)-3-hexen-1-ol (7/7)
4→ nonanal (7/7)
5→ 2,6-dimethyl-1,3,5,7-octatetraene * (2/7)
6→ (Z)-3-hexenyl butyrate (1/7)
7→ 2-ethyl-1-hexanol (7/7)
8→ decanal (7/7)
9→ hexadecane (6/7)
10→ germacrene D * (1/7)
11→ 2,6-dimethyl-3,5,7-octatriene-2-ol * (3/7)
12→ isopropyl laurate (7/7)
13→ geranyl acetone * (7/7)

* → tentatively identified

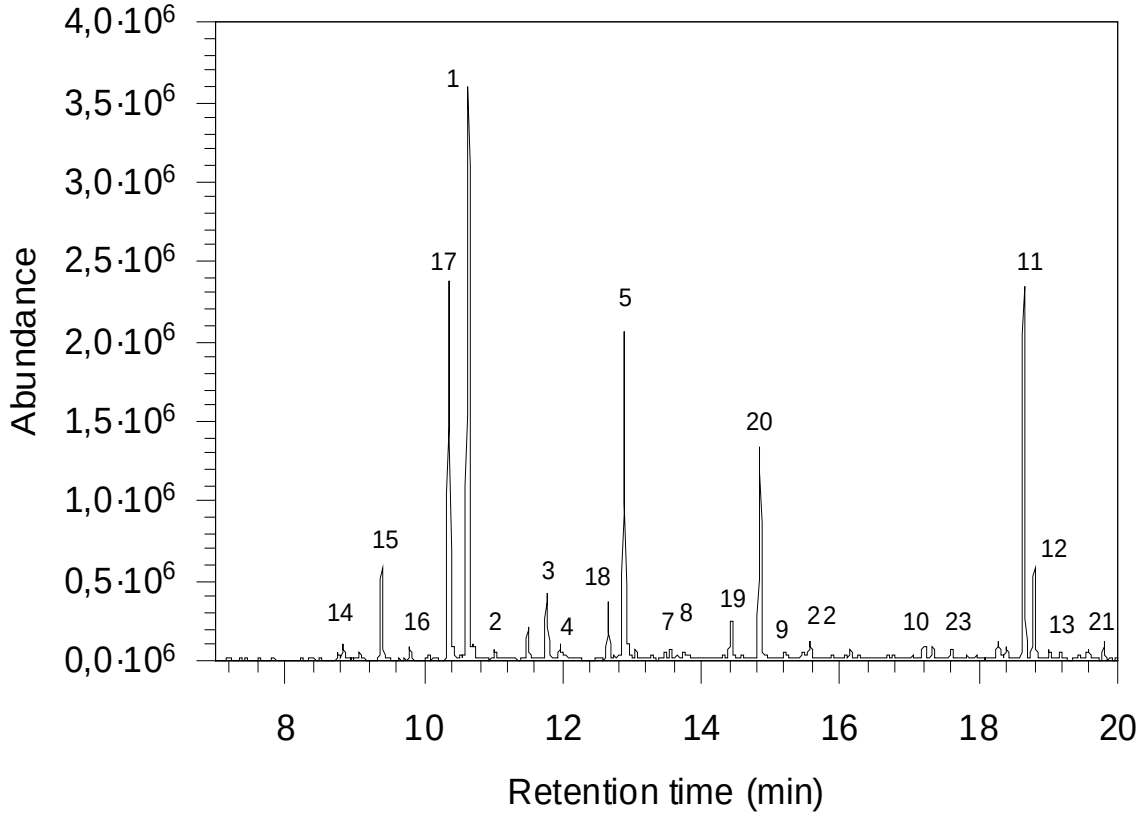

Figure A.1 b: shoot volatiles of *Quercus* sp., plants damaged by aboveground feeding of Aphididae, Thripidae and Tetranychidae, and infestation of mildew (N=7)

1→ (Z)-3-hexenyl acetate (7/7)
2→ 6-methyl-5-hepten-2-one (7/7)
3→ (Z)-3-hexen-1-ol (7/7)
4→ nonanal (7/7)
5→ 2,6-dimethyl-1,3,5,7-octatetraene *(7/7)
7→ 2-ethyl-1-hexanol (7/7)
8→ decanal (7/7)
9→ hexadecane (2/7)
10→ germacrene D * (2/7)
11→ 2,6-dimethyl-3,5,7-octatriene-2-ol * (6/7)
12→ isopropyl laurate (7/7)
13→ geranyl acetone * (7/7)
14→ 1,8-cineol (2/7)
15→ β-ocimene * (6/7)
16→ hexyl acetate (7/7)
17→ unidentified compound (7/7)
18→ unidentified compound (6/7)
19→ unidentified compound (4/7)
20→ unidentified compound (5/7)
21→ benzyl alcohol (7/7)
22→ β-caryophyllene (1/7)
23→ α-farnesene * (6/7)

* → tentatively identified

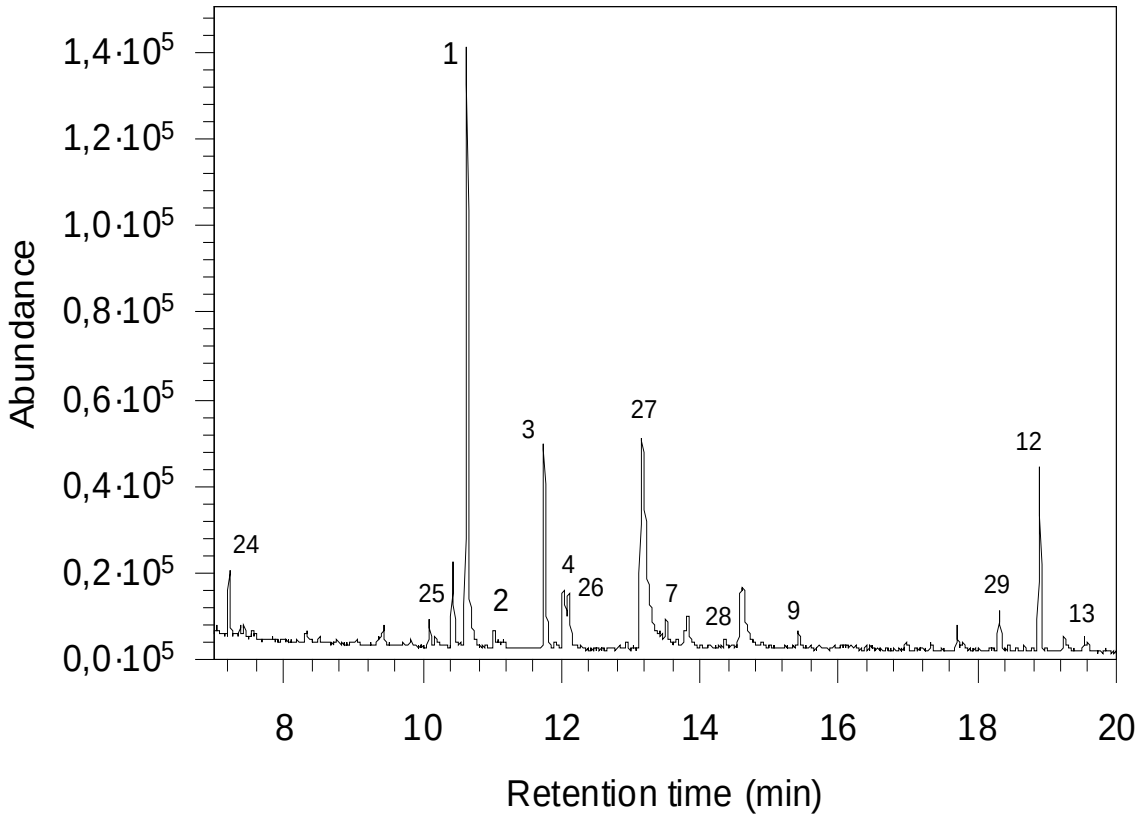

Figure A.1 c: shoot volatiles of *Quercus* sp., plants damaged by aboveground feeding of Aphididae, Thripidae and Tetranychidae, and mildew infestation with additional belowground feeding of *M. hippocastani* larvae, (N=9)

1→ (Z)-3-hexenyl acetate (9/9)
2→ 6-methyl-5-hepten-2-one (8/9)
3→ (Z)-3-hexen-1-ol (9/9)
4→ nonanal (9/9)
7→ 2-ethyl-1-hexanol (9/9)
9→ hexadecane (8/9)
12→ isopropyl laurate (5/9)
13→ geranyl acetone * (9/9)

24→ 2-pentanol * (5/9)
25→ trimethyl benzene* (9/9)
26→ 2-butoxy ethanol * (7/9)
27→ acetic acid * (9/9)
28→ β-bourbonene (4/9)
29→ methyl salicylate (7/9)

* → tentatively identified

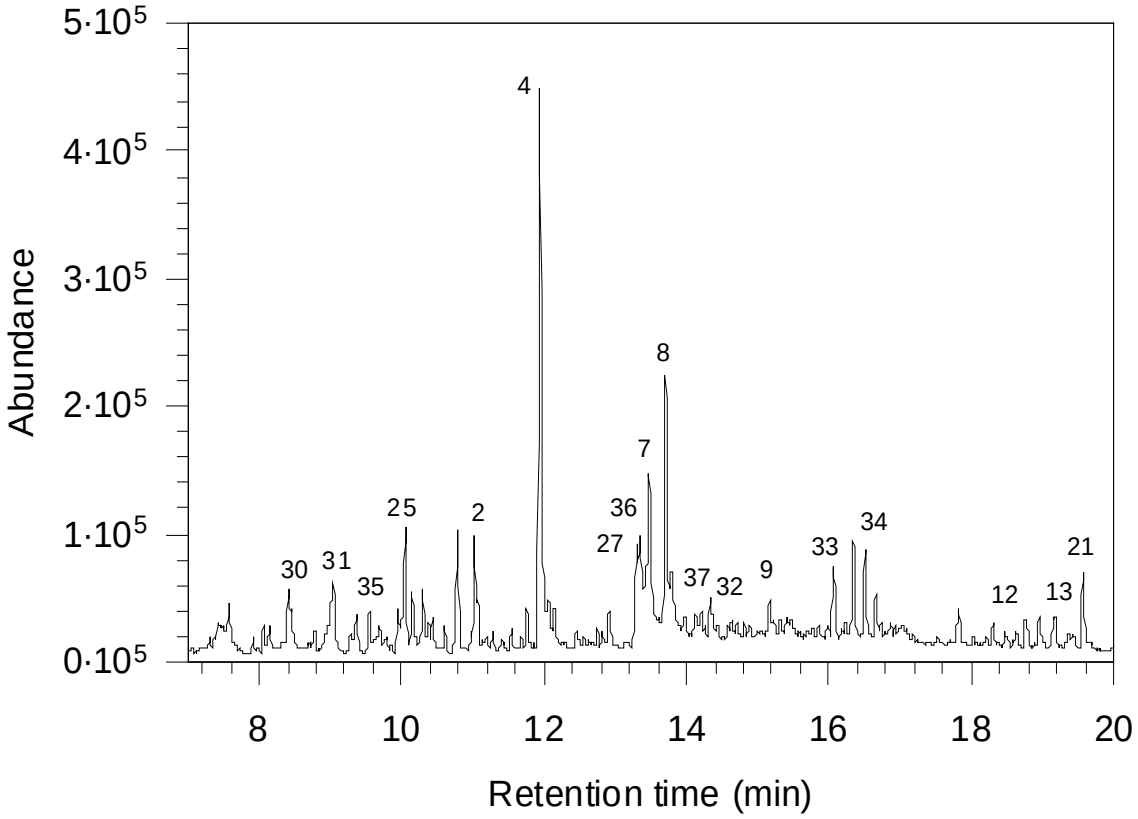

Figure A.1 d: root volatiles of *Quercus* sp., undamaged plants (N=9)

2→ 6-methyl-5-hepten-2-one (9/9)
4→ nonanal (9/9)
7→ 2-ethyl-1-hexanol (9/9)
8→ decanal (9/9)
9→ hexadecane * (7/9)
12→ isopropyl laurate (9/9)
13→ geranyl acetone * (9/9)
21→ benzyl alcohol (8/9)
25→ trimethy lbenzene* (9/9)
27→ acetic acid * (2/9)
30→ heptanal * (4/9)
31→ 3-ethyl toluene * (8/9)
32→ benzaldehyde * (9/9)
33→ sabina ketone * (5/9)
34→ diethoxy methane* (9/9)
35→ 3-octanone (9/9)
36→ linalool oxide* (5/9)
37→ camphor (9/9)

* → tentatively identified

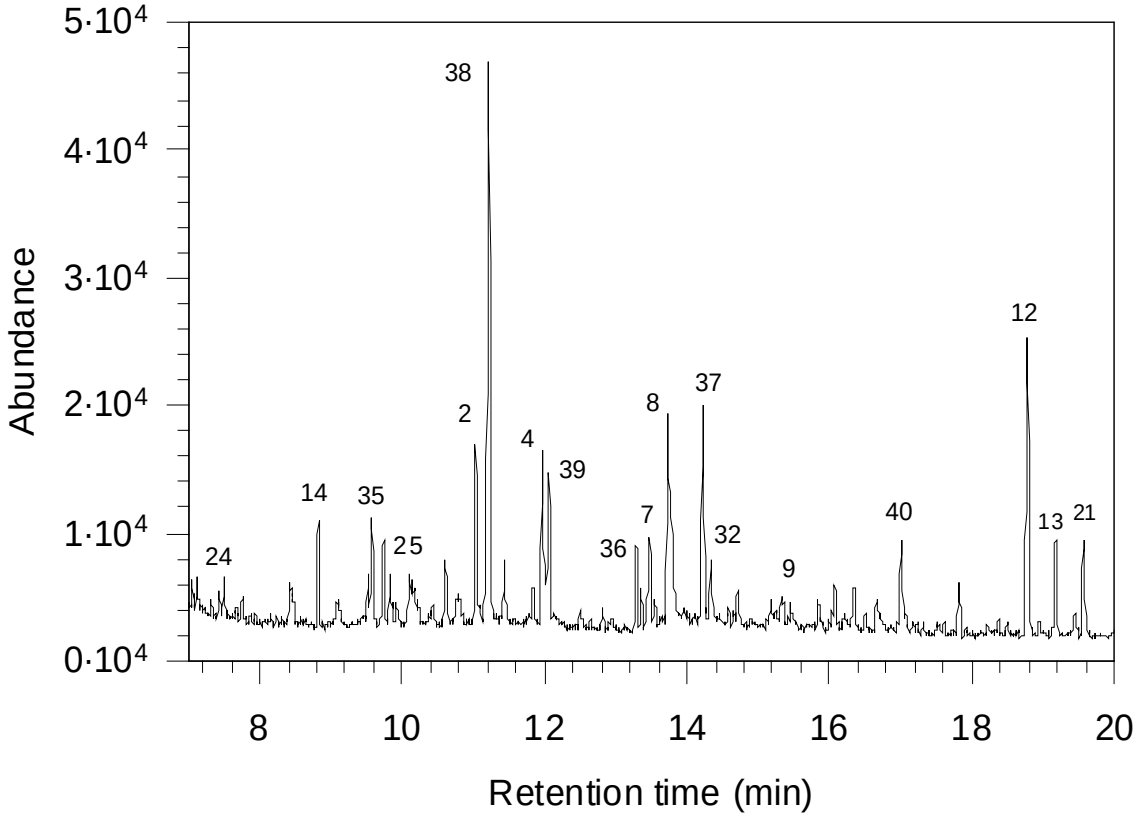

Figure A.1 e: root volatiles of *Quercus* sp., shoots damaged by aboveground feeding of Aphididae, Thripidae and Tetranychidae, and infestation of mildew, additionally root damaged by larval feeding of *M. hippocastani* (N=11).

2→ 6-methyl-5-hepten-2-one (10/11)
4→ nonanal (10/11)
7→ 2-ethyl-1-hexanol (11/11)
8→ decanal (10/11)
9→ hexadecane * (5/11)
12→ isopropyl laurate (10/11)
13→ geranyl acetone * (7/11)
14→ 1,8-cineol (8/11
21→ benzyl alcohol (9/11)
24→ 2-pentanol * (5/11)
25→ trimethyl benzene* (11/11)
32→ benzaldehyde * (8/11)
35→ 3-octanone (9/11)
36→ linalool oxide* (9/11)
37→ camphor (10/11)
38→ anisol * (10/11)
39→ methyl benzyl ether * (4/11)
40→ borneol * (8/11)

* → tentatively identified

A 1.2 *Aesculus hippocastanum*

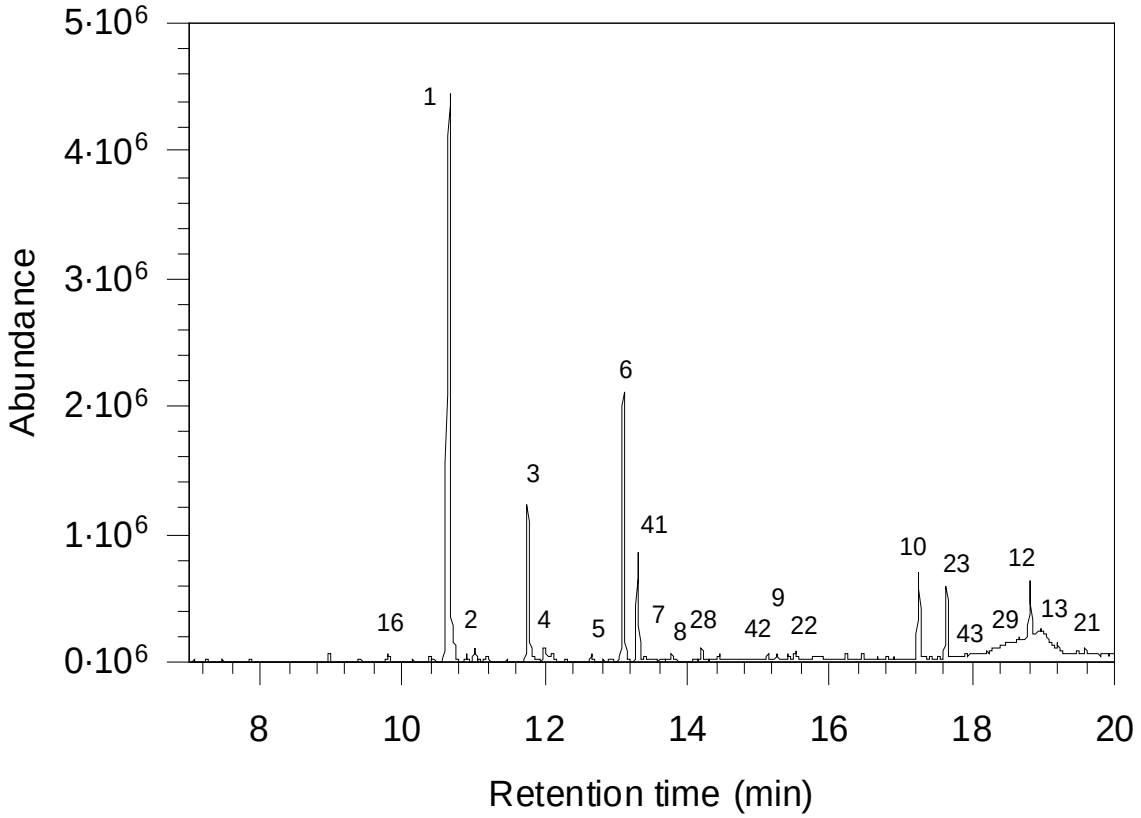

Figure A.2 a: shoot volatiles of *Aesculus hippocastanum*, undamaged plants (N=2)

1→ (Z)-3-hexenyl acetate (2/2)
2→ 6-methyl-5-hepten-2-one (2/2)
3→ (Z)-3-hexen-1-ol (2/2)
4→ nonanal (2/2)
5→ 2,6-dimethyl-1,3,5,7-octatetraene * (1/2)
6→ (Z)-3-hexenyl butyrate (2/2)
7→ 2-ethyl-1-hexanol (2/2)
8→ decanal (2/2)
9→ hexadecane * (2/2)
10→ germacrene D * (2/2)
12→ isopropyl laurate (2/2)
13→ geranyl acetone * (2/2)
16→ hexyl acetate (2/2)
21→ benzyl alcohol (2/2)
22→ β-caryophyllene (2/2)
23→ α-farnesene * (2/2)
28→ β-bourbonene * (2/2)
29→ methyl salicylate (1/2)
41→ 3-hexenyl isolvalerate * (2/2)
42→ unidentified compound (2/2)
43→ δ-cadinene (2/2)

* → tentatively identified

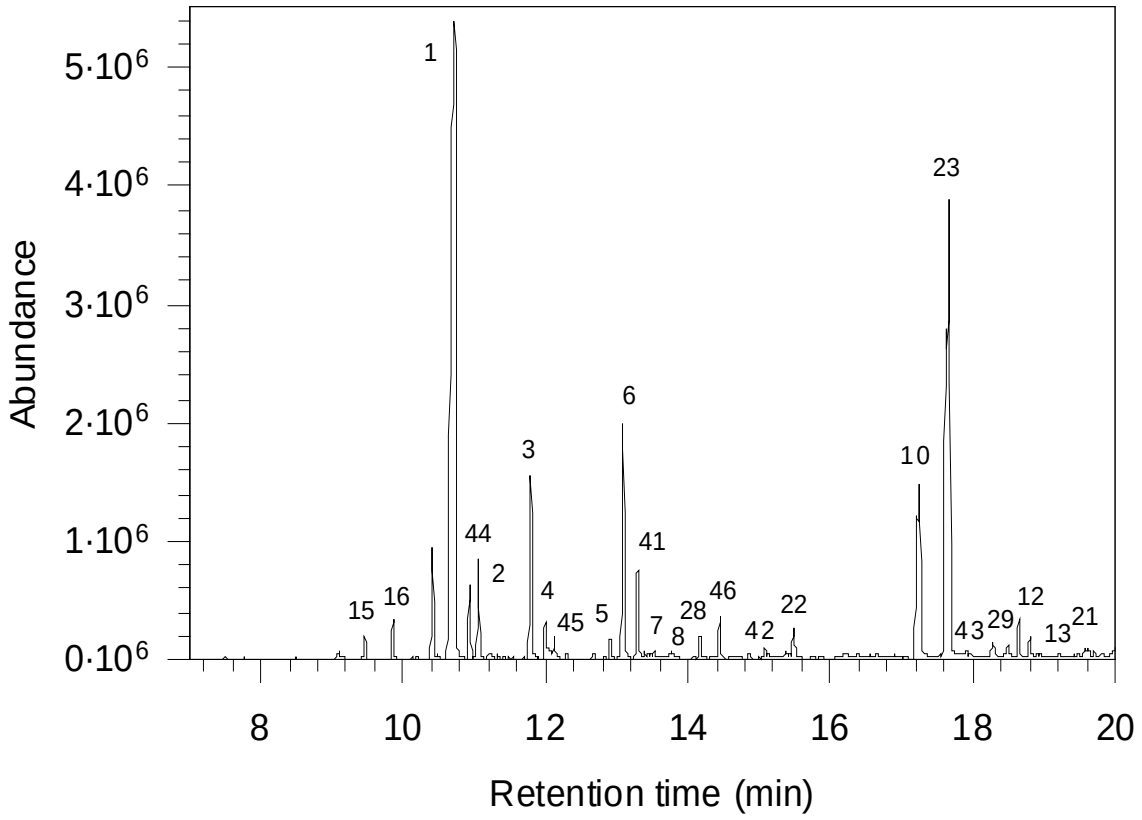

Figure A.2 b: shoot volatiles of *Aesculus hippocastanum*, plants damaged by aboveground feeding of Aphididae, Thripidae and Tetranychidae (N=2)

1→ (Z)-3-hexenyl acetate (2/2)
2→ 6-methyl-5-hepten-2-one (2/2)
3→ (Z)-3-hexen-1-ol (2/2)
4→ nonanal (2/2)
5→ 2,6-dimethyl-1,3,5,7-octatetraene * (2/2)
6→ (Z)-3-hexenyl butyrate (2/2)
7→ 2-ethyl-1-hexanol (2/2)
8→ decanal (2/2)
10→ germacrene D * (2/2)
12→ isopropyl laurate (2/2)
13→ geranyl acetone * (2/2)
15→ β-ocimene * (2/2)
16→ hexyl acetate (2/2)
21→ benzyl alcohol (2/2)
22→ β-caryophyllene (2/2)
23→ α-farnesene * (2/2)
28→ β-bourbonene * (2/2)
29→ methyl salicylate (2/2)
41→ 3-hexenyl isolvalerate * (2/2)
42→ unidentified compound (2/2)
43→ δ-cadinene (2/2)
44→ 2-hexenyl acetate * (2/2)
45→ 2-hexen-1-ol (2/2)
46→ unidentified compound (2/2)

* → tentatively identified

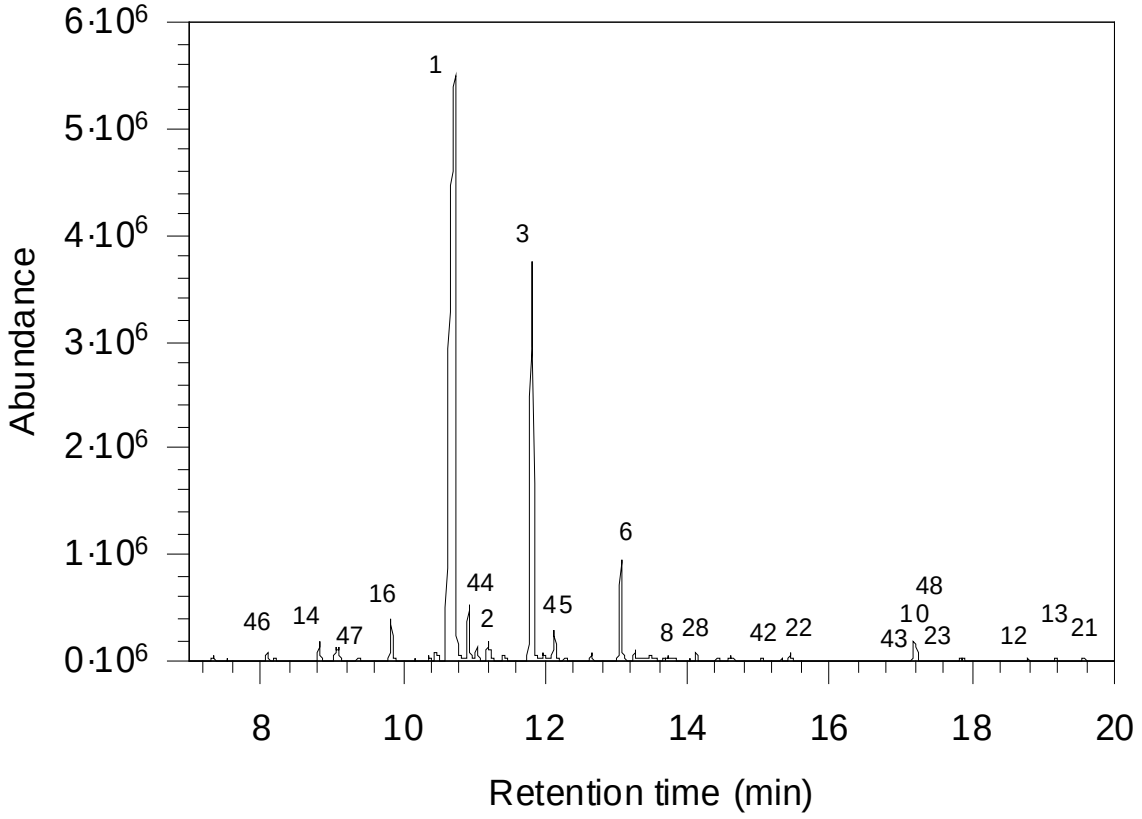

Figure A.2 c: shoot volatiles of *Aesculus hippocastanum*, plants damaged by aboveground feeding of Aphididae, Thripidae and Tetranychidae, with additional belowground feeding of *M.hippocastani* larvae (N=4)

1→ (Z)-3-hexenyl acetate (4/4)
2→ 6-methyl-5-hepten-2-one (4/4)
3→ (Z)-3-hexen-1-ol (4/4)
6→ (Z)-3-hexenyl butyrate (4/4)
8→ decanal (4/4)
10→ germacrene D * (4/4)
12→ isopropyl laurate (4/4)
13→ geranyl acetone * (4/4)
14→ 1,8-cineol (4/4)
16→ hexyl acetate (4/4)
21→ benzyl alcohol (4/4)
22→ β-caryophyllene (2/4)
23→ α-farnesene * (2/4)
28→ β-bourbonene * (3/4)
42→ unidentified compound(1/4)
43→ δ-cadinene (1/4)
44→ 2-hexenyl acetate * (4/4)
45→ 2-hexen-1-ol (4/4)
46→ 1-penten-3-ol * (3/4)
47→ 2-hexenal * (4/4)
48→ cubebene (3/4)

* → tentatively identified

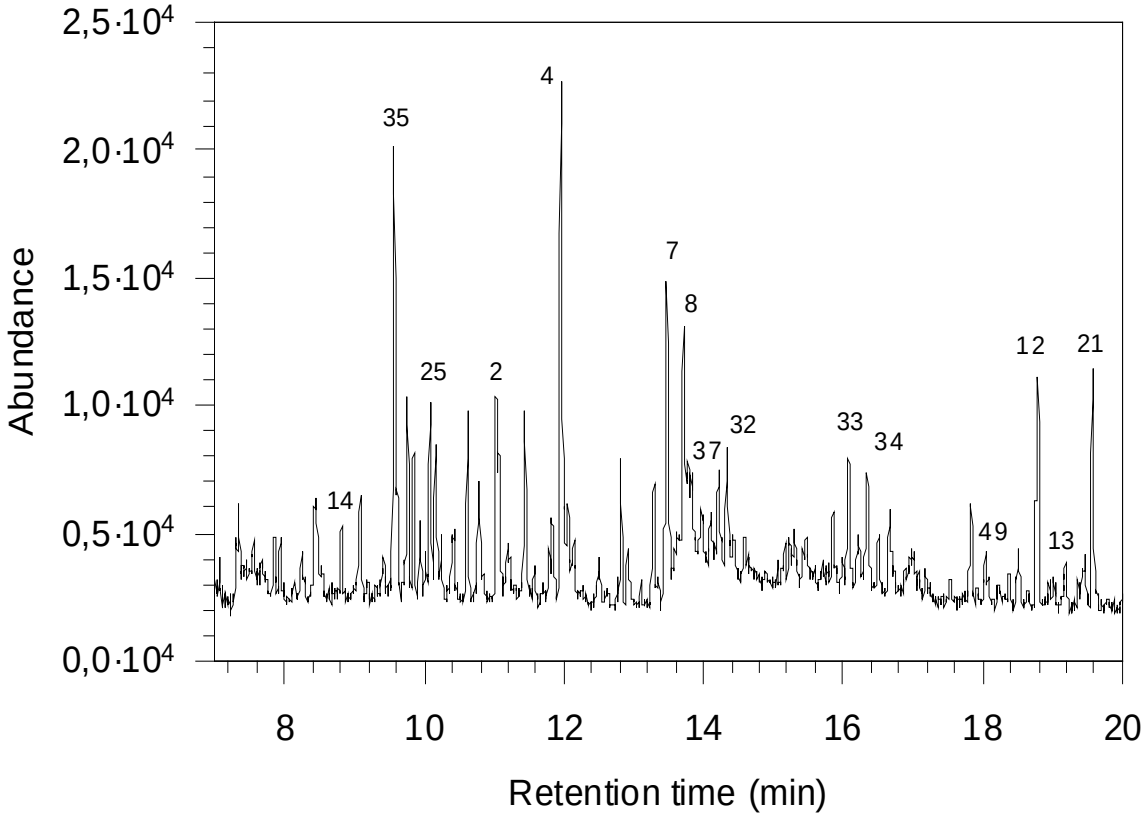

Figure A.2 d: root volatiles of *Aesculus hippocastanum*, undamaged plants (N=4)

2→ 6-methyl-5-hepten-2-one (4/4)
4→ nonanal (4/4)
7→ 2-ethyl-1-hexanol (4/4)
8→ decanal (4/4)
12→ isopropyl laurate (4/4)
13→ geranyl acetone * (3/4)
14→ 1,8-cineol (1/4)
21→ benzyl alcohol (4/4)
25→ trimethyl benzene* (4/4)
32→ benzaldehyde * (4/4)
33→ sabina ketone * (4/4)
34→ diethoxy methane* (4/4)
35→ 3-octanone (4/4)
37→ camphor (3/4)
49→ curcumene* (3/4)

* → tentatively identified

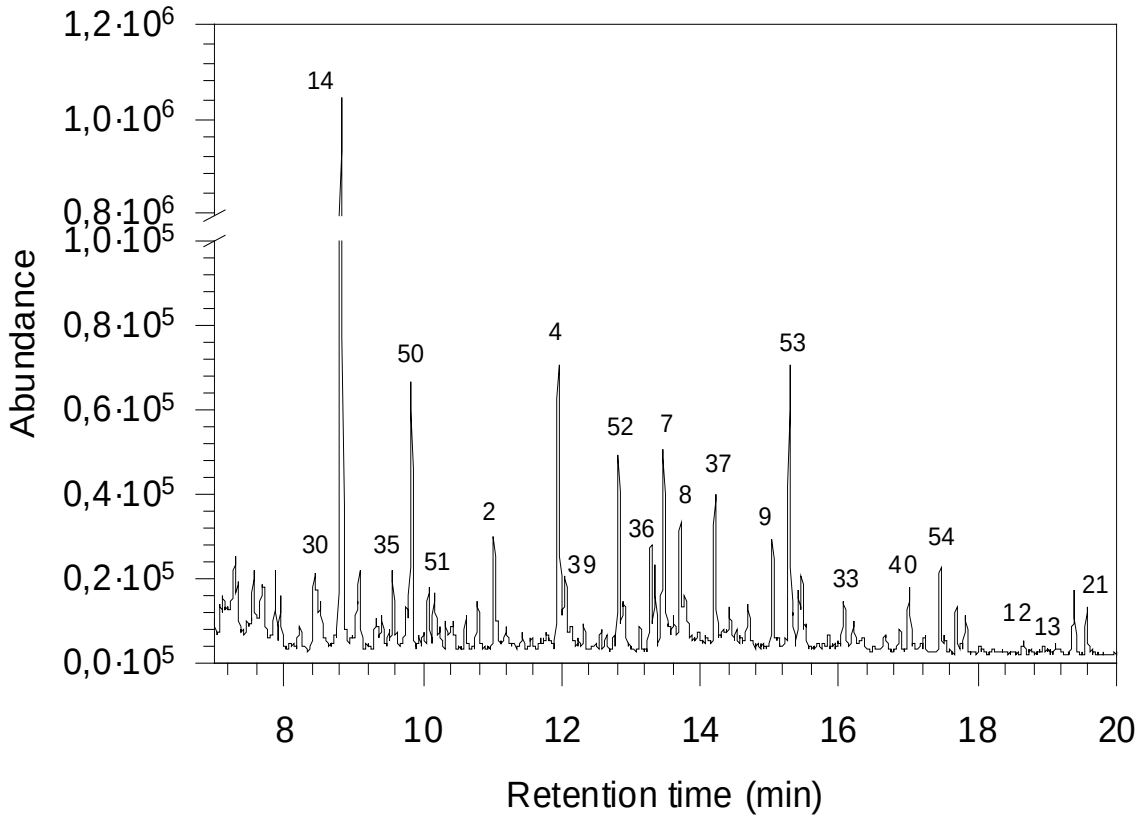

Figure A.2 e: root volatiles of *Aesculus hippocastanum*, plants damaged by aboveground feeding of Aphididae, Thripidae and Tetranychidae, with additional belowground feeding of *M. hippocastani* larvae (N=4)

2→ 6-methyl-5-hepten-2-one (4/4)
4→ nonanal (4/4)
7→ 2-ethyl-1-hexanol (4/4)
8→ decanal (4/4)
9→ hexadecane * (2/4)
12→ isopropyl laurate (2/4)
13→ geranyl acetone * (2/4)
14→ 1,8-cineol (3/4)
21→ benzyl alcohol (4/4)
30→ heptanal * (3/4)
33→ sabina ketone * (3/4)
35→ 3-octanone (4/4)
36→ linalool oxide* (2/4)
37→ camphor (2/4)
39→ methyl benzyl ether * (2/4)
40→ borneol * (3/4)
50→ cymol * (4/4)
51→ octanal (4/4)
52→ p-methyl anisol * (4/4)
53→ thymol methyl ether * (3/4)
54→ unidentified compound (1/4)

* → tentatively identified

Because of the small number of single measurements in the different treatments (2 to 4) the following interpretations have to be handled with care. Further investigations with more replications are needed to get meaningful results.

The table A.1 shows the substances occuring only in one of the five different treatments. Numbers given behind compounds show how often the compound was present in all samples of the treatment above detection threshold. These compounds occurred as so-called “marker-substances” for the particular treatment.

Table A.1: Compounds emitted only of one of the five different treatments by *Aesculus hippocastanum*. The numbers behind the compounds indicate how often the compound was present in all samples of one treatment above the detection threshold. SUA Shoot measured, plant undamaged; SDA Shoot measured, colonised aboveground by arthropods and infestation of mildew; SDB Shoot measured, colonised aboveground by arthropods with additionally infestation of mildew as well as root feeding of *M. hippocastani;* RDA Root measured, plant undamaged; RDB Root measured, shoot damaged aboveground by arthropods and infestation of mildew with additionally root feeding of *M. hippocastani.*

VOLATILE EMISSIONS ABOVEGROUND

SDA
β-ocimene 2/2

SUA + SDA
2,6-dimethyl-1,3,5,7-octatetraene 1/2 + 2/2
methyl salicylate1/2 + 2/2
3-hexenyl isovalerate 2/2 + 2/2

SDB
2-hexenal 4/4
cubebene 3/4

VOLATILE EMISSIONS BELOWGROUND

RDA
trimethyl benzene 4/4
benzaldehyde 4/4
diethoxy methane 4/4
curcumene 3/4

RDB
heptanal 3/4
linalool oxide 2/4
methyl benzyl ether 2/4
borneol 3/4
cymol 4/4
octanal 4/4
p-methyl anisol 4/4
thymol methyl ether 3/4

The table A.2 shows the substances occuring only in *Quercus* sp. or in *A. hippocastanum* above- or belowground.

Table A.2: Compounds emitted only by *Quercus* sp. or *Aesculus hippocastanum* either above- or belowground.

	ABOVEGROUND	BELOWGROUND
***Quercus* sp.**	2,6-dimethyl-3,5,7-octatriene-2-ol 2-butoxy ethanol	3-ethyl toluene anisol
Aesculus hippocastanum	3-hexenyl isovalerate δ-cadinene 2-hexenyl acetate 2-hexen-1-ol 1-penten-3-ol 2-hexenal cubebene	curcumene cymol octanal p-methyl anisol thymol methyl ether

The table A.3 shows the emitted volatile compounds of *Quercus* sp. and *Aesculus hippocastanum* of the five different treatments:

Table A.3: Compounds emitted by *Quercus* sp and *A. hippocastanum* in the five different treatments. Compounds, appearing in less than 50% of the single measurements are marked with O, those appearing in more than 50% of the single measurements are marked with X. RT...Retention time, Nr...Numbers referring to the identification in the chromatograms, SUA...Shoot measured, undamaged, SDA...Shoot measured, damaged aboveground by arthropod feeding and infestation of mildew, SDB...Shoot measured, damaged aboveground by arthropod feeding and infestation of mildew with additionally root feeding of *M. hippocastani*, RDA...Root measured, plant undamaged, RDB... Root measured, shoot damaged aboveground by arthropod feeding and infestation of mildew (only in *Quercus* sp.) with additionally root feeding of *M. hippocastani.*

Quercus						*Aesculus hippocastanum*						
SUA	**SDA**	**SDB**	**RDA**	**RDB**	**Compound**	**RT**	**Nr**	**SUA**	**SDA**	**SDB**	**RDA**	**RDB**
X	X	X			3-hexenyl acetate	10,62	1	X	X	X		
X	X	X	X	X	6-methyl-5-hepten-2-one	11,04	2	X	X	X	X	X
X	X	X			3-hexen-1-ol	11,7	3	X	X	X		
X	X	X	X	X	nonanal	11,97	4	X	X		X	X
O	X				2,6-dimethyl-1,3,5,7-octatetraene	18,64	5	O	X			
O					3-hexenyl butyrate	13,06	6	X	X	X		
X	X	X	X	X	2-ethyl-1-hexanol	13,48	7	X	X		X	X
X	X		X	X	decanal	13,74	8	X	X	X	X	X
X	O	X	X	O	hexadecane	15,17	9	X				O
O	O				germacrene D	17,25	10	O	X	X		
O	X				2,6-dimethyl-3,5,7-octatriene-2-ol	18,64	11					
X	X	X	X	X	isopropyl laurate	18,79	12	X	X	X	X	O
X	X	X	X	X	geranyl acetone	19,19	13	X	X	X	X	O
	O				1,8-cineol	8,82	14			X	O	X
	X				β-ocimene	9,4	15		X			
	X				hexyl acetate	9,8	16	X	X	X		
	X		X	X	benzyl alcohol	19,58	21	X	X	X	X	X
	O				β-caryophyllene	15,48	22	X	X	O		
	X				α-farnesene	17,63	23	X	X	O		
		X		O	2-pentanol	7,21	24					
		X	X	X	trimethyl benzene	10,1	25				X	
		X			2-butoxy ethanol	12,11	26					
		X	O		ethylic acid	13,17	27					
		O			β-bourbonene	14,61	28	X	X	X		
		X			methyl salicylate	18,32	29	O	X			
			O		heptanal	8,41	30					X
			X		3-ethyl toluene	9,04	31					
			X	X	benzaldehyde	14,34	32				X	
			X		sabina ketone	16,07	33				X	X
			X		diethox ymethane	16,35	34				X	
				X	3-octanone	9,6	35				X	X
			X	X	linalool oxide	13,29	36					O
			X	X	camphor	14,22	37				X	O
				X	anisol	11,22	38					
				O	methyl benzyl ether	12,06	39					O
				X	borneol	17,02	40					X
					3-hexenyl isolvalerate	13,29	41	X	X			
					δ-cadinene	12,66	43	X	X	O		
					2-hexenyl acetate	10,94	44		X	X		
					2-hexen-1-ol	12,13	45		X	X		
					1-penten-3-ol	8,1	46			X		
					2-hexenal	9,1	47			X		
					cubebene		48			X		
					curcumene	12,82	49				X	
					cymol	9,84	50					X
					octanal	10,79	51					X
					p-methyl anisol	12,83	52					X
					thymol methyl ether	15,04	53					X

A.2 Identification of root volatiles of several potential host plants of *M. hippocastani*

Root volatiles of different potential host plants are sampled in may of 2005. Volatiles were obtained by circulating the air through adsorbent traps loaded with 1.5 mg charcoal (Daumazan sur Arize, France), by miniature pumps (Fürgut, Tannheim, Germany). The sampling time was four hours with a flow rate of 1l/min, for details see chapter 5.3.4. After elution the samples were immediately analysed by using a gas chromatograph coupled to a mass spectrometer (6890N and 5973, Agilent, Palo Alto, USA, technical information see Weißbecker et al. 2004) For chemical identification, an apolar column was used (HP-5MS, length 30 m, ID 0.25 mm, and film thickness 0.25 µm, Agilent). The GC was operating in the following temperature program: start: 40 °C, hold for 2.5 min, ramp 6.2 °C/min to 250 °C, hold for 10 min. Helium (purity 99.999%) was used as carrier gas with a flow rate of 1 ml/min. For more details see chapter 5.3.5. The volatiles were identified by comparing retention time and mass spectra with the Mass Spectral Search library of the National Institute of Standards and Technology (NIST, Gaithersburg, USA) and the database of MassFinder 3.0 software in conjunction with the library “Terpenoids and Related Constituents of Essential Oils” (Hochmuth, König, Joulain, Hamburg, Germany).

The following total ion chromatograms (figures A.3 a-h) show the volatile patterns of some plant roots, which are described in the literature as potential host plants (see chapter 1.5). They want to give an impression of the diversity of emitted volatiles by roots.

N indicates the number of samples of each plant species. Only one chromatogram of each plant is shown as an example. Within the chromatograms, peaks of volatile compounds are labelled by arbitrarily chosen numbers. A list below each chromatogram translates these numbers to the compound names. Several compounds were not detected in every single sample. Behind each name, the number of samples showing an abundance of this compound above detection threshold, is given versus the total number of samples measured.

Different y-axis scaling factors were used due to different total abundances of

emitted volatiles.

The root volatiles from all investigated plants are collected from undamaged plants. The classification was done visually. Slightly mechanical damage by digging out the roots and washing them with tap water was inevitable. The root volatile emissions differ relatively strong in their volatile patterns between plant species. Only a few number of samples of each plant are measured.

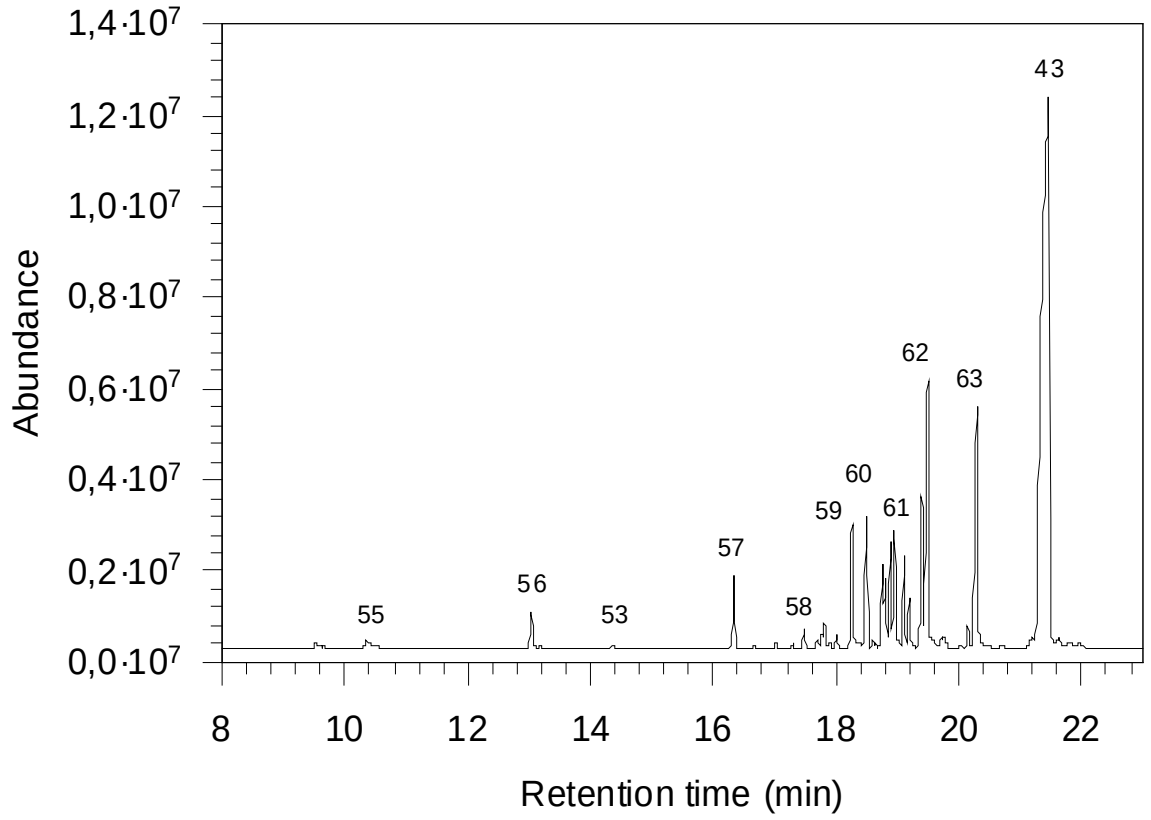

Figure A.3 a: root volatiles of *Achillea millefolium* (N=2)

55→ pseudolimonene (2/2)
56→ unidentified compound (2/2)
53→ thymol methyl ether (2/2)
57→ δ-elemene (2/2)
58→ α-cedrene (2/2)
59→ β-bisabolene (2/2)
60→ isoledene (2/2)
61→ α-elemene (2/2)
62→ β-sesquiphellandrene (2/2)
63→ cyclofenchene (2/2) ?
43→ δ-cadinene (2/2)

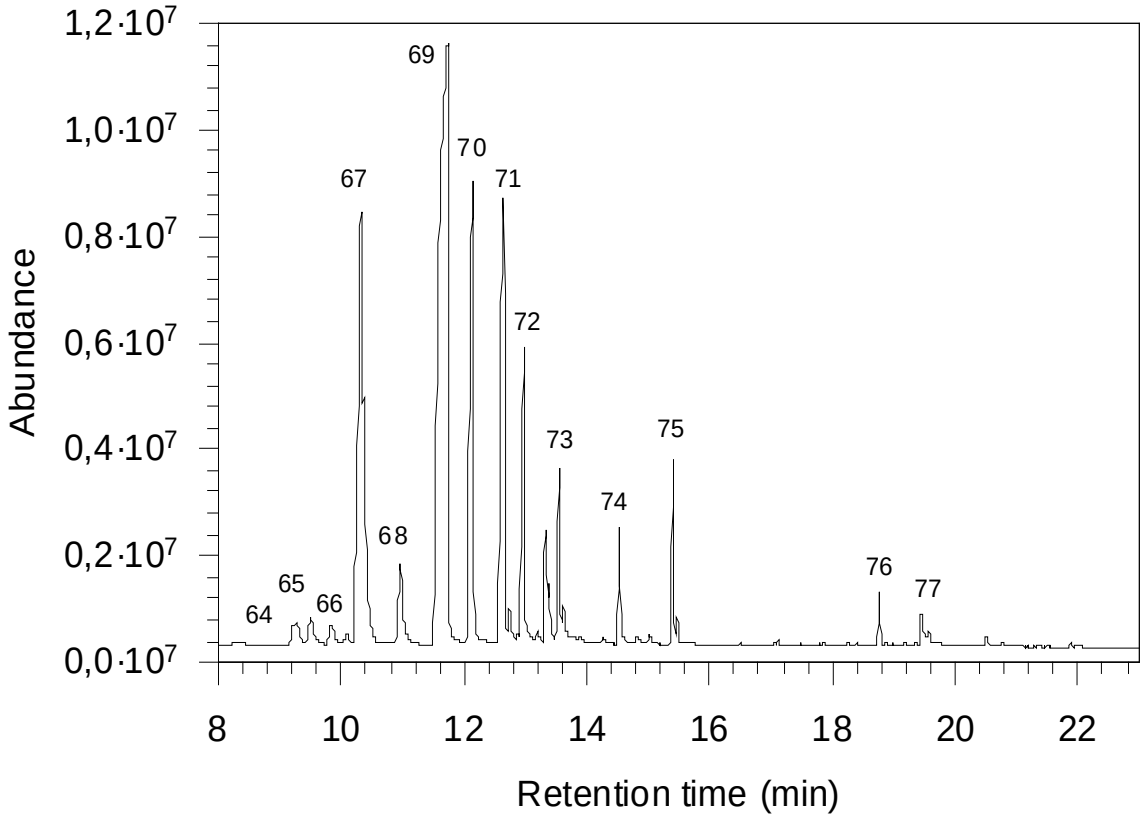

Figure A.3 b: root volatiles of *Daucus carota* (N=4)

64→ β-pinene (3/4)
65→ β-myrcene (3/4)
66→ α-phellandrene (4/4)
67→ p-cymene (4/4)
68→α-terpinene (4/4)
69→ terpinolene (4/4)
70→ 1,3,8-p-menthatriene (4/4)
71→ 5-ethyl-m-xylene (4/4)
72→ 4-isopropenyl toluene (3/4)
73→ unidentified compound
74→ 2-methyl coumaran
75→ bornyl acetate (4/4)
76→ di-epi-α-cedrene (4/4)
77→ myristicine (2/4)

There are some similarities between the volatile emissions of *Daucus carota ssp. sativus* (see chapter 3.4) and those of *Daucus carota*.

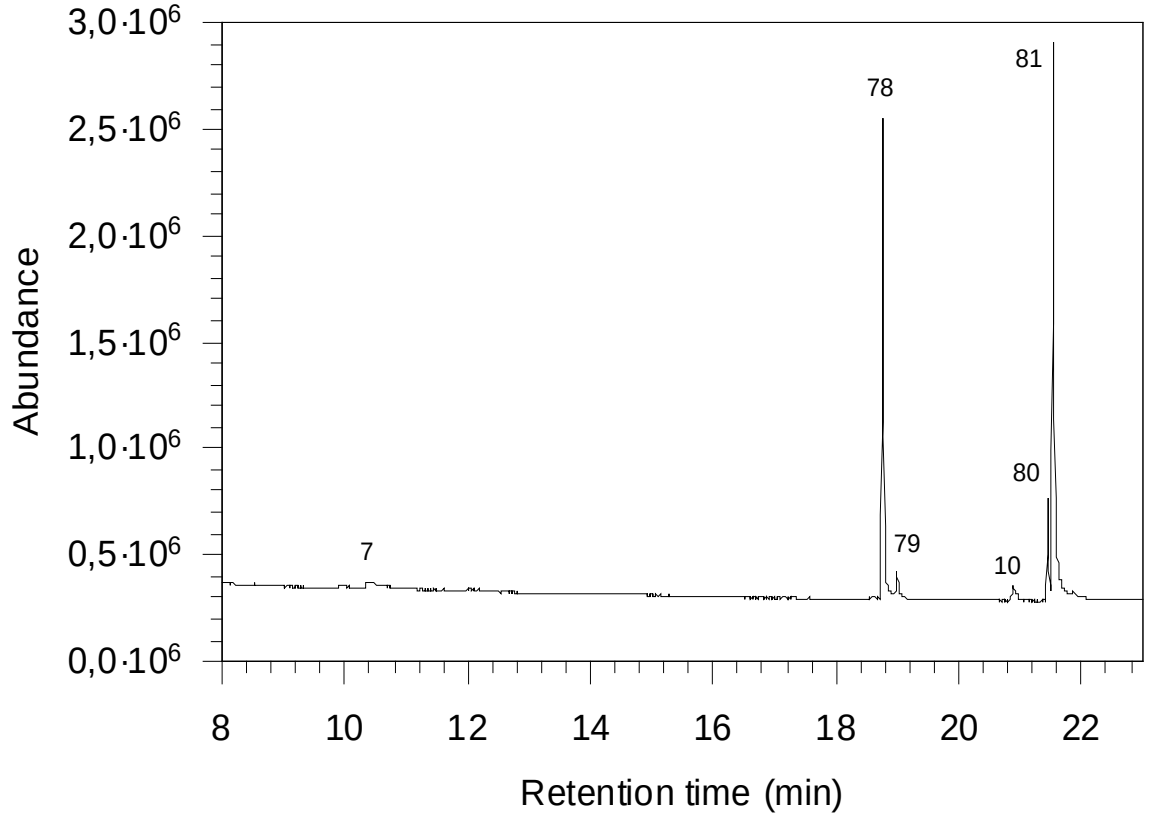

Figure A.3 c: root volatiles of *Cirsium arvense* (N=6)

7→ 2-ethyl-1-hexanol (4/6)
78→ 1-pentadecene (6/6)
79→ β-eudesmene (3/6)

10→ isopropyl laurate (2/6)
80→ unidentified compound (6/6)
81→ 7,10,13-hexadecatrienal (4/6)

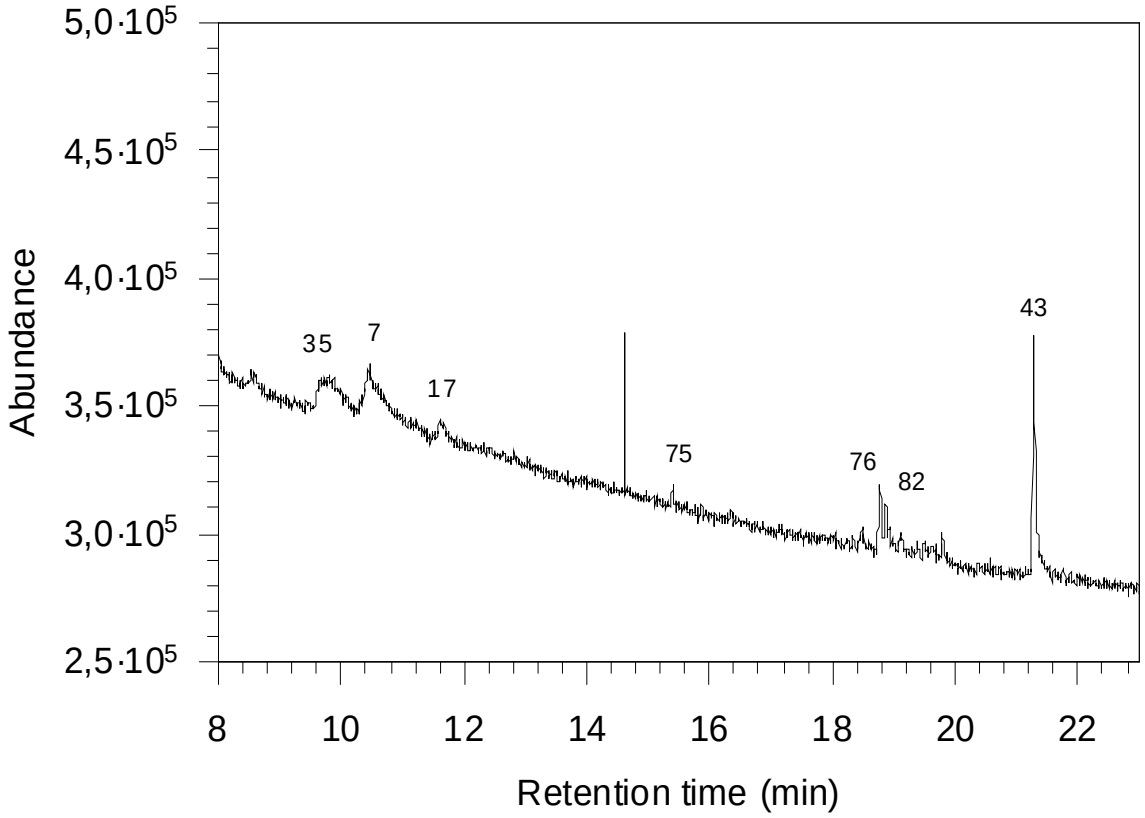

Figure A.3 d: root volatiles of *Plantago lanceolata* (N=3)

7→ 2-ethyl-1-hexanol (1/3)
17→ terpinolene (1/3)
35→ 3-octanone (2/3)
43→ δ-cadinene (1/3)
75→ bornyl acetate (2/3)
76→ di-epi-α-cedrene (1/3)
82→ β-farnesene (1/3)

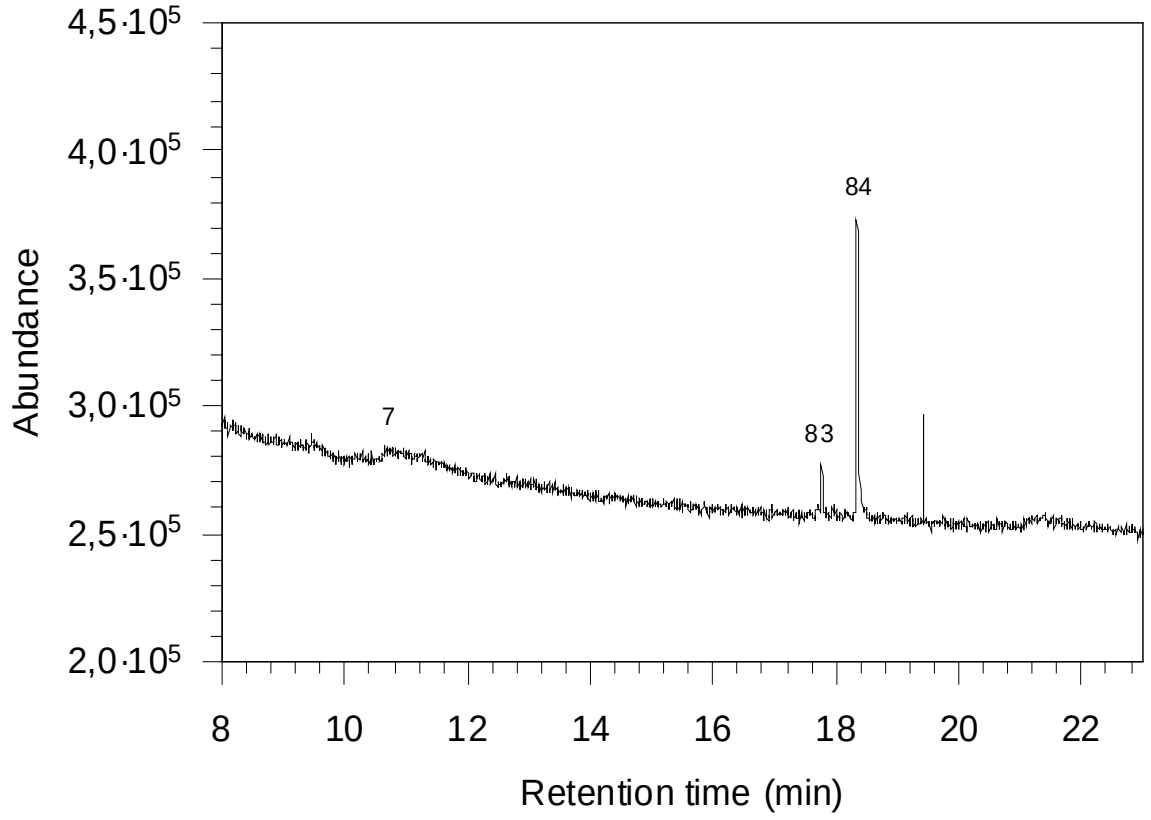

Figure A.3 e: root volatiles of *Taraxacum officinale* (N=2)

7→ 2-ethyl-1-hexanol (1/2)
83→ 6-camphenol (1/2)
84→ unidentified compound (2/2)

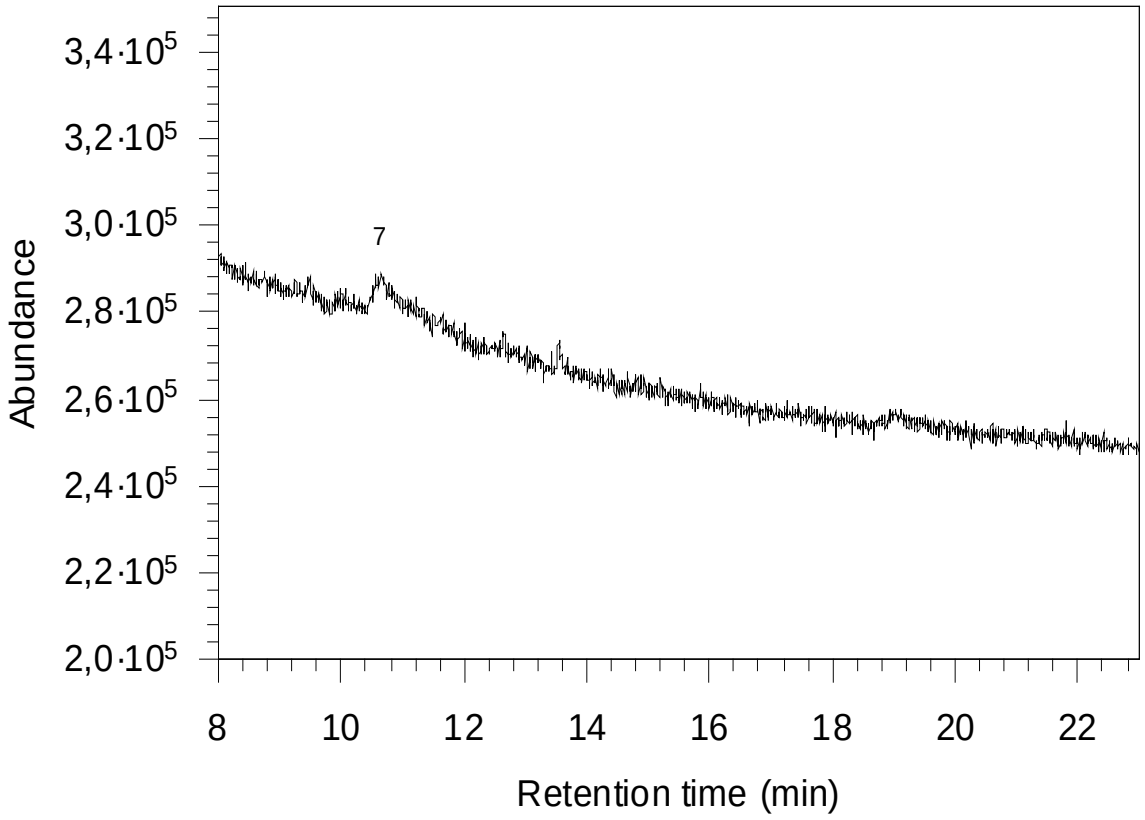

Figure A.3 f: root volatiles of *Solanum tuberosum* (N=9)

7→ 2-ethyl-1-hexanol (9/9)

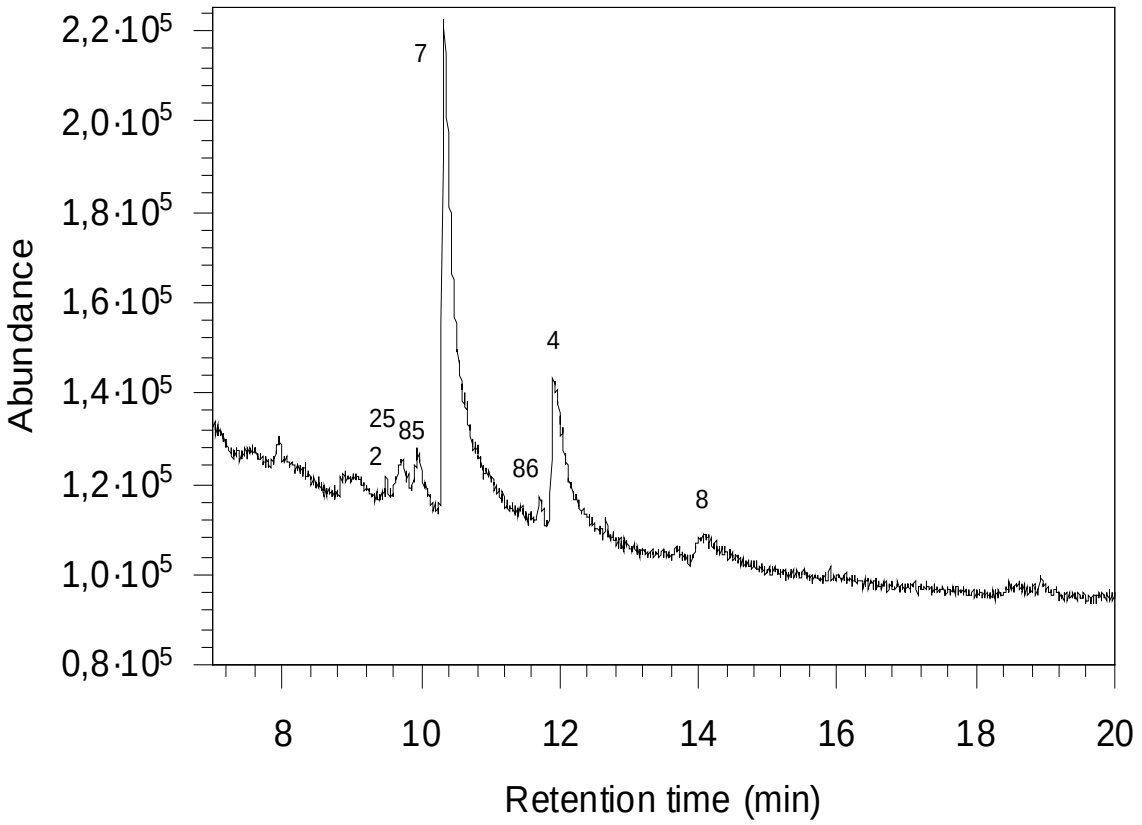

Figure A.3 g: root volatiles of *Solanum tuberosum*, damaged by larval feeding of *M. hippocastani* (N=8)

2→ 6-methyl-5-hepten-2-one (4/8)
4→ nonanal (8/8)
7→ 2-ethyl-1-hexanol (8/8)
8→ decanal (7/8)
25→ trimethyl benzene (2/8)
85→ octanal (6/8)
86→ undecane (3/8)

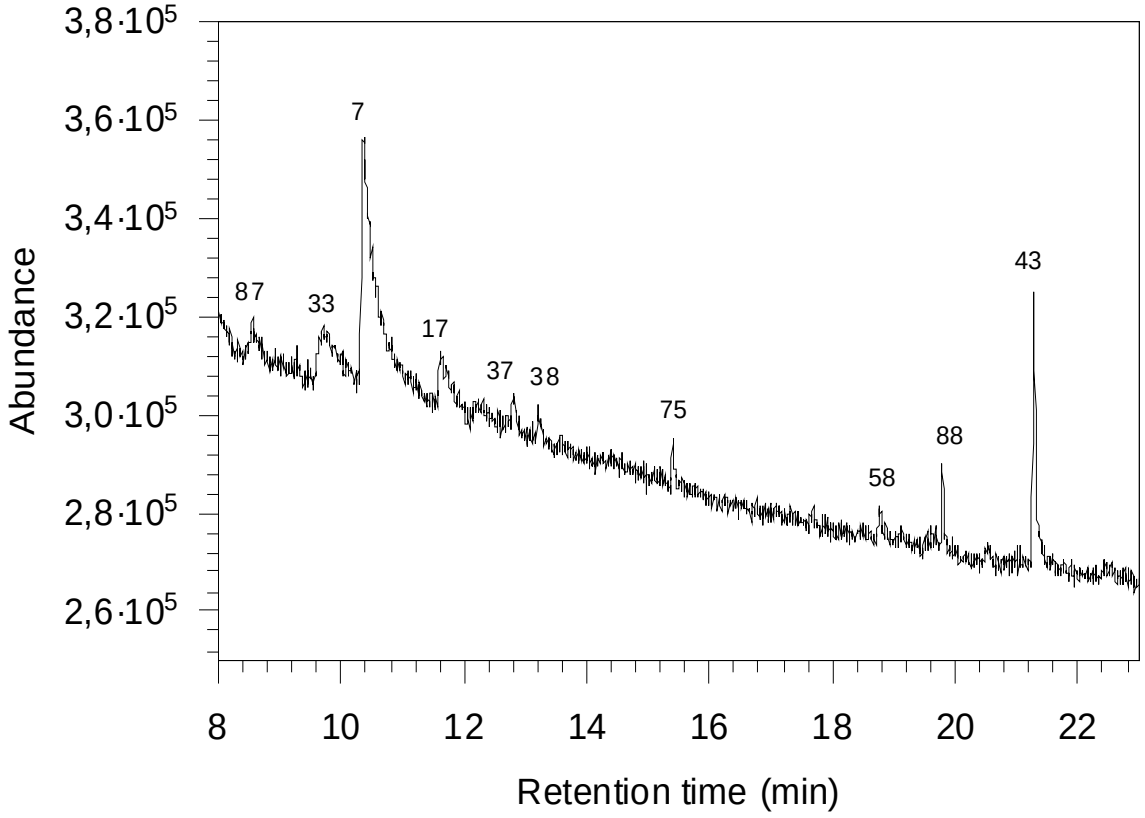

Figure A.3 h: root volatiles of *Calamagrostis* sp. (N=1)

7→ 2-ethyl-1-hexanol (1/1)
17→ terpinolene (1/1)
33→ 3-octanone (1/1)
37→ camphor (1/1)
40→ borneol (1/1)
43→ δ-cadinene (1/1)
58→ α-cedrene (1/1)
75→ bornyl acetate (1/1)
87→ unidentified compound (1/1)
88→ unidentified compound (1/1)

Calamagrostis sp. is not described as a preferred host plant of *Melolontha* sp. larvae, but as a weed, as it is mentioned by Hauss & Schütte (1976), which is able to reduce the mortality of larvae in the first larval stage. Further investigations are necessary to obtain more data about the emitted volatiles of potential host plants of *M. hippocastani* larvae. However, because of the small number of some plants (especially in *Achillea millefolium*, *Daucus carota*, *Plantago lanceolata*, *Taraxacum officinale* and in particular *Calamagrostis* sp.), the obtained data have to be handled with care.

A.3 Further notes on the detected VOCs in roots of *Quercus* sp.

In this sub-chapter I want to highlight the volatile compounds detected in the volatile emission of oak root samples in their context in insect-plant relation as discussed in the literature.

Several of the volatile compounds detected in the treatment “roots of *Quercus* sp. damaged by larval feeding” were tested upon their electrophysiological, antennal response (see chapter 4.4.2) and upon their behavioural effect on the larvae (see chapter 4.4.5). The volatile compounds are discussed sequentially following the order in table 5.4.1.

The alcohol **1-octen-3-ol** and the keton **3-octanone** as representatives of the eight-carbon volatiles are supposed to be typical indicators for fungal growth. Both volatiles have a sweet aroma, but whereas 1-octen-3-ol smells like mushroom, 3-octanone remembers of fruits, lavender, smelling musty/mouldy (Combet et al 2006).1-octen-3-ol, if associated with acetone or carbon dioxide, acts as an attractant for several biting flies (see also chapter 1.6). 1-octen-3-ol appeared only in some of the samples, therefore it was not tested in the electrophysiological and behavioural experiments. 3-octanone was present in almost all samples damaged by larval feeding. The larvae of *M. hippocastani* were neither attracted nor repelled in the behavioural tests from 3-octanone.

The ketone **6-methyl-5-hepten-2-one** possibly acts as a marker of basidiomycetes, found also in soil samples collected 2005 (Weissteiner, unpublished data). It is described as an alarm pheromone as well, which was found to significantly decrease the percentage of egg hatching and increase the larval mortality of *Spodoptera littoralis* (Emara 2004). As it did not appear in the roots damaged by larvel feeding and it did not cause antennal response in the GC-MS/GC-EAD-experiments, this compound was tested neither in the electrophysiological nor in the behavioural experiments.

The potential marker of microorganisms **2-ethyl-1-hexanol** appears only in mechanical damaged oak-roots and could in this case be rather an indicator of enhanced cell respiration and degradation because of the mechanical infraction. It is also known as an anthropogenic volatile. Moreover, it is described to play a

role in plant-insect interaction in the Mediterranean fruit fly *Ceratitis capitata* W. (Gonçalves et al. 2006). In the experiments the electrophysiological and the behavioural responses to 2-ethyl-1-hexanol were not tested.

1,8-cineol is a very well investigated component in different plants, e.g. *Eucalyptus* sp. and *Rosmarinus officinalis* and can act as a natural pesticide (Batish et al. 2008 and references therein). It has significant bioactivity as mosquito feeding deterrent and ovipositional repellent (Klocke et al. 1987), and in higher concentration is repellent and toxic against stored-grain beetles (Obeng-Ofori et al. 1997). The christmas beetles *Anoplognathus* spp. (Scarabaeidae) choose an exotic plant species (*Schinus molle*) instead of potential *Eucalyptus* host plants, which could be related to the absence of 1,8-cineol (Steinbauer & Wanjura 2002). The phytochemistry of *Eucalyptus* spp. and its role in insect-host-tree selection was studied by Li (1993). An antimicrobial effect was shown e.g. by Trivedi & Hotchandani (2004) and Hendry et al. (2009), antiviral effects were shown by Schnitzler et al. (2001), and an acaricidal activity is described by Saad et al. (2006). In addition, an antifungal (Zuzarte et al 2009) and a nematicidal activity (Ibrahim et al. 2006) of eucalyptus oil could be confirmed. However, in the literature 1,8-cineol is described as an attractant for the banana weevil *Cosmopolites sordidus* as well (Ndiege et al. 1996). In our experiments, the concentration 10^{-2} had a clear attractant effect on the behaviour of *M. hippocastani* larvae, as well as the concentration 10^{-4}.

In the study of Ômura et al. (2000) **furanoid trans-linalooloxide** elicited relatively strong EAG responses in the white cabbage butterfly *Pieris rapae crucivora* B.. The volatile compound acted as weak deterrent in the Proboscis Extension Reflex (PER, Laloi et al. 1999) and as weak repellent in flower-visiting tests (*Osmanthus fragrans*) as well. In other EAG experiments in female and male mosquitos, furanoid trans-linalooloxide evoked the strongest response among the tested compounds (Jhumur et al. 2008). The furanoid trans-linalooloxide has neither an attractant nor a repellent effect on the behaviour of the larvae of *M. hippocastani.*

Nonanal and **decanal** are among others indicator substances for degradation processes. So decanal can be detected during the senescence of leaves (Schütz 2001). Nonanal and decanal were found in increased abundances in the laboratory air during the measurement process. This may originate from the

measurement setup because all the work (preparing and the measurement itself) is done in the laboratory. Decanal is also described to be induced by mechanical and herbivore damage (Schütz et al. 1997, Weißbecker et al. 1999, Dicke et al. 2003). In EAG experiments by Chinta et al. 1994, nonanal and GLVs were the most active odourants tested in females and males of *Lygus lineolaris* P. Nonanal and decanal were tested neither in the electrophysiological nor in the behavioural experiments.

Camphor is a chiral compound with (1R)-camphor and (1S)-camphor as the two possible enantiomeres. Roller et al. and Zuzarte et al. (both 2009) mentioned that camphor has antimicrobial and antifungal properties. Arakaki et al (2009) found in behavioural experiments that (1R)-camphor was an attractant for the Scarabaeidae *Protaetia pryeri pryeri*. Donkin (1999) described (1R)-camphor as the dominant form in natural plant products. In In the behavioural experiments performed in this work, neither (1R)-camphor nor (1S)-camphor had an attractive or repellent effect in both concentrations 10^{-2} and 10^{-4} on the choice behaviour of the larvae.

The terpene **borneol** can be synthesized by reduction of camphor, and it is a component of many plants (e.g. *Achillea millefolium* L., *Salvia officinalis* L., Duke 1992). In *Aedes aegypti* L. the oviposition rate increased, if borneol and camphor were present (Waliwitiya 2009). Borneol is known as an insect repellent as well. The electrophysiological and the behavioural responses to borneol were not tested in the thesis.

The alcohol **anisol** (also called methoxybenzene) was detected by Vrkočová et al. (2000) in high contents of *Quercus robur* twigs, if they were attacked by females and males of *Scolytus intricatus* by maturation feeding. In the behavioural experiments, anisol was attractive for the larvae of *M. hippocastani* in the concentration 10^{-2}, but not in the concentration 10^{-4}.

The terpene **geranyl acetone** is a wound response “alarm” volatile that functions as an attractant to herbivore natural enemies (Sing et al. 2005). It potentially derived from phytoene, phytofluene or carotene (Simkin et al. 2004). The compound could be found in volatile emissions of the dalmatian toadflax *Linaria dalmatica* L., if they were infested with the larvae of the curculionid *Mecinus janthinus* G., whereas in uninfested plant emissions was absent.

Geranyl acetone is common in volatile blends from other plants (like *Rosa*, Dobson et al. 1987) and animals (Chung & Cadwallader 1993). Takáks et al. (1997) showed in their experiments that the 1 : 1 mixture of nonanal and geranyl acetone, but not the single compounds alone was as attractive as the volatile emissions of infested beaver belt with the larvae of the casemaking clothes moth *Tinea pellionella* L. for the braconid parasitoid *Apanteles carpatus*.

Jumean et al. showed 2004 that a blend of synthetic (E)-2-octenal, (E)-2-nonenal, sulcatone, and geranyl acetone, in combination with either 3-carene and/or three saturated aldehydes (octanal, nonanal, decanal), elicited behavioral responses from *Cydia pomonella* larvae.

Anopheles gambiae antennae showed strong EAG response among the tested volatiles (indole, 3-methyl indole and p-cresol) (Biessmann et al. 2010).

Geranyl acetone was tested neither in the electrophysiological nor in the behavioural experiments.

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Danksagung

Ich möchte mich ganz herzlich bei der Bischöflichen Studienförderung, dem Cusanuswerk für die finanzielle und ideelle Unterstützung meiner Promotion durch ein Stipendium bedanken. Die zahlreichen Tagungen und Workshops ermöglichten darüberhinaus eine willkommene Abwechslung zum Labor- und Büroalltag sowie einen ungemein bereichernden “Blick über den eigenen Tellerrand hinaus in die Welt”.

Mein Dank gilt Herrn Prof. Dr. Stefan Schütz, für die Aufnahme in die Gruppe, die Arbeitsmöglichkeiten mit modernstem Messequipment in sehr gut ausgestatteten Labors und sein allzeit offenes Ohr. Besonders bedanken möchte ich mich bei Herrn Dr. Bernhard Weißbecker, für seine zuverlässige und kompetente Hilfe bei allen technischen sowie fachlichen Problemen.

Ein ganz besonders herzlicher Dank geht an alle Mitarbeiter der Abteilung “Forstzoologie und Waldschutz, einschließlich Wildbiologie und Jagdkunde”, hier vor allem an Ulrike Eisenwiener, Kira Duntemann, Miriam Rameckers, Marina Horstmann, Stefan Rath, Reinhold Dankworth, Jörg Berger, August Capelle, Dr. Sergio Angeli, Dr. Prodpran Thakeow, Dr. Pavel Plasil, Gerrit Holighaus, Michael Ksinsik, Monique Weidner, Sara Nicke, Maria Vlaic, Martin Scholz, Marta Paczkowska, Julian Heiermann und Dr. Bettina Johne für das wirklich sehr angenehme Arbeitsklima, die vielen aufschlussreichen Diskussionen, nützlichen Ratschläge sowie die unvergesslichen Erlebnisse außerhalb des Instituts. Stefan und Michael, Ihr fehlt!
Ebenso möchte ich mich bei Jessica Gross, Andrea Möller und Henrik Ziegenhagen für die tolle Zusammenarbeit und dem teilweisen Mitwirken an meiner Arbeit bedanken.

Bei Dr. Roberto Romani, Universitá di Perugia und Prof. Dr. Joachim Schachtner, Universität Marburg bedanke ich mich ganz herzlich für die sehr angenehme Zusammenarbeit und ihren Beitrag an dieser Arbeit.

Bedanken möchte ich mich auch bei Frau Monika Göbel und Herrn Michael Göbel (Revierförsterei Griesheim) für Ihre Hilfe beim Ausgraben der Maikäferlarven. Weiters bei den Mitarbeitern der Revierförsterei Ebergötzen, für die Möglichkeit, die Eichen aus dem Wald holen zu dürfen. Volker Meng (Forstbotanischer Garten, Göttingen) stand jederzeit für gartenbauliche Fragen zur Verfügung.

Ein ganz besonderer Dank gilt an dieser Stelle Herrn Prof. Dr. Wolfgang Schaller, der nicht nur sprachliche Vorschläge zur Verbesserung der Arbeit gemacht hat.

Bei Frau Christa Rogalla und Herrn Hans-Jürgen Rogalla bedanke ich mich ganz herzlich für die Zeit, die sie mit Florian verbracht haben.

Frau Elke Schulz danke ich ganz besonders herzlich für ihr Da sein, ihre Ruhe und Geduld in turbulenten Tagen.

Mein allergrößter Dank gilt meiner Familie. Meinen Eltern Rosa und Walter Weissteiner für ihre Liebe, ihr Verständnis und ihre Unterstützung bei all meinen Entscheidungen. Meiner “einigen” kleinen Familie Matthias und Florian für ihr Verständnis, ihre Nachsicht und Geduld. Danke, Matthias, für Deine große Hilfe, Deinen Einsatz und Beistand besonders aber nicht nur in der letzten Phase!