Perception of host plant volatiles contributes to an optimised strategy for reproduction in the European grapevine moth, *Lobesia botrana*

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Par

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IMPRIMATUR POUR LA THESE

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„Die Natur ist das einzige Buch,
das auf allen Blättern großen Gehalt bietet”

J. W. v. Goethe

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Summary

The European grapevine moth *Lobesia botrana* is one of the most damaging insect pests of grapevine. The larvae feed on flower buds and fruits and the damage they cause favours the development of bacteria and fungi. In the absence of adequate control measures *L. botrana* can cause big loss at harvest. Mating disruption is a semiochemical-based control method for *L. botrana* but has to become more reliable and cost-efficient. Host plant volatiles hold promise for improving mating disruption of *L. botrana*.

For phytophagous insects the ability to perceive plant compounds is important since plant volatiles lead the insects to a suitable host plant, which can serve as an oviposition-, a feeding- and a mating site. It has also been shown that the sexual behaviour of phytophagous insects is often linked with their host plants. Host plant volatiles were found to enhance insect responses to sex pheromones. Our hypothesis is that the grapevine moth males use volatile host plant cues in mate-finding behaviour, and that the perception of these plant products contributes to an optimised strategy for reproduction.

Using gas chromatography linked electroantennogram detection 15 plant volatiles were identified that elicit electroantennogram responses in grapevine moth males and are common to at least three different host plant headspace collections. In a wind tunnel the attractiveness of underdosed and optimal sex pheromone levels to *L. botrana* males was increased when the pheromone was presented in a background of volatile emissions of vine plants, with more males contacting the pheromone source than with the pheromone alone. Two-component blends of sex pheromone and of either (E)-β-caryophyllene, 1-hexanol, (Z)-3-hexenyl acetate or 1-octen-3-ol had strong effects on the attractiveness of the pheromone by increasing the entire male behavioural sequence from activation to source contact. (E)-β-caryophyllene was also biologically active in the field, in that traps emitting (E)-β-caryophyllene and pheromone captured more grapevine moth males than traps emitting only the pheromone. Furthermore, *L. botrana* males engaged upwind flights to single plant volatiles in the absence of pheromone products. (E)-β-caryophyllene, 1-hexanol, (Z)-3-hexenyl acetate or 1-octen-3-ol elicited oriented upwind flights when presented singly in the wind tunnel with behavioural responses

persisting down to release rates at biological relevant picogram levels. Blending (E)-β-caryophyllene and 1-hexanol had a strong synergistic effect on male response.

Overall, our findings demonstrate the importance of plant volatiles in the biology of *L. botrana* and the new insights suggest that plant volatiles hold great promise for improving mating disruption of *L. botrana*.

Keywords: Insect-plant interaction, mate-finding, sex pheromone, host plant, plant volatiles, behaviour, electroantennogram, mating disruption, (E)-β-caryophyllene, European grapevine moth, *Lobesia botrana*

Résumé

Le ver de la grappe, eudémis, *Lobesia botrana* est un des insectes causant le plus de dommages sur la vigne. Les larves se nourrissent des boutons floraux et des grappes causant ainsi des dégâts qui favorisent le développement d'infections bactériennes et fongiques. Sans mesures de contrôle adéquates, les pertes de production de la vigne peuvent être énormes. La technique de confusion sexuelle basée sur la phéromone sexuelle de *L. botrana* est couramment employée dans les vignobles suisses. Elle présente cependant des faiblesses face à des hautes populations d'eudémis et génère des coûts élevés. A ce titre, les composés volatiles des plantes hôtes présentent un intérêt particulier afin d'améliorer la technique de confusion sexuelles chez *L. botrana*.

Les composés volatiles des plantes guident les insectes vers une plante hôte appropriée. Celle-ci fournit à l'insecte une source de nourriture, mais aussi un endroit idéal pour la reproduction suivie de l'oviposition. Il a aussi été démontré que le comportement sexuel des insectes phytophages est souvent lié à leur plantes hôtes. Il a également été montré que les composés volatiles des plantes hôtes amplifient la sensibilité des insectes aux phéromones sexuelles. En conséquence, notre hypothèse est que les mâles utilisent les composés volatiles des plantes hôtes comme indice afin de mieux pouvoir localiser les femelles. La perception de ces composés devrait contribuer à une optimisation de leur stratégie de reproduction.

Nous avons analysé cinq collections d'émissions volatiles provenant de quatre plantes hôtes avec l'electroantennographie liée à un chromatographe en phase gazeuse. Nous avons ainsi pu identifier 15 composés volatiles de plantes hôtes qui sont perçus par les cellules olfactives des L. botrana males et présents dans au moins trois différentes collections d'émissions. En chambre de vol, l'ajout de composés volatiles émis par les plantes hôtes a augmenté l'attraction des mâles à des niveaux de phéromone sexuelle sous-dosé ou optimal. Les mâles ont montré une attraction plus élevée à la phéromone sexuelle en présence des émissions volatiles de la vigne. Des mélanges synthétique constitués de deux composants, contenant toujours la phéromone sexuelle accompagnée soit de (E)-β-caryophyllene, de 1-hexanol, de (Z)-3-hexenyl acetate ou de 1-octen-3-ol, ont augmenté l'attractivité de la phéromone en amplifiant entièrement la séquence de comportement entièrement depuis l'activation jusqu'au

contact avec la source d'odeur. Les effets du (E)-β-caryophyllene ont également pu être démontrés en milieu extérieur. Les pièges émettant du (E)-β-caryophyllene accompagnés de la phéromone ont capturé plus de mâles que lorsqu'ils émettaient uniquement la phéromone. En chambre de vol, les mâles ont aussi montré une forte attraction aux composés volatiles seuls sans la phéromone. Le (E)-β-caryophyllene, le 1-hexanol, le (Z)-3-hexenyl acetate ou le 1-octen-3-ol ont induit un vol en direction de la source d'odeur. Cette attraction a persisté jusqu'à des concentrations émises de l'ordre du picogramme, montrant ainsi leur importance biologique. En particulier, un mélange de (E)-β-caryophyllene et 1-hexanol a eu un effet synergétique fort sur la réponse des mâles.

En conclusion, nos découvertes démontrent l'importance des composés volatiles émis par les plantes hôtes dans la biologie de *L. botrana*. Nos résultats offrent de nouvelles perspectives sur l'amélioration des techniques de confusion sexuelle en vue de la diminution des effets néfastes de ces insectes ravageurs de la vigne.

Mots clés: Interaction insecte-plante, recherche de partenaire sexuel, phéromones sexuelles, plant hôte, composés volatiles de plantes, comportement, électroantennographie, technique de confusion, (E)-β-caryophyllene, vers de la grappe, eudémis, *Lobesia botrana*

Zusammenfassung

Der bekreuzte Traubenwickler, *Lobesia botrana*, ist einer der Hauptschädlinge in Europas Weinkulturen. Die Larven fressen auf Blütenknospen und Trauben und fördern damit die Verbreitung von Bakterien und Pilzen, was zu ernsthaften Ernteausfällen führt, wenn wirksame Bekämpfungsmassnahmen fehlen. Eine häufig verwendete Bekämpfungsmethode in Schweizer Rebbergen ist die Verwirrungs-Technik, welche auf der Verwendung des Sexualpheromons des bekreuzten Traubenwicklers basiert. Um weiter mit herkömmlichen Insektiziden konkurrieren zu können, muss die Bekämpfunsmethode aber effizienter und kostengünstiger werden. Die Integrierung von Pflanzenduftstoffen in Pheromon-Dispenser könnte die bestehende Verwirrungs-Technik verbessern.

Die Fähigkeit Pflanzenduftstoffe wahrzunehmen ist für phytophage Insekten von grosser Bedeutung, da Pflanzenduftstoffe die Insekten zu geeigneten Wirtspflanzen führen, welche entweder Nahrung, Orte für die Reproduktion oder Oviposition darstellen. Weiter ist bekannt, dass Pflanzenduftstoffe Verhaltensreaktionen zu Sexualpheromonen verstärken können. Unsere Hypothese ist, dass Männchen des bekreuzten Traubwicklers neben dem Sexualpheromon auch Pflanzenduftstoffe zur Lokalisierung von Weibchen benützen und dass die Wahrnehmung von Pflanzenduftstoffen die Wahrscheinlichkeit sich fortzupflanzen erhöht.

Fünf Duftstoffemissionen von vier Wirtspflanzen wurden mittels Gaschromatographie gekoppelt mit Elektroantennographie analysiert. 15 Pflanzenduftstoffe wurden gefunden, welche von olfaktorischen Rezeptorzellen in *L. botrana* Antennen wahrgenommen wurden und in mindestens drei Duftstoffemissionen enthalten waren. In einem Windkanal zeigte sich eine erhöhte Attraktivität von unterdosierten und optimalen Pheromon-Levels, wenn das Pheromon mit Emissionen von Weinpflanzen kombiniert wurde. Die Beigabe von den Pflanzenduftstoffen (E)-β-Caryophyllen, 1-Hexanol, (Z)-3-Hexenyl acetat und 1-Octen-3-ol zum Pheromon führte zu einer höheren Anzahl von Traubenwickler, welche zur Duftquelle hin flogen und steigerten signifikant die Attraktivität des Pheromons. (E)-β-caryophyllen war auch in Feldversuchen biologisch aktiv. Fallen, welche Pheromone- und (E)-β-Caryophyllen-Dispenser beinhalteten, fingen mehr Traubenwickler Männchen als Fallen, welche nur mit Pheromon-Dispensern ausgestattet waren. Des Weiteren wurden Flüge von Traubenwickler

Männchen zur Duftquelle hin beobachtet, wenn entweder (E)-β-Caryophyllen, 1-Hexanol, (Z)-3-Hexenyl acetat oder 1-Octen-3-ol alleine als Stimulus präsentiert wurden. Synergistische Effekte wurden beim mischen von (E)-β-Caryophyllen and 1-Hexanol gefunden, da signifikant mehr Traubenwickler Männchen auf der Duftquelle landeten als wenn die Pflanzenduftstoffe einzeln präsentiert wurde.

Zusammenfassend zeigen unsere Resultate die wichtige Rolle welche Pflanzenduftstoffe in der Biologie des bekreuzten Traubenwicklers einnehmen. Diese neuen Erkenntnisse sind ein weiterer Schritt zu einer Integrierung von Pflanzenduftstoffen in bestehende Verwirrungs-Techniken, welche nur auf dem Sexualpheromon allein basieren.

Schlagwörter: Insekten-Pflanzen Interaktion, Sexualpheromon, Wirtspflanze, Pflanzen-duftstoffe, Verhalten, Elektroantennogram, Verwirrungs-Technik, (E)-β-Caryophyllen, bekreuzter Traubenwickler, *Lobesia botrana*

CHAPTER 1

General Introduction

1.1 Insect – plant interactions

Animal life, including that of insects, depends to a large extent on green plants, which serve as the primary source of energy-rich compounds for heterotrophic organisms. Nearly half of all existing insect species feed on living plants. Thus, more than 400'000 phytophagous insect species live on roughly 300'000 vascular plant species. One of the most striking features of insect-plant relationships is the high degree of food specialization among herbivorous insects. This phenomenon defines the core of these relationships. Insects that in nature occur on only one or a few closely related plant species are called monophagous (Bernays and Chapman, 1994). Oligophagous insects, such as the cabbage white butterfly *Pieris brassicae* and the Colorado potato beetle *Leptinotarsa decemlineata*, feed on a number of plant species, all belonging to the same plant family, the Brassicacea and the Solanaceae, respectively (Feltwell, 1982; Jacques, 1988). Polyphagous insect species seem to exercise little choice and accept many plants belonging to different plant families. However, to reveal how phytophagous insects locate and identify suitable plants from a distance in the natural environment where host and non-host plants grow side by side is a great challenge for the today's researchers. The important role that volatile chemicals play in this relationship is well accepted but how phytophagous insects filter out, code or interpret complex sensory stimuli into specific signals of behavioural relevance remains unclear.

Insect-plant interactions are of crucial importance from an applied point of view. Insects remain the major pests of crops and stored products, despite expensive and environmentally hazardous control measure. There is an irrefutable need to better understand the factors

governing the relationship between insects and plants. Such knowledge is fundamental in attempting to create biologically safe control strategies intended to prevent insect pest outbreaks.

1.2 The European grapevine moth *Lobesia botrana*

The grapevine moth *Lobesia botrana* (Denis and Schiffermüller; Order Lepidoptera, Family Tortricidae, Subfamily Olethreutinae) is one of the most damaging insect pests of grapevine (Fig. 1. 1). The grapevine moth occurs throughout the European wine-growing area as well as in adjacent Mediterranean countries of North Africa and Asia Minor (Gabel and Roehrich, 1995). Grapevine moth larvae can cause big loss at harvest in the absence of adequate control measures. The larvae feed on flower buds and fruits and the damage they cause favours the development of bacteria and fungi, especially the grey mould *Botrytis cinerea*. In Switzerland, the grapevine moth was first discovered in the Valais in 1880 and first damage was observed in 1910 (Bovey et al., 1972). The grapevine moth is a polyphagous insect, not limited to the grapevine as a host plant, and it can develop on plants belonging to different families (Katerinopoulos et al., 2005; Thiery and Moreau, 2005). In Mediterranean regions up to 4 generations can occur per year where climatic conditions are suitable. In Switzerland the emergence of hibernating pupae takes place from the end of April and beginning of May when first generation moths are on the wing to find a mating partner and lay eggs (Fig. 1. 2). The females lay their eggs (around 40 – 50 per female) on flower buds and pedicels of the host plant.

Fig. 1. 1: *Lobesia botrana* larva and adult on vine *Vitis vinifera*.

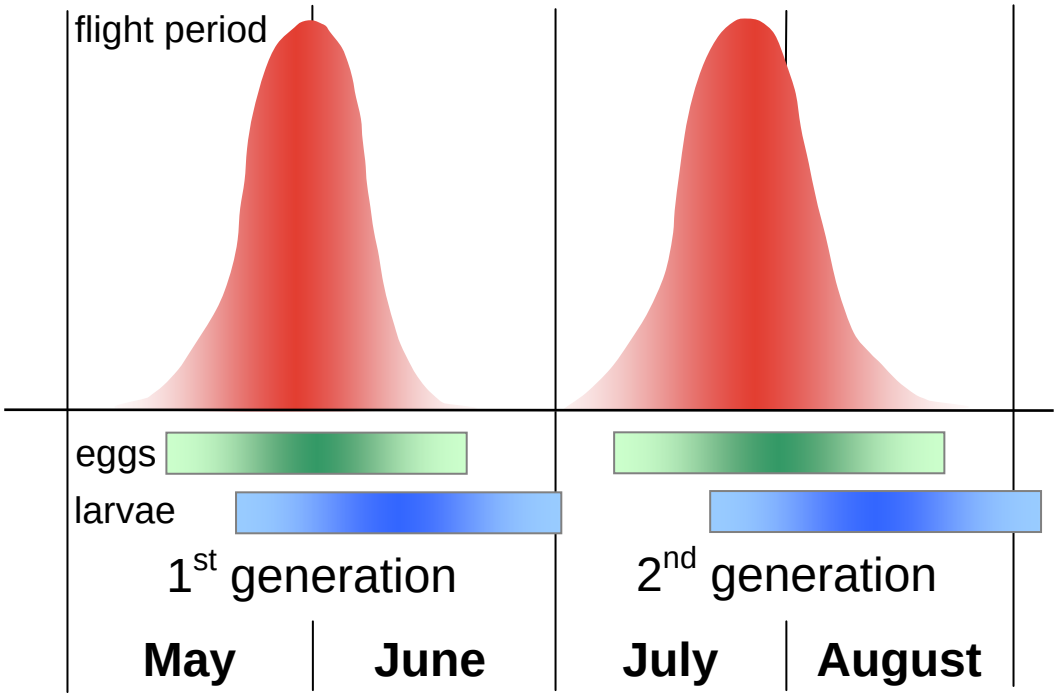


Fig. 1. 2: Phenology of *L. botrana* in Switzerland. 2nd generation larvae normally pupate in September and hibernate until end of April the next year.

After 7 – 11 days the larvae hatch and begin to feed on the flower buds. After developing through the five larval stages, which takes between 21 - 28 days, the larvae enter pupation. After 10 - 14 days, normally in the middle of July, adults of the second generation emerge. Their flight lasts around 10 – 20 days and they lay their eggs on grapes. Because of the increased temperature the larvae already hatch after 4 – 6 days. They develop inside the grape and hibernate as pupae. Under optimal conditions sometimes a third generation emerges in Switzerland and the flight occurs at the end of August (Bovey et al., 1972).

1.3 Insect sensory ecology

Semiochemicals

Semiochemicals (Greek: semeon, a signal) are chemicals that mediate interactions between organisms. Semiochemicals are subdivided into allelochemicals and pheromones depending on whether the interactions are interspecific or intraspecific, respectively (Klowden, 2002). Allelochemicals are chemicals that are significant to individuals of a species different from the source species. Allelochemicals are subdivided into several groups depending on the

benefit for the emitter and the receiver of the chemical. One group of allelochemicals, the kairomones, comes into play in the context of grapevine moths using host plant volatiles in mate-finding behaviour. A kairomone is defined as a chemical substance produced or acquired by an emitter organism that contacts a heterospecific receiver organism and evokes the receiver's behavioural or physiological response that is adaptively favorable to the receiver, not the emitter. Pheromones (Greek: phereum, to carry; horman, to excite or stimulate) are released by one member of a species to cause a specific interaction with another member of the same species (Chapman, 1999). Pheromones may be further classified on the basis of the interaction mediated, such as alarm, aggregation or sex pheromone. *L. botrana* relies on a mating system using sex pheromones for long-distance mate finding with the female releasing a mixture of long-chain hydrocarbons, such as (E,Z)-7,9-dodecadienyl acetate.

Olfaction in insects

Insects can recognize and discriminate chemical signals in the environment, which provide essential information for survival and strongly influence their behaviour. Chemical cues are used to detect and locate food, mating partners, prey and predators. The olfactory system has a difficult task: it has to filter the thousands of odour molecules surrounding the relevant information from background. Some invertebrates rely on olfaction as the principal sense and their olfactory systems have evolved to a level of extreme sensitivity and specificity. This enables them to identify minute concentrations of essential chemical compounds. Remarkable chemosensory abilities have been demonstrated in insects in that a single pheromone molecule may be sufficient to elicit an action potential in a male olfactory receptor neuron of the silkworm *Bombyx mori* (Schneider et al., 1968).

Studies in the field of insect olfaction have progressed greatly in the last decade where new methods have added important data about morphological, physiological and behavioural aspects of the olfactory system. A considerable amount of work focuses on signal integration in the insect antennal lobe and the molecular basis of signal transduction in olfactory sensilla. Unfortunately, behavioural experiments are often neglected, and conclusions drawn from observations on physiological functioning in insect brains have a weak point in that the behavioural response of the insect, which is the most critical, is not studied. We combined electrophysiological methods with a behavioural approach that complement one another.

Morphology of sensory organs and olfactory detection and transduction

Sensory organs are so-called sensilla and consist of olfactory receptor neurons (ORNs), accessory cells, and a cuticular structure (Fig. 1. 3). The dendrites are usually located in specialized cuticular structures, which are classified on the basis of external form. They include hair-like varieties (sensilla trichoidea), pegs and cones (sensilla basiconica, often involved in plant odour perception), pegs or cones sunk in shallow depressions (sensilla coeloconica), and pore-plate organs (sensilla placodea). Typically there are two to five ORNs in an olfactory sensillum. ORNs are mostly bipolar and their axons run to the CNS. Every ORN expresses one or few of a large variety of olfactory receptor gene products. Olfactory receptors (ORs) are G-protein coupled receptors, present in the membrane of ORN dendrites.

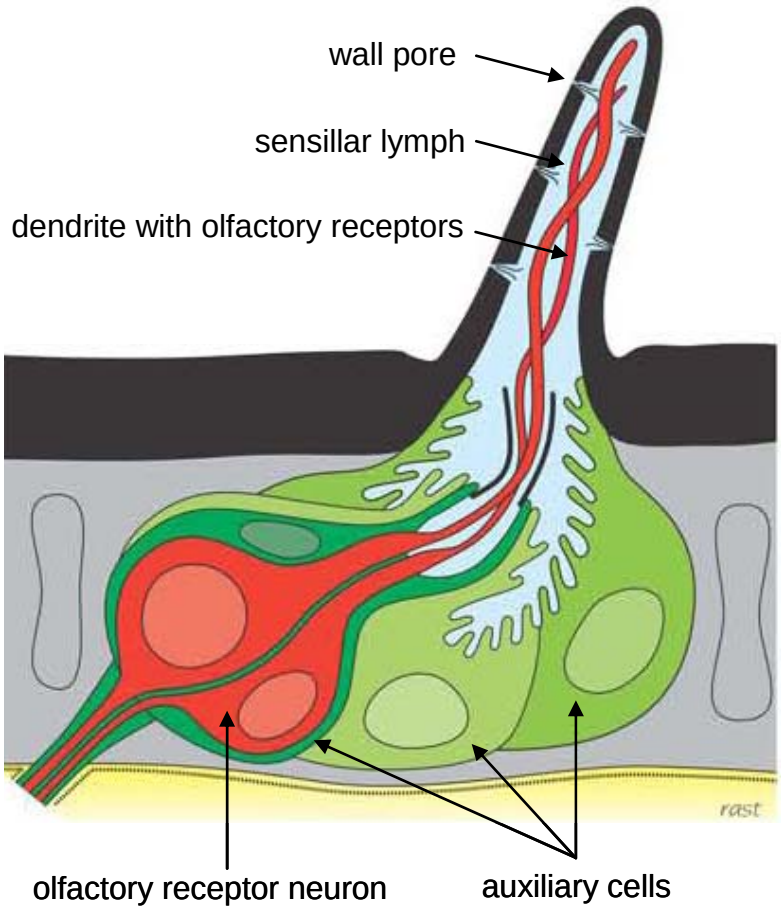


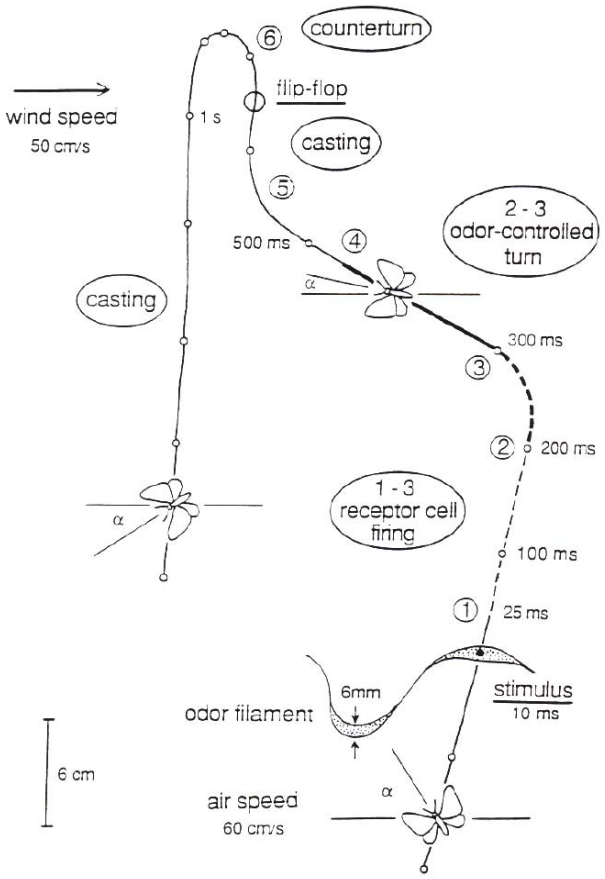
Fig. 1. 3: Scheme of an olfactory sensillum trichoideum with two receptor cells (red) and three auxiliary cells (green). Modified from Kaissling (2004)

The ORs are specialized to respond to the chemical stimulus with a graded potential called the receptor potential. When this potential reaches a value above a certain threshold it gives rise to action potentials in the ORN. Olfactory sensilla are multiporous, the entire sensillum wall or plate is perforated by up to thousands of minute pores. The dendritic tips are close to the pores, but are protected from desiccation by the receptor lymph, which is secreted into the sensillum lumen by the auxiliary tormogen and trichogen cells at the sensillum base. On an antennae of a male moth up to 100'000 sensilla belonging to different classes can be present (Steinbrecht, 1970). The process by which quality and quantity of the chemical stimulus is converted into a receptor potential and eventually into action potentials involves a sequence of steps. First, volatile molecules diffuse into the sensillum lumen via the pores in the sensillum wall and bind to water-soluble odourant binding proteins (OBPs), which serve to transport the volatile molecules through the sensillar lymph. Either the stimulus molecule or the complex of OBP and stimulus molecule then binds to ORs. Activated ORs induce the opening of ion channels that leads to a depolarization of the dendritic membrane. The activity of stimulus molecules is most probably terminated by odour-degrading enzymes present in the sensillar lymph. ORNs project to the primary olfactory centre in the brain, the antennal lobes in insects (Vosshall et al., 2000). ORNs do not only code the identity of odourants but also concentration and time course of the stimulus through action potential frequency. The antennal lobe (AL) consists of structurally and functionally distinct subunits, the glomeruli. Each glomerulus receives input from only one ORN class expressing one olfactory receptor. Therefore the response to each odour is reflected in a specific pattern of activated glomeruli in the AL respectively (Joerges et al., 1997). This combinatorial code is further processed in higher brain centres.

The electroantennogram (EAG) introduced by (Schneider, 1957) permits the measurement of the electrophysiological response of an insect antenna to stimulation with a chemical stimulus. In our case a cut antenna held between a reference grounded electrode and a recording electrode, connected to a high impedance preamplifier, permitted to record EAG potentials after odour stimulation of the antenna. The EAG permits to investigate the sensitivity of grapevine moths to different chemostimuli and give insights in the moth's olfactory capabilities

Behavioural response to volatile compounds

When a flying grapevine moth male is searching for a female, he engages in positive anemotaxis, that is, he flies upwind using the actual direction of air flow as a cue (Kaissling, 1997). Mechanoreceptors located on his antennae and over the body serving as anemoreceptors provide this directional information. His flight path can be described as a regular zigzag of limited amplitude (Fig. 1. 4). How do the pheromone molecules emitted by the calling female come into play since the male tries to localize the female using his anemoreceptors? The odour plume emitted by the female is filamentous and does not provide a reliable concentration gradient for the male to follow.



(1) receptor cells begin firing

(2) beginning of anemotactic manoeuvre, resulting in a small net upwind progress

(3) receptor cell firing ceases;

(4) end of upwind surge

(5) downwind turn and onset of casting

(6) counterturn, induced by a change of sign of an internal turning tendency (flip-flop), casting continues

Fig. 1. 4: Imaginary flight track of a moth showing the chain of events elicited by crossing of a single odour filament (at time t=0, α=course angle). Picture by Kaissling (1997)

It has been shown that such filamentous odour packets can travel a considerable distance and diffusion can be neglected (Murlis and Jones, 1981). The role of the pheromone is therefore not to provide a reliable trace, but to trigger the behavioural response. First, the pheromone molecules induce the moth to take off from a resting state. Once in flight, he may pick up an odour plume released from a female, and his subsequent flight path is then determined mainly by trying not to lose the odour plume. When, over a certain minimum time interval, olfactory receptor cells are no longer stimulated by an adequate chemical stimulus, the male engages in a so-called "casting" flight where moth reduces speed and increases the amplitude of counterturns. The moth then flies more cross-wind and regresses in a downwind direction. If during casting, pheromone molecules are again perceived by olfactory receptor cells in the sensilla of the antennae, upwind flight is resumed. This behavioural sequence is designated as odour-conditioned positive anemotaxis and may be repeated until the final approach to the calling female (Kaissling and Kramer, 1990).

A current view of the mechanisms steering this behaviour maintains that the serial counterturning is controlled by a motor programme in the CNS that is triggered by odour detection, but afterwards is continued automatically (self-steered, Olberg, 1983). The switch from zigzagging to casting, however, is controlled by olfactory information: absence of activity changes in the olfactory receptor cells over a certain minimum timespan causes casting behaviour. Upwind progress is made possible by optomotor feedback, that is, mainly input from the ground which controls the motor response via a feedback loop (Kaissling, 1997). Positive odour-conditioned optomotor anemotaxis is presently considered not only to be the main mechanisms used during pheromone-mediated upwind flight in insects but also for host-plant searching in both specialized and polyphagous herbivorous insect species

1.4 Insect Pests and Integrated Pest Management

Great developments have occurred in agriculture in the last half century. Automation and new technologies has led to a completely different ways of producing foodstuffs. Nowadays intensive agriculture is widespread worldwide, whereas in the past a strategy of self-sufficiency was common. Intensive agriculture signifies monocultures, and a rise of pests and parasite populations accompanies such practices. In species-rich and richly structured ecological systems outbreaks of pests and parasites hardly occur due to small numbers of breeding sites and ecological interactions (Kogan, 1998). Under certain climatic and culture conditions outbreaks of pests are often favoured. There are a lot of species with common or

occasionally explosive population dynamics that permit them to become well known pests all around the world. Phytophagous insects constitute a major threat for agricultural crops. The Tortricidae, which belong to the order *Lepidoptera*, is comprised of a great number of insect pests of plant cultures with the larvae deforming shoots, attacking young growth, flowers and fruits. Most significant in Europe on fruit are the summerfruit tortrix *Adoxophyes orana*, the codling moth *Cydia pomonella*, the grapevine moth *Lobesia botrana* and the grape berry moth *Eupoecilia ambiguella*.

In Europe the total area under vineyards is about 4 million hectares. Classic pest control consists in the use of pesticides, such as insect growth regulators, neurotoxic insecticides and insecticides based on biologicals (e.g. *Bacillus thuringiensis* and viruses). Despite the reliance on insecticides for the efficient control of insect pests of crops, biological control methods are progressing. The use of insecticides faces more and more problems because reliance on these chemicals leads to resistance in insect pests. Due to the high costs of developing new pesticides and strict registration procedures it will become more difficult to bring new pesticide formulations on the market. Furthermore, the use of insecticides can have a bad impact on the health of farmers and workers who deploy them on crops (Baldi et al., 2006; Viel et al., 1998). Late applications of insecticides can also result in high residues on grapes, which can still be present in the crop at harvest and be potentially harmful to consumers (Cabras and Angioni, 2000). The public has been sensitized to ecological considerations and the preservation of the environment. In the long term, agriculture has to take an ecological approach to be sustainable.

Integrated pest management (IPM) is a way of farming that is socially acceptable, environmentally responsible and economically viable. The factors that cause pest outbreaks depend on the complex interactions between crops, pests, physical habitats and the natural biota. No schedule of pesticides, fertilizers or planting techniques is perfect. IPM is a process, defined for each particular situation. The key to success has been in developing new ways to understand and make decisions about pest management. It refers to a way of farming that aims to protect and conserve natural resources, while responding to the needs of the growers, consumers and farmers. IPM includes cultural, biological and chemical methods of pest control. Pesticides are important in IPM programs, but are used in a rational manner and are often the last resort to combat a pest (De Opender et al., 2004).

1.5 Semiochemicals in Integrated Pest Management

The use of semiochemicals in agriculture has increased in the last 30 years where they find applications for insect pest control in different cultures such as cotton, corn, pome fruit and many more. Semiochemicals are used for pest monitoring as well as in the direct control of insect pests. The most successful approach using semiochemicals for insect pest control over the last few decades has been the release of synthetic pheromone from dispensers in a crop in an effort to prevent or delay mating. This strategy is called mating disruption and is the most highly developed and widely adopted semiochemical-based control method for insects (Gut et al., 2004). Mating disruption is based on the male's response to the sex pheromone of the female. A female-produced pheromone plume is a filamentous structure of varying internal concentrations detected by males as a series of stimulus pulses. The intensity at which pheromone molecules are detected by the receptor cells determines the rate at which action potentials are generated and induce upwind flight in grapevine moth males. The disruption of any or the entire above-described plume following behaviour is referred to as mating disruption or pheromone confusion. The major mechanism of mating disruption is assumed to be "false-trail-following". This signifies the competitive attraction of males between calling females and point sources of pheromone (Miller et al., 2006). The principle is to inhibit the male moth to follow the odour-trails to females and therefore disrupt female fertilization to keep the size of the population at a tolerable level. This method has the following advantages compared to treatment with insecticides: high specificity to the target insects, no toxicity for users and no residues on fruits. Several studies underline the success of this method (Charmillot and Pasquier, 2000, 2004), but also point out the drawbacks. Firstly, the method affects only the behaviour of males and not that of gravid females. In addition, it is only active on the adult stages. Even if mating is disrupted, immigrating gravid females can lay eggs and cause damage. To avoid the problem of immigration, vineyards treated with pheromones must be either isolated or have a minimum size of 5 – 10 hectares with borders well protected. Also, if the population density is too high the possibility of males encountering females increases and so mating disruption fails. Moreover, when compared with insecticides, mating disruption costs 2 to 3.5 times more (Degen et al., 2005).

Despite this, in Switzerland the area under vineyards treated by mating disruption against *L. botrana* augmented from 3.1% in 1996 up to 33.2% in 2000 (Charmillot and Pasquier, 2000). Nowadays, the treated area is about 6000 ha, which accounts for around 45% of Swiss vineyards (Degen et al., 2005), and in most cases mating disruption achieves better results

than classical insect pest control. Mating disruption is a good method to combat the grapevine moth, but has to become more reliable and cost-efficient.

1.6 Potential for the use of plant compounds in Integrated Pest Management

A large number of volatile plant compounds leave the plant and are transported away from the source of production. General plant odour components are formed via biosynthetic pathways commonly present in plants, whereas specific odour components arise from biosynthetic pathways peculiar to a plant family or species (Schwab et al., 2008; Taiz and Zeiger, 2002). From a chemical point of view, volatile compounds found in plants include terpenoids, aliphatics and aromatics (Knudsen et al., 1993). Ovipositing females generally seem to utilize plant volatiles as cues for orientation (Honda, 1995). Additionally, the host plant can also serve as a mating site for the insects, with sexual behaviour occurring principally or exclusively on host plants (Visser, 1986), i.e. encounter sites are focused on resources attractive to females (Thornhill and Alcock, 1983). Since host plants are an attractive resource, it is likely that males should also use volatile host cues to locate appropriate reproduction sites. Therefore, odours of oviposition sites may be attractive to females as well as males. Dickens et al. (1993) studied the antennal responses of beet armyworm, *Spodoptera exigua*, males and females to plant odour components and found that male moths were more responsive to plant odours than females.

Responses of males to female pheromones may also be directly influenced by host plant compounds. Several chemicals from host plants have been identified to synergize or enhance insect responses to sex pheromones (Landolt and Phillips, 1997). By these means, host plants may be used by insects to regulate or mediate sexual communication. In a study by Rahn (1968), male leek moths, *Acrolepsis assectella*, were released in different parcels of land together with 4 females per parcel. Most males were recaptured in the parcels with leek (61%) and many less in parcels with grass (9.2%) or beet (12.6%), and almost none in parcels with only soil (0.8%), suggesting that the presence of the host plant conditions the meeting of the sexes. Deng et al. (2004) recorded enhancement of attraction of beet army worm males, *Spodoptera exigua*, to the sex pheromones by adding volatile compounds produced by host plants. In a wind tunnel lures baited with synthetic sex pheromone plus benzaldehyde, phenylacetaldehyde, (Z)-3-hexenyl acetate, or linalool, respectively, increased the landing rate of beet army worm males by 101.4%, 79.6%, 60.6%, and 34.3%, respectively, compared to

sex pheromone alone. In field tests, traps baited with pheromone plus either (E)-2-hexenal, phenylacetaldehyde, (Z)-3-hexenyl acetate or (Z)-3-hexenol enhanced moth catches by 38.8%, 34.6%, 24.6%, and 20.8%, respectively, compared to traps baited with the pheromone alone. Light et al. (1993) found that green leaf volatiles (GLVs; C6 aldehydes, alcohols and their esters) influence the behaviour of corn earworm males by enhancing the response to the sex pheromone. In experiments in the field these authors captured significantly more adult corn earworm males with traps containing sex pheromone and (Z)-3-hexenyl acetate than with the sex pheromone alone. Dickens et al. (1993) showed the same phenomenon with the tobacco budworm, *Heliothis virescens,* in that a mixture of (Z)-3-hexenyl acetate and (E)-2-hexenyl acetate were shown to enhance responses of tobacco budworm males to the sex pheromone in the field. The influence of green leave volatiles on the sex pheromone response of males has also been shown for the diamondback moth, *Plutella xylostella*, (Reddy and Guerrero, 2000): In a wind tunnel, mixtures of GLVs combined with the sex pheromone attracted 80-100% of the males tested, significantly more than the effect induced by the sex pheromone alone. Light et al. (2001) showed the potential of plant compounds in insect pest management when they tested a pear-derived volatile, ethyl (2E, 4Z)-2,4-decadienoate (pear ester) on the codling moth, *Cydia pomonella*. These authors found that pear ester attracts both *C. pomonella* males and virgin and mated females and that at the same low microgram loads on rubber septa as codlemone, the combined gender capture of *C. pomonella* in pear ester-baited traps was similar to the capture rate of males in traps baited with codlemone. The particular attribute of attracting *C. pomonella* females renders pear ester a novel tool for monitoring population flight, the mating and oviposition status of females, and potentially a new resource for directly controlling *C. pomonella* populations. However there is currently a major critique as pear ester does not attract codling moth in pear orchards (Light et al., 2001) and, for some unknown reasons, in field tests in Europe pear ester was not attractive to the codling moth (Ansebo et al., 2004).

1.7 Research interest and outline of the thesis

L. botrana is the most damaging insect pest on vine in Europe. Existing semiochemical based control techniques against *L. botrana* have two drawbacks: they are costly and ineffective at high population densities. Any improvement in efficacy is therefore desirable. Possible synergism between host odour and sex attractants is of great importance in the development of novel semiochemical-based lures. There is evidence that insects in natural settings perceive and exploit signals in the volatile emissions of plants, and lures based solely on pheromone

are therefore likely to be suboptimal for pest control. The enhancement of the attraction of insects to traps baited with pheromone mixed with the relatively inexpensive and environmentally safe secondary host plant compounds may be important for the development of future control strategies based on semiochemicals for insect pests. However, the moth sensory system is highly sensitive and care must be exercised in choosing potential host plant products and in the formulation of new dispensers. A deeper knowledge about the sensory ecology of grapevine moth males is indispensable in order to affect this insect's behaviour by means of plant products. Therefore, this thesis aims to elucidate the olfactory capabilities of *L. botrana* males with regard to host plant volatile perception and especially to provide new insights into the use of host plant volatiles by males in their searching behaviours for females. Our hypothesis is that the grapevine moth males use volatile host plant cues in mate-finding behaviour, and that the perception of these plant products contributes to an optimised strategy for reproduction.

First, we had to provide evidence that grapevine moth males carry olfactory receptor cells tuned to plant volatile perception. In chapter 2 we recorded electroantennogram responses from male antennae and analysed five plant volatile collections from four different *L. botrana* host plants in order to identify commonly occurring host plant products that elicit male antennal receptor cell responses. In parallel, behavioural assays were carried out to study the effects of host plant volatiles on the attractiveness of the sex pheromone. The behavioural response of *L. botrana* males to its sex pheromone presented in a background of headspace emissions from growing vine plants was recorded in a wind tunnel.

In chapter 3, after finding support for the hypothesis that host plant volatiles affect male *L. botrana* responses to its sex pheromone and identifying the chemostimuli involved, we wanted to test the attractiveness of the sex pheromone with synthetic host plant compounds and to investigate in detail dose response characteristics of plant volatiles presented with the sex pheromone, along with the effects of mixtures of host plant volatiles. With a view to future applications we carried out field trails to confirm the lead findings from these laboratory studies.

Finally, in chapter 4 we were interested in the behavioural responses of *L. botrana* males to host plant volatiles in the absence of the sex pheromone components, since males could increase their chances of mating success by locating rendez-vous sites before the females start calling. Furthermore, males have to find shelter during the day so it is likely that their

behaviour is affected by plant volatiles on their own. Subtractive bioassays permitted to identify host plant volatiles that are essential to a multi-component blend of volatile host plant products.

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CHAPTER 2

Perception and behavioural effects of host plant headspace emissions in *L. botrana* males

Abstract

Recently it has been showed that *L. botrana* females can make upwind oriented flights to a bunch of grape berries. In a wind tunnel we tested the effect of volatile emissions of *Vitis vinifera* cv. Solaris on the attractiveness of the sex pheromone to *L. botrana* males at an underdosed and an optimal pheromone release rates of 0.1pg/min and 1.0pg/min, respectively. The behavioural response of grapevine moth males was increased when the sex pheromone was presented with a background of vine plant headspace. To identify the biological active host plant compounds, five headspace collections of volatiles from four host plants were analysed using gas chromatograph linked electroantennogram detection using *L. botrana* male antennae. We identified 15 plant volatiles that elicit electroantennogram responses in grapevine moth males that are common to at least three host plant headspace collections.

Keywords: *Lobesia botrana*, mate-finding, host plant, chemoreception, headspace profile, GC-EAD, *V. vinifera* cv. Solaris, *V. vinifera sylvestris*, *Olea europea*, *Ligustrum vulgare*, *Rosmarinus officinalis*

2.1 Introduction

For phytophagous insects, like *L. botrana*, the ability to perceive plant compounds is important as plant volatiles can signal food, oviposition sites, places of refuge and mating sites. Host plant influences on sex pheromone behaviour of phytophagous insects have been reviewed in detail by Landolt and Phillips (1997) who documents: (1) insects that sequester or acquire host plant compounds and use them as sex pheromones or sex pheromone precursors; (2) insects that produce or release sex pheromones in response to particular host plant cues and (3) chemicals from host plants that enhance insect responses to sex pheromones. Grapevine moth male antennae carry sensilla trichoidea mainly that are equipped with pheromone sensitive olfactory receptor neurons (ORNs). As it was observed for other moth species it is likely that there are ORNs tuned to plant odours besides the pheromone sensitive ORNs (Røstelien et al., 2000; Stranden et al., 2003), and that males are also able to exploit the information provided by volatile emissions of plants. Since the primary goal of adults is reproduction it is likely that plant volatiles are used by males to increase their chances to find females present on host plants and therefore play a role in the male sexual behaviour.

The grapevine moth is a polyphagous insect, not limited to the grapevine as a host plant as it can develop on plants belonging to different families (Katerinopoulos et al., 2005; Thiery and Moreau, 2005). This makes it improbable that a single set of volatiles serves a key semiochemicals in the biology of grapevine moth males. A single plant can already emit hundreds of plant products and headspace volatile profiles from plants of different families are likely to be considerably different from one another. We hypothesised that a blend of commonly occurring plant products across a range of host plants provides information that may be involved in mate-finding by grapevine moth males rather than products unique to one host plant. To investigate this hypothesis we set out using chemosensory recordings from male antennae and behavioural essays to show that grapevine moth males are equipped with sensory cells to perceive plant volatiles and that the behavioural response of grapevine moth males to the sex pheromone is affected by adding plant products. Headspace profiles of *Vitis vinifera sylvestris* male flowers, *V. vinifera sylvestris* female flowers, *Olea europea*, *Ligustrum vulgare*, *Rosmarinus officinalis* were analysed for constituents that elicit male antennal receptor cell responses using gas chromatograph linked electroantennogram detection. In that way biological active compounds that occur across the four host plants could be identified. We then carried out wind tunnel assays to study the behavioural responses of grapevine moth males to an underdosed and an optimal pheromone release rate in

combination with vine headspace volatiles in order to test for any positive effect of the grape headspace volatiles on the behavioural responses of *L. botrana* males to the female sex pheromone. To our knowledge, this is the first study regarding the perception of host plant volatiles by *L. botrana* males and the effects of host plant volatile headspace emissions on the activity of the sex pheromone for males.

2.2 Material and Methods

Insects

Pupae of *L. botrana* were obtained from a laboratory culture at the Research Station Agroscope Changins-Wädenswil ACW (Switzerland). Insects were reared in a climate chamber at 65% RH and 25°C during the photophase (16 hrs) and at 85% RH and 18°C during the scotophase (8 hrs). The larval stages were reared on a semiartifical medium (Rauscher et al., 1984, Annex A). Pupae were sexed and put on a cloth mesh over a dish filled with water for emergence. Males emerged into cages (BugDorm, 30*30*30cm, MegaView Science Education Services Co., Taiwan) each day. The cages were enclosed in plastic bags. A sugar solution (10% sucrose) in glass tubes closed with cotton wool wicks was offered to the males by placing two of them on the top of the cage.

Vine plants

Potted vine plants (*Vitis vinifera* cv. Solaris) were obtained from the Research Station Agroscope Changins-Wädenswil ACW (Switzerland). The vine plants were held in an environmental cabinet with a 13L:11D light cycle and a constant temperature of 22°C. The plants were watered three times a week. Vine plants used for headspace collections and behavioural experiments measured 45±5cm and had a wet weight of leaves of 20±2g.

Synthetic chemicals

The synthetic pheromone compounds used were (E,Z)-7,9-dodecadienyl acetate (E7Z9-12:Ac, >97%), (E,Z)-7,9-dodecadien-1-ol (E7Z9-12:OH, >94%) and (Z)-9-dodecenyl acetate (Z9-12:Ac, 99.9%) purchased from Plant Research International (Netherlands). The synthetic plant volatile (Z)-3-hexen-1-ol (>98%) was purchased from Fluka (Switzerland).

Piezo sprayer

A piezo sprayer was installed according to (El-Sayed et al., 1999a) to release synthetic pheromone blends. The ultrasound evaporator consisted of a syringe pump (type CMA 400, CMA Microdialysis AB, Solna, Sweden) that pumped the test solution at 10 µl/min from a 5 ml gas tight syringe (containing an alcohol solution of known amounts of the test products, Hamilton type 81527, Milian SA, Meyrin, Switzerland) into PTFE micro tubing (1.5 m long, 1.02 mm o.d., 0.56 mm i.d., Hamilton type 90674) connected by a PTFE micro tubing connector (3 cm long, 1.57 mm o.d., 0.97 mm i.d., Hamilton type 20919) to a borosilicate

glass capillary (50 mm long, 1 mm o.d., type GC100-10, Clark Electromedical Instruments, Pangbourne, England) with a drawn out tip (10-20 mm tip length, 30-40 µm i.d. tip opening). A frequency generator (Wavetek FG-5000A, Willtek Communications GmbH, Ismaning, Germany) producing a square-wave signal (ca. 80 kHz, 40V amplitude) was connected to a piezo-ceramic disc (25 mm diameter, Philips PXE5 25/2.0) that was connected by a clip (a modified piano chord) with the glass capillary. This caused the latter to oscillate and produce an aerosol of the test solution that broadened to ca. 10 mm at 50 mm from the source. The oscillating glass capillary was installed in the middle upwind end of the wind tunnel at 30 cm from the floor. A metal grid cylinder (15 mm long, 30 mm o.d.) placed over and in the same axis as the capillary protected the latter against damage by moths attempting to contact the chemical source. Oscillations of the capillary tip dispersed the solution as micro-droplets that evaporated within a few cm downwind of the release point to create an invisible plume at 10-15 cm from the source. The capillary tip was protected from the approaching moths by a small metal mesh that was cleaned after exposure to each treatment. Ethanol (pro Analysis, Merck, Germany) was used as solvent for the pheromone as it has been shown by (El-Sayed et al., 1999a) to have no influence on the behaviour of *L. botrana* males.

Four release rates of the ternary blend admixed at a ratio of 100:5:1 proposed by El-Sayed et al. (1999b) (0.1pg/min, 1.0pg/min, 10pg/min and 100pg/min of the main pheromone component E7Z9-12:Ac) and eight release rates of the ternary pheromone blend proposed by Arn et al. (1988) (0.01pg/min, 0.1pg/min 1.0pg/min, 10pg/min and 100pg/min, 1.0ng/min, 10ng/min, 100ng/min) were tested. Release rates of the ternary pheromone blend refer to their E7Z9-12:Ac content hereafter.

Vine headspace emission presentation

Volatile vine plant emissions were presented in the following manners: the vine plant placed directly inside the wind tunnel (setup 1), grape headspace was introduced through Teflon tubing into the wind tunnel (setup 2) and headspace extracts dissolved in ethanol were sprayed into the wind tunnel by means of the piezo nebulizer (setup 3). For setup 1 a plastic platform (12 x 60cm) was placed horizontally at a height of 12cm between the charcoal filter and the perforated steel at the upwind end of the wind tunnel. Eight potted vine plants were distributed regularly on the platform. The pots were enclosed in tin foil to reduce volatile emissions from the soil. As a control, the vine plants were replaced by plant imitates, consisting of clean plastic pots, metal wire and transparent plastic to mimic leaves. The

number and size of the artificial leaves was identical with the vine plants. Present vine headspace from two vine plants held outside the wind tunnel (setup 2), each was held under a glass cylinder (10 cm diameter, 50 cm high). Air was first pushed through a flowmeter to measure and regulate the air flow, and a charcoal filter to purify the air. The pure air then entered the two glass cylinders close to the base at 600 ml/min and passed over the plants that were placed in the cylinder through the open bottom. A Teflon disk placed against the bottom of the cylinder covered the soil. The disk consisted of two halves: a guillotine-like metal plate was attached to one half and this could be pushed into a groove in the other half. A hole of 1 cm diameter was left in the centre of the disk for the stem of the plant. The two halves were pushed together around the lower part of the plant's stem, which left most of the plant and all of its leaves inside the cylinder while the pot remained outside. This contributed that only odours from the plant were present in the cylinder. The Teflon disk had a 1-cm-wide and 5-mm-deep groove which fitted precisely the widened base of the glass cylinder. Approximately 2.5 cm below the top of the cylinder a glass port with screw caps and Teflon-sealed O-rings permitted attachment of Teflon tubing that was brought into the wind tunnel and fixed above the capillary of the piezo nebulizer upwind of the perforated steel plate. In order to be able to present grape headspace by means of the piezo nebulizer, headspace volatile emissions of vine plants had to be collected, analysed and then admixed with ternary pheromone blend first (see following sections). Two solutions were formulated, one containing 120pg/µl of plant volatiles, another of 1200pg/µl plant volatiles, both in combination with an underdosed pheromone level of 0.01pg/µl of E7Z9-12:Ac and released at 10µl/min by the piezo sprayer.

The wind tunnel

The wind tunnel (flight section 60 : 60 : 195 cm) is constructed of non-reflecting glass. Air was blown by a centrifugal fan (Fischbach GmbH, Neunkirchen, Germany) through a section of charcoal cartridges and a perforated steel screen (1mm thick, 3mm round holes, 51% of air passage, Schäfer, Neunkirchen, Germany) which resulted in a laminar airflow at 30 cm/s. The outcoming air was sucked by another fan and cleaned by additional sets of charcoal filters. Females, pheromone mixtures and plant volatiles were presented in different configurations as odour sources at the upwind end of the wind tunnel. Overhead illumination is provided by high frequency fluorescent lights (36W, >1kHz, Philips) running the length of the tunnel. The light intensity was regulated with a potentiometer to produce ca. 10 lux along the wind tunnel floor. Light was dispersed using a Perspex Prisma® crystal-clear plastic sheet under the fluorescent tubes and by placing crepe paper on the roof of the wind tunnel. Below the tunnel

floor, black shapes of irregular sizes and forms were placed on a white sheet and used as optomotor cues. The wind tunnel was housed in a walk-in climate chamber (Schaller Uto AG, Bern, Switzerland) that allowed the air stream to be maintained at 18 ± 0.5°C and 85 ± 2% RH during experiments in the wind tunnel.

At the beginning of the scotophase, three day-old males were placed individually in glass tubes (125 mm long, 24.2 mm o.d., 21 mm i.d.) which were closed with cotton wool-plugs and transferred into the climate chamber housing the wind tunnel. Grapevine moth males had 10 minutes to adapt to the conditions before the experiments started. Male moths were individually presented to the odour source on a stand (30 cm high) placed 30 cm upwind of at the downwind end of the wind tunnel. Moth behaviour was scored for (1) no activation, (2) activation, (3) wing fanning, (4) take off, (5) upwind flight, (6) passing the midline of the wind tunnel, (7) close in within 10 cm of the source and (8) contacting the source by means of the OBSERVER software package (version 5.0, Noldus Information Technology, Wageningen, The Netherlands). The behavioural response of male moths was recorded for two minutes. Males that were not activated within one minute or landed on a wall (min. 5 s rest) were removed from the wind tunnel.

Headspace collections

Headspace collections from entire vine plants

Volatiles emitted by two vine plants (*V. vinifera* cv. Solaris) were collected with a modified system described before in the section "Vine headspace emission presentation". During 18hrs air was pulled by means of a vacuum pump through a charcoal filter into two glass cylinders (see above). Approximately 2.5 cm below the top of the cylinder, a glass port with screw caps and Teflon-sealed O-rings allowed for the attachment of a collection trap. A flow meter allowed regulation of the airflow to 400ml/min. For solventless headspace collections a commercial Tenax™ GR cartridge (6 mm OD, Gerstel®, Switzerland; conditioned under N_2 at 200°C for 90min before) was submitted to thermal desorption and gas chromatograph-mass spectrometry analysis immediately after the vapour collection. For headspace collections using solvent desorption, the volatiles were trapped on 25mg of 80/100 mesh PorapakQ adsorbent (Altech Assoc., Deerfield, Ill., USA; conditioned under N_2 at 200°C for 90min before). Plant volatiles were desorbed from filters with 500µl hexane (Acros Organics, New

Jersey, USA). Headspace extracts were reduced under N_2 to 100ml and transferred into glass ampoules that were sealed and stored in the freezer at -20°C until analysis.

Headspace collections from plant parts

Headspace collections were made of male and female flowers of *Vitis vinifera sylvestris* (Vitaceae), flowers of *Ligustrum vulgare* (Oleaceae), leaves of *Olea europea* (Oleaceae) and leaves of *Rosmarinus officinalis* (Lamiaceae). Fresh plant material was placed in 250ml gas-wash bottles. A vacuum pump pulled air at 500ml/min for 2 hours through a charcoal filter into the gas wash bottle and finally through 25mg PorapakQ trap (conditioned under N_2 at 200°C for 90min as above. Trapped volatiles were eluted with 100µl dichloromethane (DCM; analytical grade, Merck, Germany) into glass ampoules that were sealed and stored in the freezer at -20° C until analysis.

Gas chromatography linked electroantennographic detection (GC-EAD)

The headspace extracts were analysed by means of gas chromatography linked to electroantennogram detection (GC-EAD) (Arn et al., 1975). A grapevine moth male antenna was suspended between two glass electrodes filled with 0.1M KCl and served as a biological detector. The antenna was held in a humidified charcoal filtered air stream (90–100% RH, 23 ± 2°C) delivered at 1 m/s via a glass water-jacketed tube (7 mm ID) whose outlet was about 1 cm from the preparation. Constituents of headspace extracts were separated by means of a gas chromatograph (5300, Carlo Erba Instruments, Italy), equipped with a polar high resolution capillary column (BGB-FFAP, length 30m, I.D. 0.25mm, film thickness 0.25µm) and an on-column injector. The oven was programmed to 40°C for 5min, a ramp of 10°C/min to 230°C held for 5min. The column effluent was split (50:50) between the flame ionization detector (FID) and to a heated (230°C) outlet from the chromatograph that led to the humidified air stream that led to the antenna. The signal of the EAG response was recorded on a computer via a high impedance preamplifier (Syntech, NL), a DC amplifier (UN-03, Syntech, NL) and an analog-digital converter (USB-IDAC box, Syntech, NL) at the same time as the FID response that was also connected to the analog-digital converter. The responsiveness of the antenna was tested with an air puff from 10µg of (Z)-3-hexenol on a filter paper strip in a 5ml stimulus syringe (Taneja and Guerin, 1997). If the antennal response to this 1s air puff (1ml/s) from this stimulus syringe was less than 0.7mV another antenna was mounted. At the start and end of each run the antennal preparations was subjected to an odour loaded air puff as described above, and recorded EAG responses were normalised according to the decline in

order to compensate for the drop in antennal response over time. All headspace extracts were tested at least on three different *L. botrana* male antennae from different individuals. Only compounds eliciting an EAD-response of at least 80µV from at least two antennae at a particular retention time were considered, and Kovat's retention indices were calculated for these products. GC peak area was determined using the GcEAD Software (Syntech, NL).

Gas chromatography linked mass spectrometry (GC-MS)

Headspace collections dissolved in dichloromethane and hexane

Identification of headspace volatiles was done on a Hewlett Packard 5890 Series II gas chromatograph (GC) equipped with an on-column injector. DCM samples were injected at a GC-starting temperature of 40°C, hexane samples at 60°C. After 5 min at the starting temperature, the GC was programmed to heat at 15°C/min to 230°C held 5 min. The mass spectra were identified with reference to the mass spectra of products two libraries (Nist98, Wiley). Amount of compounds in injected samples were estimated by comparison with known amounts of synthetic standards.

Thermal desorption

For the analysis of solventless headspace samples the injector of the GC was equipped with a thermal desorption unit (TDS 2, Gerstel®, Germany) and a cooled injection system (CIS 3, Gerstel®, Germany) installed on the head of a high-resolution capillary column. The outlet of the GC was coupled to a Hewlett Packard mass selective detector 5971A (MS) with electron impact ionization at 70 eV. Helium (30 cm/s) was used as carrier gas. To separate and identify the constituents of headspace samples a polar column (FFAP, length 30m, i.d. 0.25mm, film thickness 0.25µm, BGB Analytik, Switzerland) was used. Headspace volatiles initially trapped on a Tenax™ GR cartridge were thermally desorbed via the TDS 2 programmed to increase in temperature at a rate of 60°C/min from 30°C to 220°C for 2 minutes and a total flow of helium of 50ml/min. Analyses were carried out with the TDS 2 in split mode. A heated (constant at 240°C) transfer line evacuated the volatiles to the CIS 3 cooled to -80°C with liquid nitrogen where volatiles were cryotrapped. At the end of the TDS 2 transfer and after an equilibrium time of 0.2min, the CIS was subjected to a ballistic increase of 12°C/s in temperature to reach 230°C for 3 min to transfer the volatiles in splitless mode (1 min) onto

the high resolution capillary column. The GC was programmed from 40°C for 5min, heated at 15°C/min to 230°C and held for 5 min.

Statistical analysis

The responses of male *L. botrana* to different treatments were compared by fitting a generalised linear model (GLM) with a logit link function (logistic regression) to the responses, assumed to be binomially distributed, using the statistical package R (Version 2.4.1). Analysis of deviance based on the asymptotic X^2 distribution was used to test whether the flight responses were significantly dependent on the odour sources ($p<0.05$). When the GLM was significant ($p<0.05$) multiple comparisons (R-package: Multcomp) were made using Tukey-contrasts.

2.3 Results

2.3.1 Behavioural response of *L. botrana* males to the ternary pheromone blend

Flight response to the ternary blend 100:5:1

Grapevine moth males responded in a dose dependent manner to the ternary pheromone blend admixed at 100:5:1 (Fig. 2. 1). The ternary blend attracted most males at a release rate of 1.0pg/min, with 60% males taking off, 37.5% flying upwind and 31.3% contacting the source. Increasing the release rate of the ternary blend to 10 or 100pg/min resulted in a decrease of attractiveness to males with lower percentages of responding males for all behavioural elements with significantly fewer of males engaging an upwind flight (12.8% and 15.6%) and contacting the source (8.5% and 6.3%).

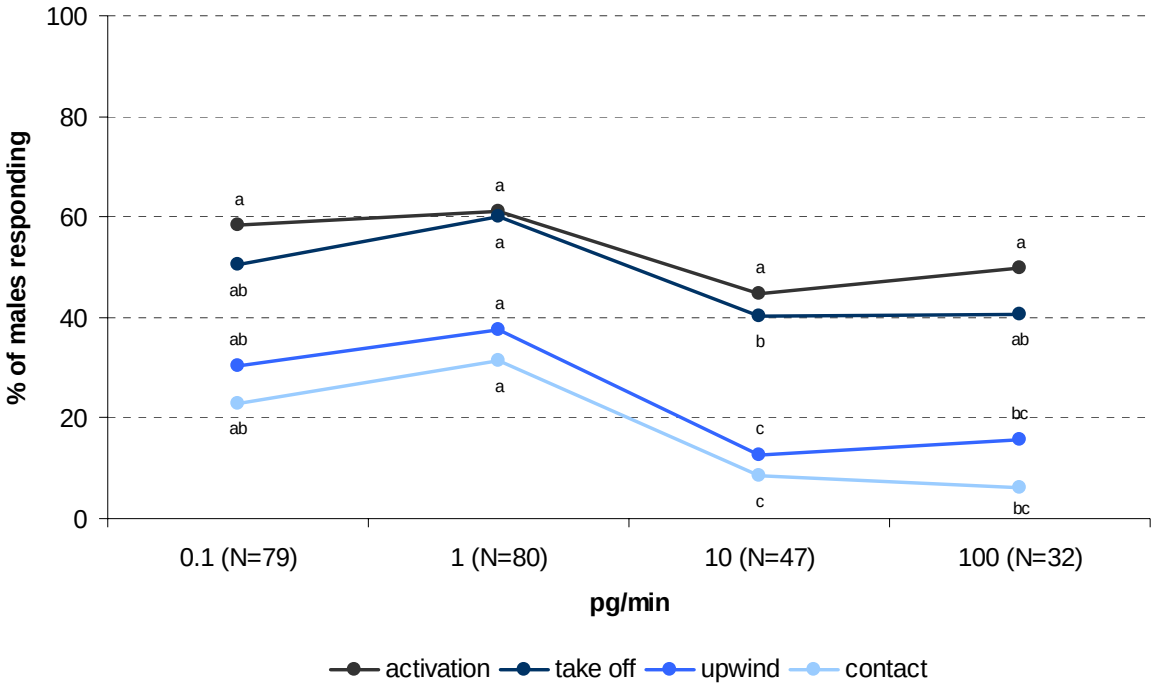

Fig. 2. 1: Behavioural response of *L. botrana* males towards the ternary pheromone blend of E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac admixed at 100:5:1 (El-Sayed et al., 1999b) released into the wind tunnel at different rates by means of a piezo sprayer. Release rates refer to the main pheromone component E7Z9-12:Ac. Most males engaged in upwind flight and contacted the pheromone released at 0.1 or 1 pg/min. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).

Decreasing the release rate to 0.1pg/min led also to a decrease in the attractiveness with 30.4% of the male moths flying upwind and 22.8 contacting the source, but statistical analyses showed no significant differences.

Flight response to the ternary blend 100:20:5

The ternary blend mixed at 100:20:5 attracted grapevine moth males in a dose dependent manner (Fig. 2. 2). By increasing the release rate of the ternary blend the percentage of activated males increased as well with a significant shift from 48.8% at 0.1pg/min to 80% at 1.0pg/min. A maximum activation rate of 91.3% was obtained at a release rate 10pg/min remaining at this level also for higher release rates.

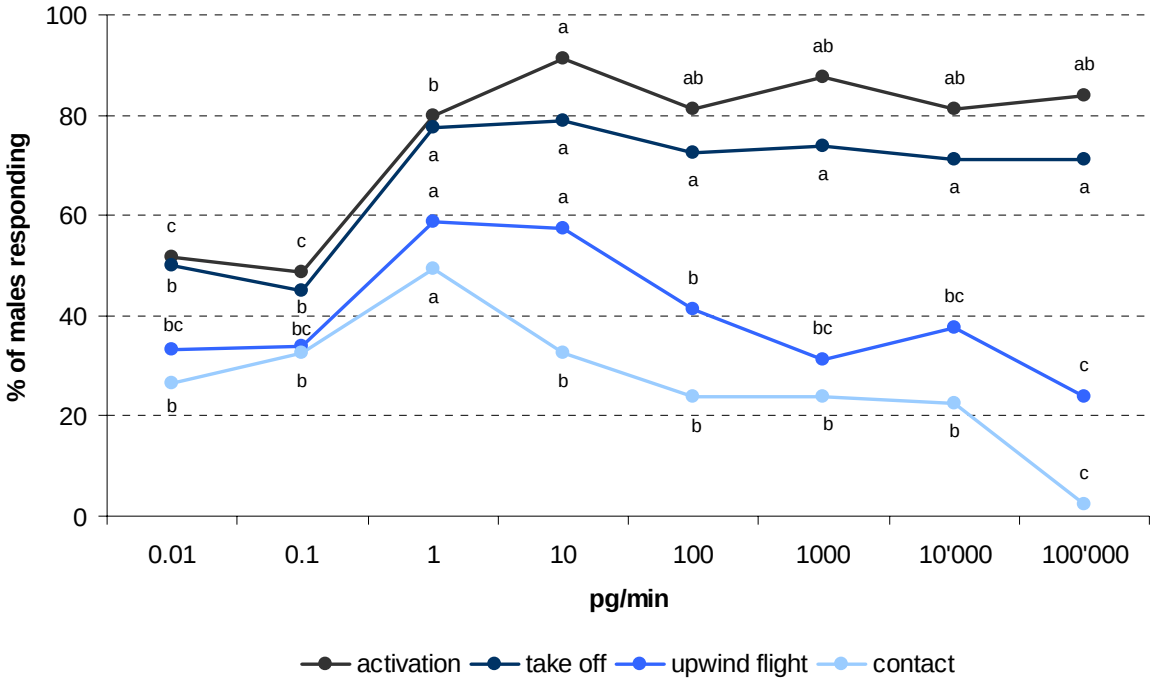

Fig. 2. 2: Behavioural response of *L. botrana* males towards the ternary pheromone blend of E7Z9-12:Ac, E7Z9-12:OH and Z9-12:AC at 100:20:5 (Arn et al., 1988) released into the wind tunnel at different rates by means of a piezo sprayer (N=80 each, except for 0.01pg/min N=60 and 1pg/min N=85). Release rates refer to the main pheromone component E7Z9-12:Ac. Most males engage an upwind flight and contact the odour source with the pheromone being released at 1 pg/min. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).

Tab. 2. 1: Comparison of flight response of *L. botrana* males towards two different ternary pheromone blends (100:20:5 and 100:5:1) released at four different rates by means of a piezo sprayer. Blend composition influenced significantly the flight behaviour for release rates of 1.0, 10 and 100pg/min with more males being attracted to a ternary blend admixed at 100:20:5. Asterisks assigned within a release rate and a behavioural element indicate statistically significant differences (GLM, p-values: *** ≤ 0.001 < ** ≤ 0.01 < * < 0.05).

release rate	blend ratio	activation	take off	lock on	upwind	midline	close in	contact	N
0.1pg/min	100:20:5[a]	48.8	45.0	36.3	33.8	33.8	32.5	32.5	80
	100:5:1[b]	58.2	50.6	34.2	30.4	27.8	24.1	22.8	80
1.0pg/min	100:20:5	80.0**	77.6*	58.8	58.8**	58.8**	52.9*	49.4*	85
	100:5:1	61.3	60.0	45.0	37.5	36.3	35.0	31.3	80
10pg/min	100:20:5	91.3***	78.8***	62.5***	57.5***	42.5***	33.8**	32.5**	80
	100:5:1	44.7	40.4	19.1	12.8	12.8	8.5	8.5	47
100pg/min	100:20:5	81.3**	72.5**	53.8***	41.3*	35.0*	32.5*	23.8*	80
	100:5:1	50.0	40.6	15.6	15.6	15.6	12.5	6.3	32

[a] ternary blend of E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac mixed at 100/20/5 (Arn et al., 1988)
[b] ternary blend of E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac mixed at 100/5/1 (El-Sayed et al., 1999b)

The dose-response curve for the behavioural element take off was similar to that described for activation with a significant shift from 45% at 0.1pg/min to 77.6% at 1.0pg/min, reaching a plateau for higher release rates. In contrast, the pheromone release rates of the ternary pheromone blend influenced the behavioural elements upwind flight and contact in a different manner. Again there was a significant increase in attractiveness by augmenting the pheromone release rate from 0.1pg/min (33.8% upwind, 32.5% contact) to 1.0pg/min (58.8% upwind, 49.4% contact). However, instead of remaining at the same level, the attractiveness decreased with further increases of the release rate. From 1.0pg/min upward the number of induced upwind flights and source contacts declined stepwise with increasing release rates, reaching its minimum at the highest dose tested of 100'000pg/min where only 23.8% flew upwind and 2.5% contacted the source. Therefore the dose-response curves for upwind flight and contact resemble a bell shaped curve with peaks at a pheromone release rate of 1.0pg/min.

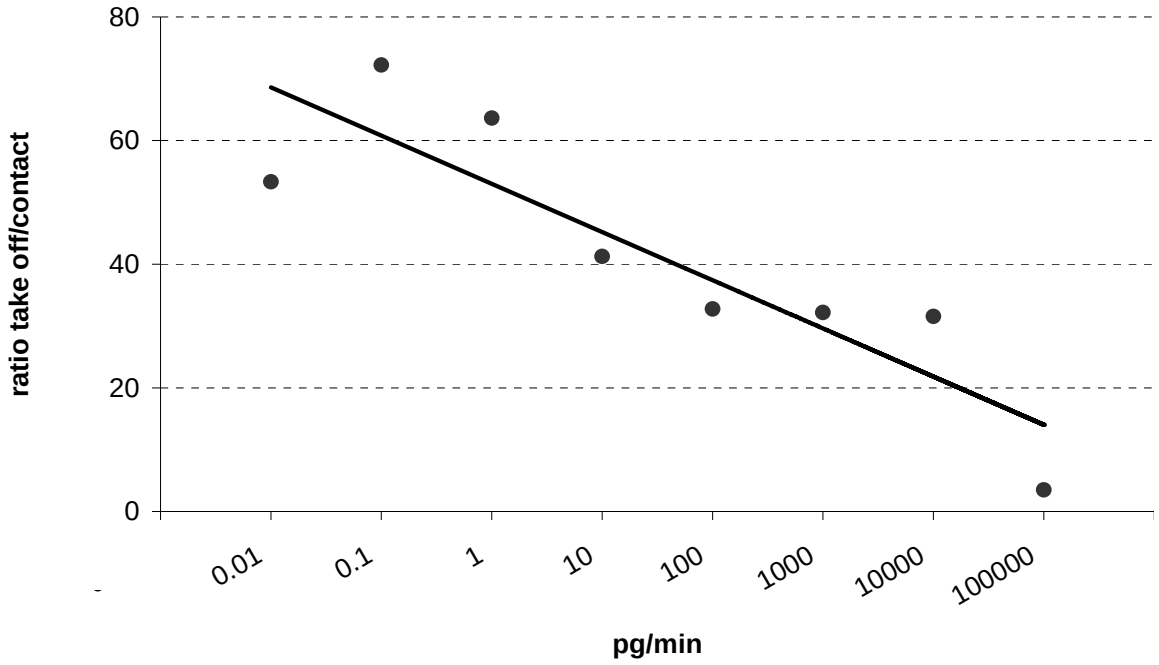

Fig. 2. 3: Proportion of males engaging consecutive behavioural elements to eight release rates of the ternary pheromone blend of E7Z9-12:Ac, E7Z9-12:OH and Z9-12:AC at 100:20:5 (Arn et al., 1988) volatilised with a piezo sprayer. The number of males succeeding to contact the pheromone source after taking off decreases with increasing pheromone release rates.

Additionally, the dose-response curves of the four behavioural elements lie close to each other for release rates between 0.01 to 1.0pg/min but diverge more and more with increasing release rates from 10 to 100'000pg/min, this is reflected in the proportion of males that succeed in taking off relative to those that performed an upwind flight up to the odour source (Fig. 2. 3). At low release rates a high proportion of males taking off succeeded to contact the source (e.g. 72.2% at 0.1pg/min). At high release rates a considerable number of males took off and engaged in upwind flight but did not succeed in contacting the odour source, resulting in a ratio of take off to source contact of 3.5% for a release rate of 100'000pg/min. Composition of the ternary blend influences the flight response of grapevine moth males (Tab. 2. 1). Comparing behavioural responses towards the two ternary blends at the same release rate resulted in significant differences for a number of behavioural elements. Increasing amounts of the secondary pheromone components E7Z9-12:OH and Z9-12:Ac increased the attractiveness of the main component E7Z9-12:Ac. For the release rates of 1.0, 10 and 100 pg/min the behavioural response of grapevine moth males towards the ternary blend 100:20:5 was statistically higher than towards the ternary blend 100:5:1 (GLM, p-value<0.05) for all behavioural elements, except for lock on at a pheromone release rate of 1.0pg/min (p-value: 0.07). At the lowest release rate of 0.1pg/min the 100:20:5 mixture initiated more males to lock on the odour plume, start an upwind flight and finally contacting the source but the differences were not statistically significant.

2.3.2 Effects of grape headspace on the flight behaviour of *L. botrana* males to their sex pheromone

Experiments were made to test the hypothesis that plant volatiles play a role in the sexual behaviour of *L. botrana* in order to establish a basis for the study. Effects of grape headspace on the behavioural response of *L. botrana* males to its pheromone were investigated in the wind tunnel. The pheromone ternary blend (E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac mixed at 100:20:5) presented using the piezo sprayer at 1.0pg/min was found to be the optimal release rate, eliciting upwind flights in 58.8% of the test males upwind flights. Vine plants (*V. vinifera* cv. Solaris) were placed behind a laminar flow screen at the upwind end of the wind tunnel in order to present the ternary pheromone blend in a background of vine headspace volatiles to the moths. Grape volatile emissions rendered the ternary blend more attractive. Compared with the control, where plant imitates rather than vine plants were presented in combination with the pheromone blend, the percentage of males responding increased for all behavioural elements (Fig. 2. 4). Significantly more males started an upwind

flight towards a plant volatile-pheromone mixture (75.3%) than to the pheromone alone (58.8%; GLM, $p<0.05$). Furthermore, the behavioural elements of passing the midline of the wind tunnel and close in on the odour source are significantly increased by the presence of grape plant volatile emissions. By presenting the grapevine and the pheromone together the contact rate of *L. botrana* males was increased by 76.5% compared to the sex pheromone in combination with artificial plants. The data indicates that the attractiveness of the ternary pheromone blend at its optimal release rate can be improved by adding plant volatiles. A more sophisticated bioassay was put in place to attempt to confirm the role that plant volatiles play in the sexual behaviour of *L. botrana*. Volatile emissions of two vine plants, each kept separately under a glass cylinder outside the wind tunnel, were introduced through a teflon tube behind the oscillating capillary of the piezo nebulizer that released the ternary pheromone blend at an underdosed level of 0.1pg/min. The behaviour of the grapevine moth males towards the pheromone was again positively affected by grape vine headspace volatiles (Fig. 2. 5).

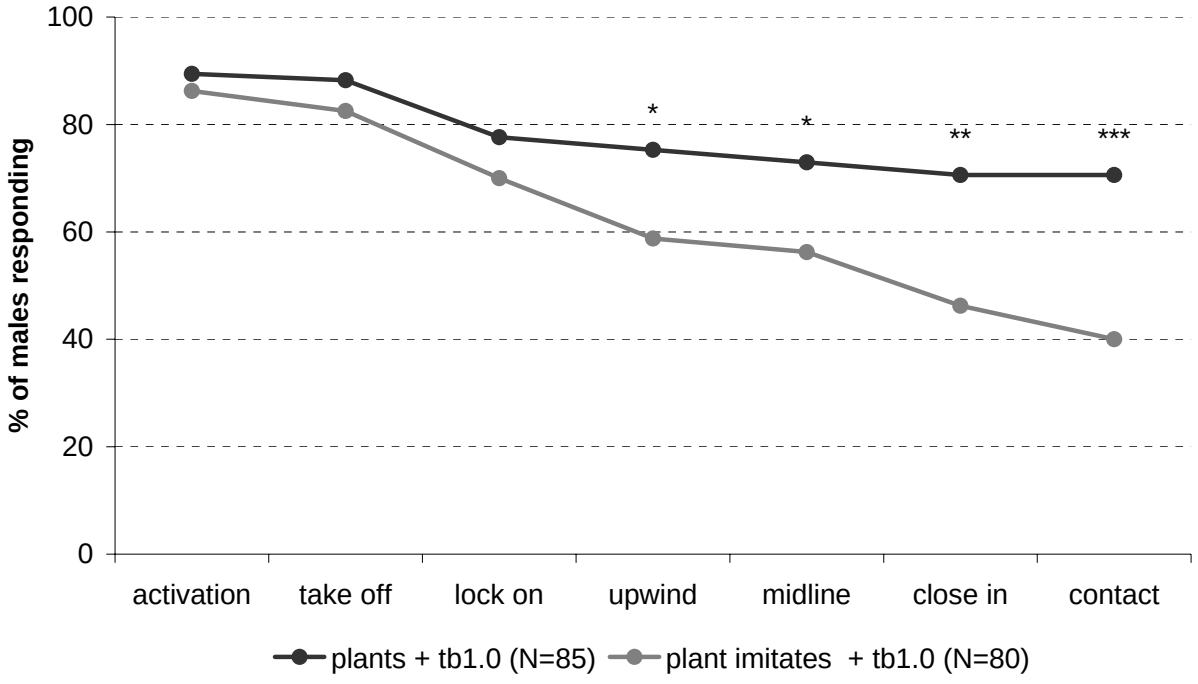

Fig. 2. 4: Behavioural response of grapevine moth males exposed to the ternary pheromone blend (released at 1.0pg/min) in combination with 8 vine plants (*Vitis vinifera* cv. Solaris) and 8 plant imitates, placed in the wind tunnel behind the laminar flow filters. Vine plant volatile emissions increase the attractiveness of the pheromone blend. Asterisks assigned within a behavioural element indicate statistically significant differences (GLM, p-values: *** ≤ 0.001 < ** ≤ 0.01 < * < 0.05).

By adding plant volatiles to the pheromone in this manner the number of males engaged an upwind flight increased to 47.5% compared to 33.8% with the pheromone presented alone. With a background of grape headspace volatiles the flight response of grapevine moth males to their sex pheromone was increased at least of 28% for all behavioural elements, except for source contact. This increase in attractiveness was significantly different for the activation behavioural element (GLM, $p<0.01$). The results confirm the insight provided by the preceding experiment and lends credence to the hypothesis that host plant compounds are used by grapevine moths to mediate sexual communication. However, we cannot rule out the possibility that alternative cues provided by the vine plants elicited the increased attractiveness of the pheromone blend. For example, carbon dioxide, humidity or other stimuli or combination of stimuli could have affected the behavioural response of males towards their sex pheromone. To demonstrate a role for plant volatile cues in the increase of attractiveness to the pheromone, grape headspace volatiles were collected and sprayed by the piezo nebulizer together with the ternary pheromone blend into the wind tunnel in order to replace any influence of other vine plant emissions.

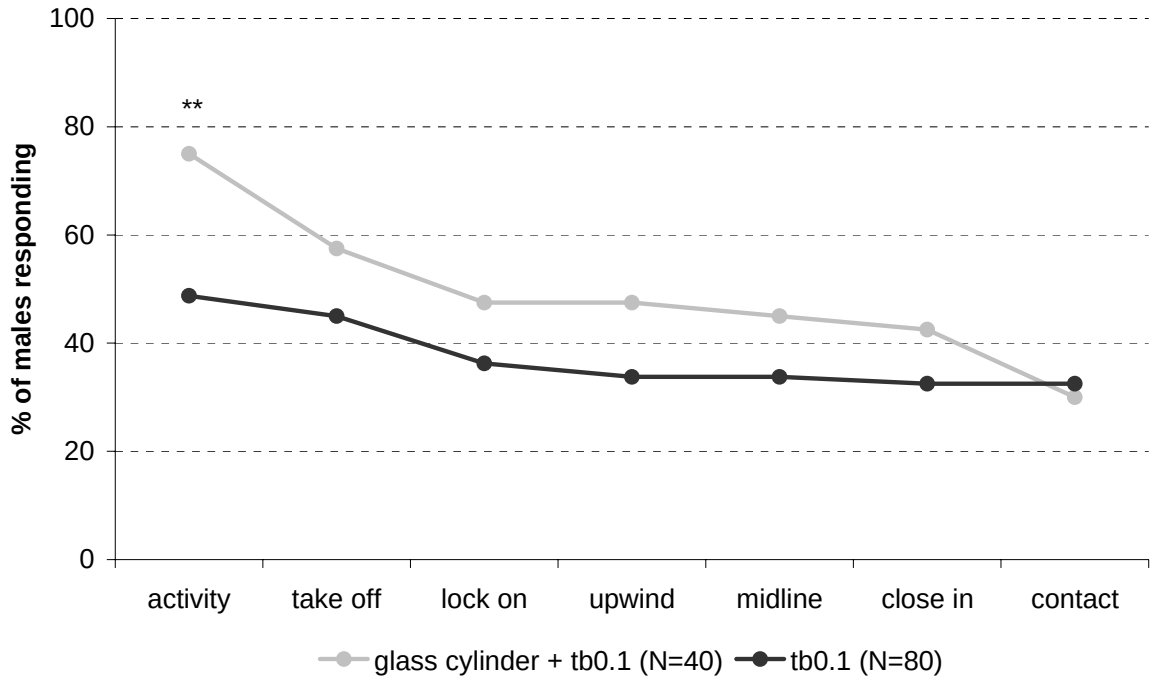

Fig. 2. 5: Behavioural response of grapevine moth males to real time headspace collected from 2 vine plants (*Vitis vinifera* cv. Solaris) kept under a glass cylinder in combination with the ternary pheromone blend released at an underdosed level of 0.1pg/min and the ternary blend alone. Grape headspace significantly increases the percentage of males getting activated. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.01$).

In advance to this experiment two headspace collection techniques were used, one using a solvent for desorbing the trapped volatiles from the porous polymer filter, the other working with thermal desorption (Fig. 2. 6) to identify constituents present in volatile emissions of vine plants by means of gas chromatograph linked mass spectrometry. A comparison of the two collection techniques revealed substantial differences both for the number of plant volatiles present in the headspace collection as well as for the ratios between the compounds present (Tab. 2. 2). Sixteen plant volatiles could be identified in vine plant headspace collections by means of the TDS technique compared to 11 with the technique using a solvent which did not contain the needed amounts for detection of benzaldehyde, 1-hexanol, (E)-2-hexen-1-ol, 1-octen-3-ol and terpinen-4-ol by mass spectrometry. In both headspace collection techniques (E,E)-α-farnesene was by far the most abundant compound. The composition was considerably different between the two collection methods. In the headspace collections using hexane to collect plant volatiles from the porous filter, (E,E)-α-farnesene was at least 12 times more abundant than other ten compounds whereas in the TDS samples the proportion was 4.5 times more of (E,E)-α-farnesene. Important variations in composition were found also for (Z)-3-hexenyl acetate, nonanal and 6-methyl-5-hepten-2-one. Our results suggest that the TDS collection technique is more sensitive and therefore allows to detect plant volatiles emitted at low release rates from vine plants.

The effect of headspace collections from vine plants on the behaviour of *L. botrana* males had been investigated at an underdosed pheromone level of 0.1 pg/min. Based on the findings of the GC-MS analysis two blends containing headspace extracts from grapevine and the pheromone were formulated, one containing 120 pg plant volatiles per µl (hs1.2) and the other one 1200 pg per µl (hs12), and released at 1.2 ng/min and 12 ng/min respectively. The effect of the headspace collections on the behavioural response of male moths towards their sex pheromone was not as strong as with the vine plants placed in the wind tunnel (Fig. 2. 7). Releasing grape headspace at 1.2ng/h with an underdosed pheromone level increased the percentage of males activated and that take off. An increase of the release rate of the grape headspace to 12ng/min led to an increase in the behavioural criteria activation, take off and lock on to the odour plume. 71.2% of males were activated when headspace collections of vine plants were admixed to the pheromone, significantly different from the 48.8% towards the ternary blend presented alone. The results again suggest a role for host plant volatiles in mate finding of *L. botrana*.

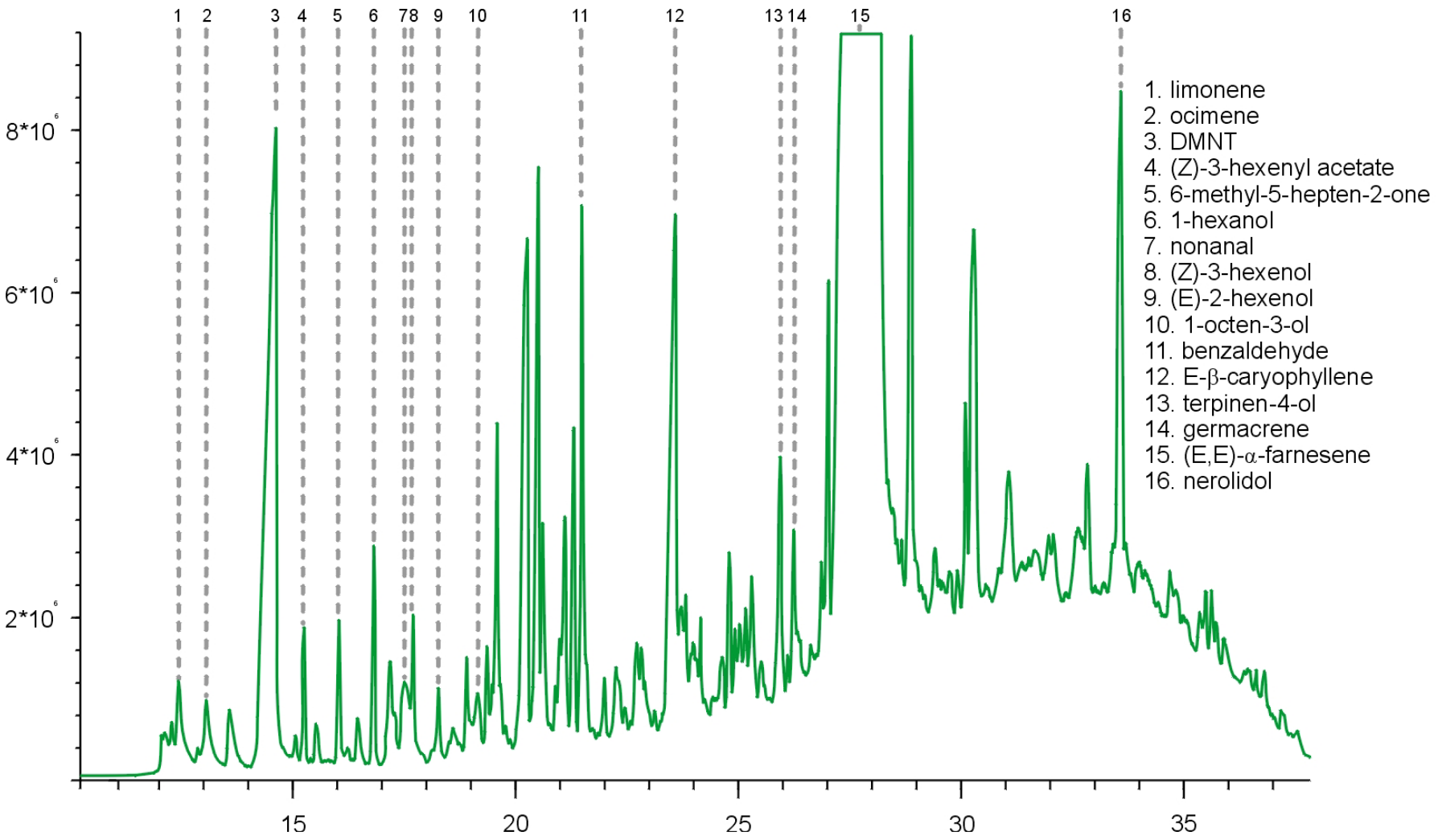

Fig. 2. 6: Gas chromatograph linked mass spectrometer analysis of a solventless headspace collection on Tenax GR from two vine plants (*Vitis vinifera cv.* Solaris) used wind tunnel studies. Numbered peaks signify plant volatiles. Identification based on Kovat's retention indices compared to synthetic standards.

Tab. 2. 2: Comparison of two headspace sampling techniques, one using a solvent (hexane, solvent) the other heat (thermal desorption system, TDS) to collect volatiles from a porous polymer (Super Q for solvent desorption and Tenax GR for TDS). Headspace collections from two vine plants (*Vitis vinifera* cv. Solaris) lasted for 24hrs. Analysis was done by gas chromatograph linked mass spectrometry. Synthetic standards were used for identification of headspace components. The TDS system detected more compounds in larger amounts. Blend composition varied considerably between the two collection methods.

			Solvent desorption		**TDS**	
chemical group	**compound**	**Kovat's index**	**proportion**	**ng/µl**	**proportion**	**ng/sample**
esters	(Z)-3-hexenyl acetate	1323	65	16.6	1	46.0
aldehydes	nonanal	1393	20	5.0	1	58.6
	benzaldehyde	1502	-[c]	-	6	216.8
alcohols	1-hexanol	1354	-	-	2	69.7
	(Z)-3-hexen-1-ol	1380	3	0.9	1	47.0
	(E)-2-hexen-1-ol	1397	-	-	1	27.6
	1-octen-3-ol	1447	-	-	1	20.1
monoterpenes	limonene	1191	4	1.1	1	51.7
	ocimene	1253	6	1.6	2	59.1
	terpinen-4-ol	1594	-	-	3	100.7
irregular terpenes	dimethyl nonatriene[a]	1313	27	7.0	20	788.9
	6-methyl-5-hepten-2-one	1342	12	3.0	1	49.9
sesquiterpenes	E-β-caryophyllene	1589	10	2.6	10	388.6
	germacrene	1694	3	0.6	1	55.0
	(E,E)-α-farnesene	1734	803	205.4	90	3486.4
	nerolidol[b]	1867	9	2.4	6	249.7

[a] (E)-4,8-dimethyl-1,3,7-nonatriene

[b] (E)-3,7,11-trimethyl-1,6,10-dodecatrien-3-ol

[c] not found in headspace sample

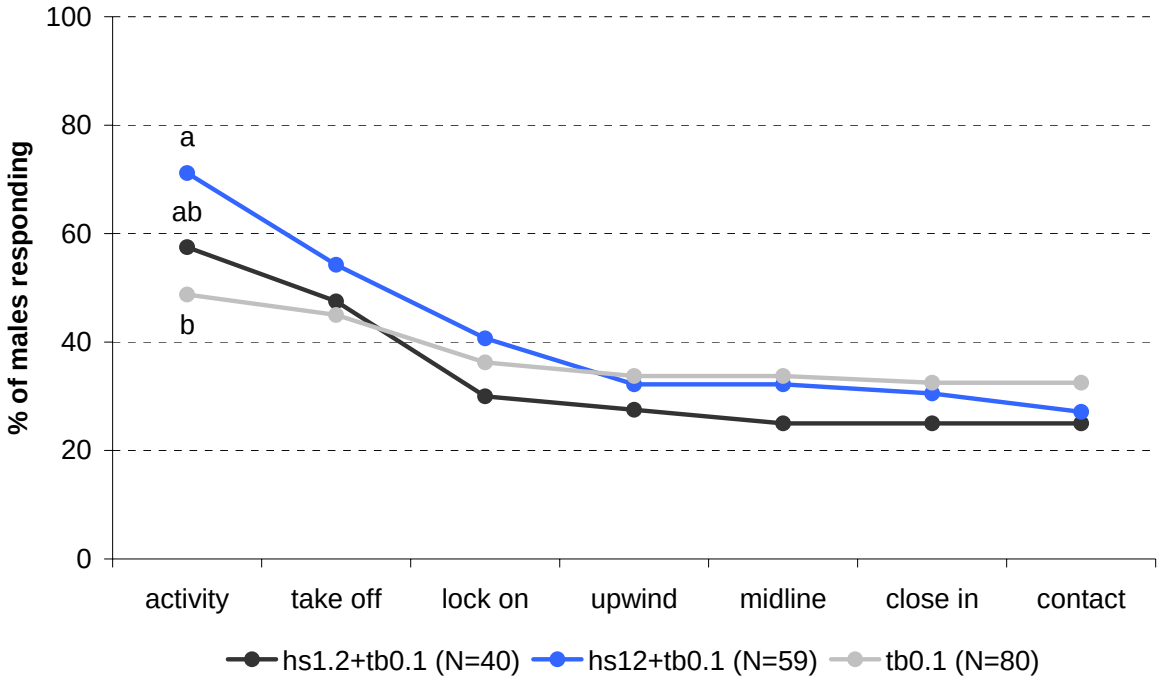

Fig. 2. 7: Influence of *Vitis vinifera* cv. Solaris headspace on attraction of *L. botrana* males to their ternary pheromone blend. Headspace collections were released at two rates (hs1.2=1.2ng/min and hs12=12.0ng/min) in combination with a pheromone release rate of 0.1pg/min. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).

2.3.3 Tracing key host plant stimuli – chemosensory approach

Five different headspace samples were analysed using gas chromatography linked electroantennogram recording to identify plant volatiles that are present in several host plants that elicit antennal receptor cell responses in grapevine moth males. In parallel, all constituents of headspace samples were identified by gas chromatography linked mass spectrometry. It must be noted that the MS detector is about 10 times less sensitive than the FID detector, such that compounds present at low amounts in headspace samples were below the threshold for valid MS-identification. Headspace collections obtained from wild vine female and male flowers (*Vitis vinifera sylvestris*) contained with 21 and 20 active constituents, respectively, the highest numbers of plant volatiles to which electroantennogram responses (EAG) could be recorded (Fig. 2. 8), followed by olive tree (*Olea europea*) headspace collections with 17 biologically active constituents (Fig. 2. 10), and finally rosemary (*Rosmarinus officinalis*) and European privet (*Ligustrum vulgare*) extracts with 16 and 15 plant volatiles respectively (Fig. 2. 9).

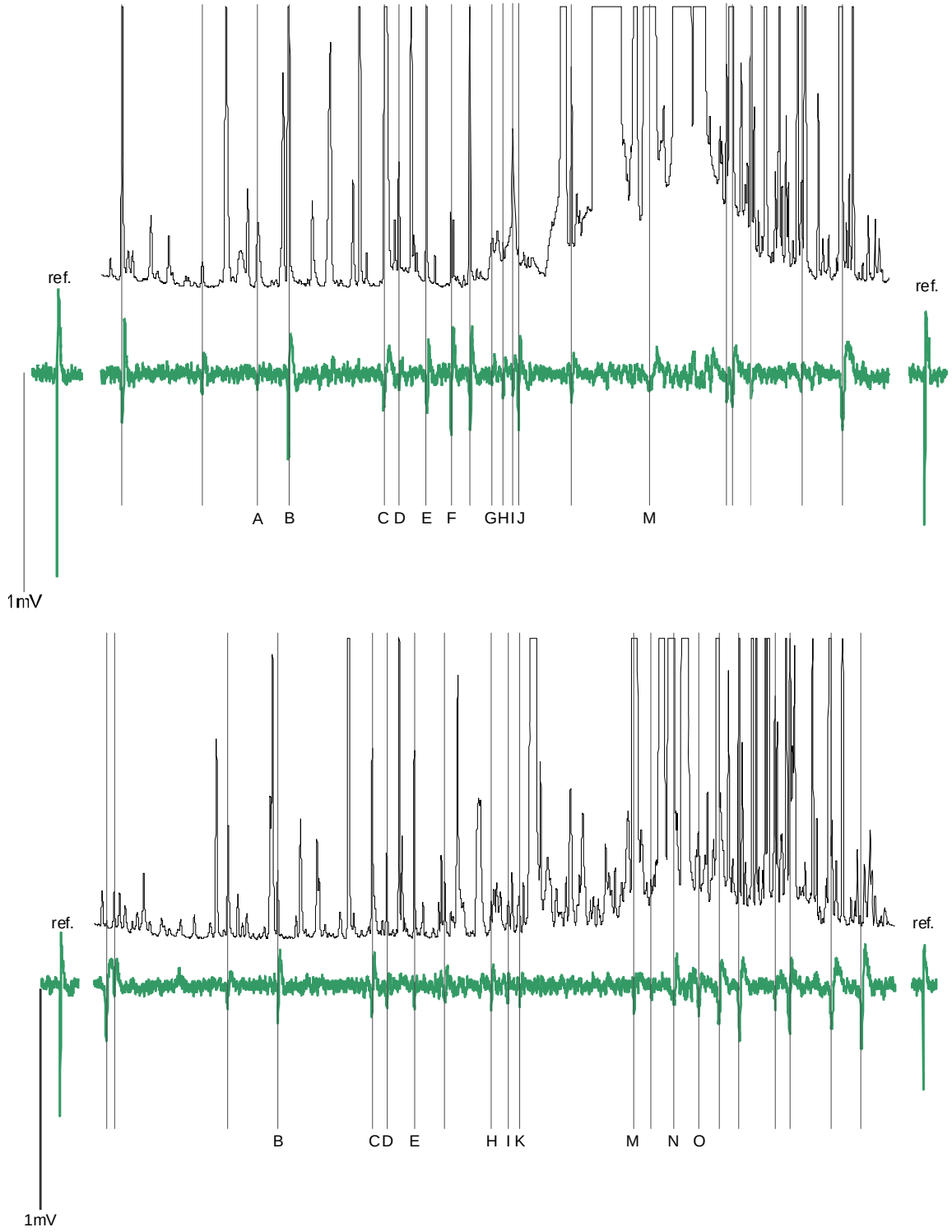

Fig. 2. 8: Antennogram responses of male *L. botrana* to 1ul of the headspace extract of *Vitis vinifera sylvestris* male flowers (above) and female flowers (below). Lines indicate constituents eliciting repeated EAG responses in three different analyses of a given extract using antennae from different individuals. Lettered constituents were found to elicit EAG response in at least three headspace collections from different host plants: A limonene, B (E)-2-hexanal, C (Z)-3-hexenyl acetate, D 6-methyl-5-hepten-2-one, E 1-hexanol, F (Z)-3-hexenol, G unknown, H 1-octen-3-ol, H unknown, I unknown, K benzaldehyde, M (E)-β-farnesene, N (E,E)-α-farnesene and O methyl salicylate.

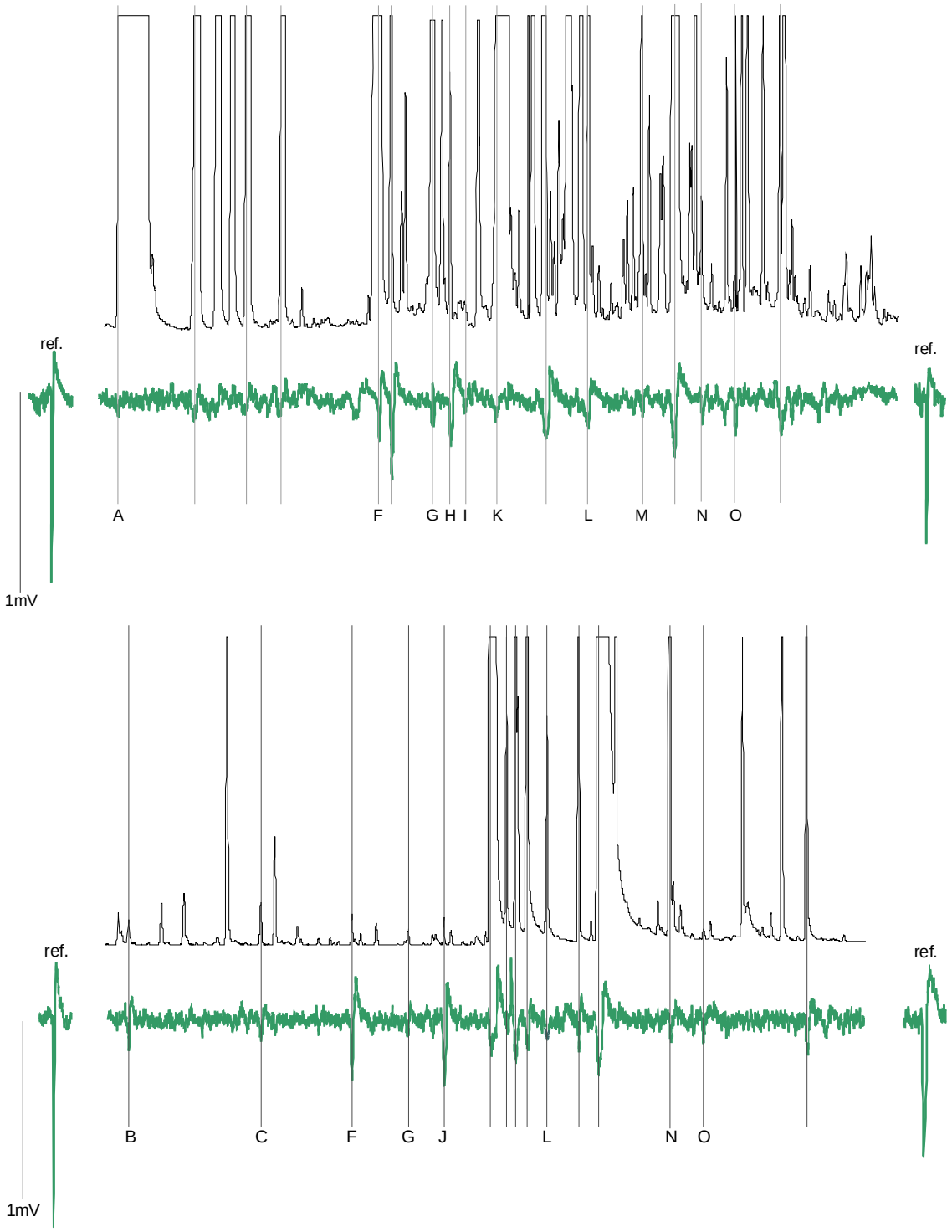

Fig. 2. 9: Antennogram responses of male *L. botrana* to 1ul of the headspace extract of *Rosmarinus officinalis* (above) and *Ligustrum vulgare* (below). Lines indicate constituents eliciting repeated EAG responses in three different analyses of a given extract using antennae from different individuals. Lettered constituents were found to elicit EAG response in at least three headspace collections from different host plants: A limonene, B (E)-2-hexanal, C (Z)-3-hexenyl acetate, F (Z)-3-hexenol, G unknown, H 1-octen-3-ol, I unknown, J unknown, K benzaldehyde, L (E)-β-caryophyllene, M (E)-β-farnesene, N (E,E)-α-farnesene and O methyl salicylate.

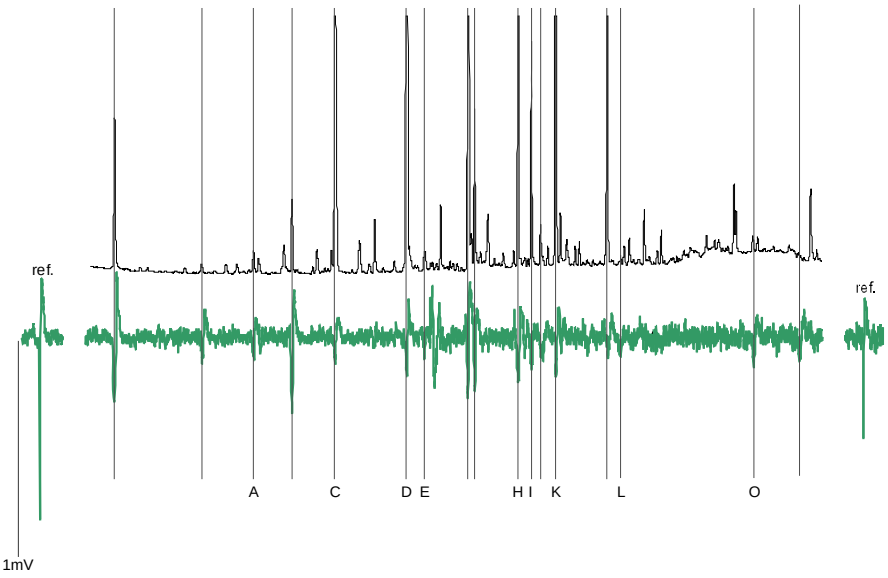

Fig. 2. 10: Antennogram responses of male *L. botrana* to 1ul of the headspace extract of *Olea europea*. Lines indicate constituents eliciting repeated EAG responses in three different analyses of a given extract using antennae from different individuals. Lettered constituents were found to elicit EAG response in at least three headspace collections from different host plants: A limonene, C (Z)-3-hexenyl acetate, D 6-methyl-5-hepten-2-one, E 1-hexanol, H 1-octen-3-ol, I unknown, K benzaldehyde, L (E)-β-caryophyllene and O methyl salicylate.

A comparison of the recordings resulted in 15 plant volatiles that occurred at least three times across the five headspace extracts and elicited EAG responses repeatedly in grapevine moth males (Tab. 2. 3). The plant volatiles can be divided into five chemical classes: (1) ester ((Z)-3-hexenyl acetate), (2) aldehydes ((E)-2-hexenal and benzaldehyde), (3) alcohols (1-hexanol, (Z)-3-hexanol and 1-octen-3-ol), (4) an aromatic compound (methyl salicylate) and (5) terpenes (limonene, 6-methyl-5-hepten-2-one, (E)-β-caryophyllene, (E)-β-farnesene and (E,E)-α-farnesene). Three of the 15 chemostimulants could not be identified. For the 15 commonly occurring chemostimulants present in 1µl of the headspace extract GC peak areas were determined (GcEAD Software, Syntech, NL) and divided in five categories based on abundance: (1) 0-200 units, (2) 201-400 units, (3) 401-600 units, (4) 601-800 units and (5) >800 units. Relative EAG responses to these constituents were compared to a 1s air puff from a 5ml stimulus syringe containing 10µg (Z)-3-hexenol on a filter paper strip (Tab. 2. 3). Except for (E)-β-caryophyllene, all plant compounds were present in the headspace extracts from wild vine. Methyl salicylate, (Z)-3-hexenol and 1-octen-3-ol were found in four of the five headspace extracts. Some compounds such as (Z)-3-hexenol, methyl salicylate and 1-hexanol elicited EAG responses at remarkably low doses (Tab. 2. 3, compounds E, F and O in Fig. 2. 8 and Fig. 2. 9).

Tab. 2. 3: Compounds eliciting EAG responses present in at least three extracts of *L. botrana* host plants. Averaged EAG responses are divided into five classes: 1=0-20%, 2=21-30%, 3=31-40%, 4=40-50%, 5>50% relative to the response to the reference (1s air puff for a 5ml stimulus syringe containing a filer paper loaded with 10µg of (Z)-3-hexenol). Peak areas are also divided into five classes: 1=0-200 units, 2=201-400 units, 3=401-600 units, 4=601-800 units, 5>800 units. Compounds with a high number for the EAG response and a low number for peak area indicate a strong chemostimulant with a strong antennal response at a low amount in the extract.

	***V. vinifera sylvestris* male flowers**		***V. vinifera sylvestris* female flowers**		***R. officinalis* leaves**		***O. europea* leaves**		***L. vulgare* flowers**	
	EAD	GC area	EAD	GC area	EAD	GC area	EAD	GC area	EAD	GC area
Ester										
(Z)-3-hexenyl acetate	2	4	2	2					3	1
Aldehydes										
(E)-2-hexenal	5	5	3	3					3	1
benzaldehyde			2	1	2	5	3	5		
Aromatic compound										
methyl salicylate			3	2	3	1	4	1	4	1
Alcohols										
1-hexanol	3	3	2	2			2	1		
(Z)-3-hexenol	4	1			3	5	2	1	4	1
1-octen-3-ol	2	1	3	1	2	4	4	4		
Terpenes										
limonene	1	2			2	3	1	1		
6-methyl-5-hepten-2-one	2	2	2	1			2	5		
(E)-β-caryophyllene					3	5	2	1	2	3
(E)-β-farnesene	2	5	3	5	3	5				
(E,E)-α-farnesene			2	5	3	2			4	5
unknown (KI 1437)	2	1			2	4			3	1
unknown (KI 1466)	2	2			2	1	3	3		
unknown (KI 1478)	4	1	4	1					4	1

2.4 Discussion

Behavioural response of grapevine moth males to the ternary pheromone blend and validation of behavioural assay methods

To validate our behavioural assay methods and to investigate the behavioural response of grapevine moth males to their sex pheromone, two blends containing the three major sex pheromone components were tested. The sex pheromone of *L. botrana*, which has been studied since the 1970s, consists of the main compound (E,Z)-7,9-dodecadienyl acetate (E7Z9-12:Ac) plus at least five other compounds eliciting EAG responses: (E,Z)-7,9-dodecadien-1-ol (E7Z9-12:OH), (Z)-9-dodecenyl acetate (Z9-12:Ac), (E)-9-dodecenyl acetate, (E)-7-dodecenyl acetate and (7E,9E,11)-7,9,11-dodecatrienyl acetate (Buser et al., 1974; El-Sayed et al., 1999b; Roelofs et al., 1973; Witzgall et al., 2005). It was found that not all these pheromone components are needed to achieve maximum attraction of *L. botrana* males (El-Sayed et al., 1999b) suggesting redundant components in the pheromone of *L. botrana*, as already described for the cabbage looper *Trichopulsia ni* (Linn et al., 1984). Two synthetic pheromone mixtures, containing E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac, are described in the literature to be highly attractive to *L. botrana* males. Based on chemical analysis of sex glands of *L. botrana* females and subsequent behavioural tests, Arn and his colleagues (1988) state that a ratio of 100:20:5 of the three main pheromone components on rubber septa to be optimal for *L. botrana* attraction in the field. Later El-Sayed et al. (1999b) showed in a wind tunnel study that a ratio of E7Z9-12:Ac, E7Z9-12:OH, Z9-12:Ac at 100:5:1 released from a piezo sprayer to be more attractive than a 100:20:5 mixture. We decided to test these two mixtures at four different release rates (0.1, 1.0, 10 and 100pg/min of E7Z9-12:Ac) and to compare the flight responses of grapevine moth males. In our wind tunnel assays the 100:20:5-blend was more attractive than the 100:5:1-blend at all release rates and could not confirm the wind tunnel data of El-Sayed and his colleagues (1999b). Analyses of female sex glands revealed a ratio of 100:25:8 (Arn et al., 1986) and 100:10:13 (Witzgall et al., 2005). Synthetic pheromone blends admixed at ratios present in female sex glands have been shown to be optimal for several moth species such as *Heliothis virescens* (Teal et al., 1986), *Cydia pomonella* (Arn et al., 1985) and *Grapholita molesta* (Baker et al., 1980; Baker et al., 1981). Some studies have demonstrated that the closer a synthetic blend is to that produced by the calling female, the straighter the flight and the faster the males approach the odour source (Palaniswamy et al., 1983; Quartey and Coaker, 1993). Since the 100:20:5 blend

is closer to the amounts in the female sex glands than the 100:5:1 blend, and attracted significantly more *L. botrana* males in the wind tunnel, we suggest used the 100:20:5 blend for all following experiments.

Grapevine moth males respond in a dose-dependent manner to the ternary pheromone blend. The optimal release rate of the ternary blend was found to be 1.0pg/min referring to the main pheromone component E7Z9-12:Ac with similar percentages of males flying upwind and contacting the odour source as for calling females (Annex B). The finding fits well with the literature. Female sex gland extracts (FE) released from a piezo sprayer as used here elicited the highest percentage of males contacting the source when released at 0.01 FE/min, which corresponds to 8 ± 4 pg/min of E7Z9-12:Ac (El-Sayed et al., 1999b; Witzgall et al., 2005). An optimal pheromone release rate in the order of 1.0pg/min is also confirmed by earlier wind tunnel data obtained using rubber septa as a pheromone dispenser: Arn et al. (1988) found a rubber septum loaded with 10µg E7Z9-12:Ac, 2.0µg E7Z9-12:OH and 0.5µg Z9-12:Ac to be the optimum lure for grapevine moth males, which results in an estimated release rate of 8pg/min of E7Z9-12:Ac (Butler and McDonough, 1979; Heath et al., 1986).

Exposing grapevine moth males to much high release rates of the pheromone (e.g. 100'000pg/min) an interesting phenomenon was observed in that a high percentage of males moths were activated and took off but did not succeed to contact the source. Instead of accomplishing the flight to the source the *L. botrana* males showed in-flight arrestment by hovering downwind from the odour release point and did no longer progress upwind. In flight arrestment has so already been documented for several moth species flying to overdose pheromone sources (Baker et al., 1981; Schmidt-Buesser, 2008) not unique to *L. botrana*. Our observation, however documents again the how the grapevine moth male olfactory and central nervous systems are extraordinarily well adapted to physiologically relevant odour plume doses. Odour plumes from females do not provide a gradient that guide males upwind. The plume from females is rather filamentous with a highly inhomogeneous odour distribution (Murlis et al., 1992). Male moths flying in such filamentous plumes will receive brief impulses of pheromone at a relatively constant concentration whenever it traverses a pheromone-loaden filament. Behavioural experiments with *Bombyx mori* confirmed that the optimal stimulus is an intermittent rate of 3Hz (Kramer, 1986). In contrast, with constant pheromone stimulation *B. mori* did not respond anemotactically although the receptor cells fired constantly (Kaissling et al., 1989). Receptor cells can resolve pheromone pulses up to a frequency of 10Hz and projection neurons of the antennal lobe show similarly high temporal

resolution (Christensen and Hildebrand, 1988), suggesting that higher-order neurons in the CNS need intermittent impulses. Releasing the grapevine moth ternary pheromone blend at the release rate of 100'000pg/min (≈10'000 times higher than what a female releases) did not provide constant pheromone stimulation for males but close to the source the odour filament density was probably very high pushing the signal analysis by male moths to its limit. Support for this was found in the very low ratio of only 3.5% grapevine moth males that took off and successfully reached the odour source at the release rate of 100'000pg/min compared to 72.2% at the pheromone release rate of 0.1pg/min. These results provide a solid basis for further experimental testing of natural and synthetic plant products in the combination with the ternary pheromone blend. Furthermore, we can test possible effects of plant volatiles at under-, optimal- and overdosed pheromone levels. This is important for a comprehensive approach taking in account possible future field applications, that are based on different pheromone concentrations used in the attract-and-kill strategy using rather low pheromone doses or mating disruption where dispensers with very high pheromone loads are deployed.

Effects of grape headspace volatiles on the sex pheromone responses of the grapevine moth *L. botrana*

The context in which the ternary pheromone blend is presented affects considerably its attractiveness to grapevine moth males. The experiments where grapevine volatile emissions were presented in the wind tunnel in combination with the pheromone showed that an increase in the behavioural response of grapevine moth males at underdosed and even at optimum pheromone levels can be obtained. This suggests that plant compounds may have a synergistic effect on the attraction of *L. botrana* males to sex pheromone. These insights provide evidence for our hypothesis that host plant compounds are used by grapevine moths to mediate sexual communication as a strategy for optimised reproduction. Effects of volatile emissions from plants on the attractiveness of the sex pheromone have been described for several herbivorous insects in laboratory and field studies. Groot and Visser (2001) showed effects of plant volatiles on the sex pheromone of the herbivorous bug *Lygocoris pabulinus,* where behavioural experiments showed, that females presented in combination with host plant material were significantly more attractive to males than females. Another case of effects of the host plant on sexual attraction was found for *Trichopulsia ni* where unmated female cabbage loopers placed in a cage in a flight tunnel induced more source contacts by males when the cage contained a bouquet of cotton foliage (Landolt et al., 1994). Light et al. (1993) could show similar effects of host plant odours in a field study where male corn earworm

moths were trapped in greater number when pheromone traps were placed near corn plants. These studies indicate an enhancement or synergism of male responses to female pheromones by plant volatiles from their respective host plants.

We cannot rule out the possibility that other cues provided by the vine plants elicited the increased attractiveness of the ternary pheromone blend in *L. botrana*. Carbon dioxide could especially affect the behavioural response of grapevine moth males towards their sex pheromone. Sensory organs that are specialised to the detection of CO_2 find their strongest expression in the almost exclusively herbivorous Lepidoptera (Bogner et al., 1986; Kent et al., 1986), with moths detecting changes in environmental CO_2 levels with an extremely high sensitivity as shown for *Helicoverpa armigera*, where the threshold for fluctuations around the atmospheric background of 350 ppm was 0.5 ppm (Stange, 1992). CO_2 has been demonstrated to influence oviposition behaviour of the pyralid moth *Cactoblastis cactorum* (Stange, 1997) and of the sphingid moth *Manduca sexta* (Abrell et al., 2005) and, furthermore, nectar foraging in *M. sexta* (Goyret et al., 2007). Supplementary wind tunnel experiments were carried out with *L. botrana* males to investigate effects of CO_2 on the attractiveness of the pheromone (Annex C). Even at low concentrations (<7.0ppm increase over the ambient CO_2 level at the moth release point) no enhancing effects on the response to the sex pheromone in *L. botrana* males were recorded. This suggests a role for host plant volatile cues for the increased attractiveness of the pheromone.

To exclude other cues, volatile emissions from vine plants were collected on Porapak Q, and the resulting extract was diluted to the main component (E,E)-α-farnesene at 120 and 1200pg/µl and was then released using the piezo sprayer at a rate of 10 µl/min with the underdosed pheromone into the wind tunnel. A significant higher number of grapevine moth males were activated when the pheromone was released at 0.1pg/min with the host plant volatile bouquet at 12'000pg/min but none of the other behavioural elements was significantly affected. The effect of the plant volatiles on the attractiveness of the pheromone was not as strong with the solvent desorbed volatiles as observed in the preceding experiments. The average amount of (E,E)-α-farnesene collected from a vine plant was 210ng/h, which corresponds to measurements by Tasin et al. (2005), who measured an amount of 280ng/h of (E,E)-α-farnesene from 200gr of green grape berries. By comparison, the release rate of the volatile extract in the wind tunnel experiment was set to 61ng/h and 610ng/h of (E,E)-α-farnesene respectively. Headspace collections on Porapak Q filters contain at least part of the odour cues responsible for the increased male attraction to the ternary pheromone

blend, as evidenced by the significantly higher response to the sprayed extract combined with the pheromone. However, both the release rate and composition of the blend were probably suboptimal. The composition of the bouquet emitted from plant material is likely to be different from the blend of compounds extracted from the Porapak Q air filters, both in terms of quality and quantity of the plant volatiles. The extract of headspace volatile collected is strongly influenced by the physical and chemical properties of the trapping agent, the concentration of the analytes in the headspace, the chemical properties of the analytes (differences in the analytes volatility affect passage through the trapping agent and differences in the analytes affinity to the glass cylinder holding the vine plants affect the ratio in the headspace), the solvent used for desorption. As a result a distortion of the blend is likely to occur (Agelopulos and Pickett, 1998; Raguso and Pellmyr, 1998). A comparison between the two host plant volatile collection techniques used in our study indeed revealed considerable variations concerning the blend composition and ratios between constituents. Firstly, in the headspace extract used for wind tunnel assays only 11 compounds were identified compared to 16 with the solventless collection method. It is therefore possible that some host plant volatiles that are essential for male attraction were absent in the headspace extract. Furthermore distorted ratios between products can be the cause of lack of biological activity. A 3-component blend of plant volatiles that induced upwind flight in grapevine moth females was non-attractive when presented in an altered ratio (Tasin et al., 2006b). The solventless headspace collection reflects more precisely the proportions of natural grape headspace constituents, since less working steps are included in their analysis and a distortion due to solvent desorption can be avoided. No single plant volatile is unique to grapevine and a mixture of compounds is expected to mediate attraction *L. botrana* males to grapevine. Furthermore it is likely that proportions are crucial and more behavioural experiments using single plant volatiles and their mixtures are essential in order to reconstruct an approximative blend emitted by host plants.

Chemosensory responses to host plant volatiles by male *L. botrana* antennal receptor cells

Having provided overall evidence for the importance of plant volatiles in the sexual behaviour of *L. botrana,* we then searched for chemostimuli in the of headspace volatile emissions of different host plants that elicit responses from males grapevine moth antennal receptor cells. Since *L. botrana* is polyphagous we hypothesize that plant compounds that occur in the headspace over different host plants can play an important role in the interactions between

plant volatiles and sex pheromones. Five headspace collections from four different host plants, *Vitis vinifera* ssp. *sylvestris, Ligustrum vulgare, Olea europea and Rosmarinus officinalis* (Galet, 1982; Katerinopoulos et al., 2005; Savopoulou-Soultani et al., 1990; Stoeva, 1982), were made and analysed using the GC-EAD technique. Our results indicate that *L. botrana* males respond selectively to some plant volatiles, since not all headspace constituents elicit EAG responses. The relative sensitivity of grapevine moth males to plant volatiles compared with the main pheromone component E7Z9-12:Ac is much lower (data not shown). To perceive female produced sex pheromones at very low concentrations nature has equipped male moths with an array of antennal receptor neurons located in long sensilla trichodea (Schneider and Steinbrecht, 1968; Steinbrecht, 1970). However, wall-pore sensilla basiconica containing receptor neurons for plant volatiles are also present on male moths antennae (Pophof et al., 2005) but grapevine moth male antenna probably carry primarily sensilla trichoidea as shown for other moth species (George and Nagy, 1984; Steinbrecht et al., 1995). Our results show that grapevine moth males sense a broad spectrum of plant volatiles, with 15 products that elicit EAG responses present in at least three out of the five headspace extracts. The chemostimuli belong to different chemical classes: aliphatic esters, alcohols and aldehydes, aromatic compounds and terpenes. None of the compounds is unique to the host plants of *L. botrana* as they are all commonly occurring in plants belonging to different families (Knudsen et al., 1993). Twelve out of the 15 plant volatiles could be identified. For *L. botrana* females EAG responses were already documented in *L. botrana* females for 8 out of the 12 host plant volatiles identified here (Tasin et al., 2005; Tasin et al., 2007). Responses of male moth antennal olfactory receptor cells to plant compounds have also been documented for *Cydia pomonella* (Ansebo et al., 2004), *Spodoptera exigua* (Dickens et al., 1993) and *Manduca sexta* (Fraser et al., 2003). Schmidt-Buesser (2008) analysed the same headspace extracts using male antennae of *Eupoecilia ambiguella*, a sister species to *L. botrana*, and documented EAG responses to 11 of the 12 compounds identified as chemostimulants for *L. botrana*. Our results suggest that the two species, that share at least 13 host plants (Galet, 1982), use many of the same secondary plant volatiles for host localization. These results indicate that host plant volatiles could influence the sex pheromone response of *L. botrana*, since we show that male antennal olfactory receptor cells respond selectively to plant volatiles. The solventless headspace collections from the vine plants that rendered the ternary pheromone blend more attractive to *L. botrana* males contained nine out of the 12 commonly occurring plant compounds identified as chemostimulants, i.e.

(Z)-3-hexenyl acetate, benzaldehyde, 1-hexanol, (Z)-3-hexenol, 1-octen-3-ol, limonene, 6-methyl-5-hepten-2-one, (E)-β-caryophyllene and (E)-β-farnesene.

The insights presented here show that *L. botrana* males are sensitive to host plant volatiles and that such chemicals from host plants enhance responses to the female sex pheromones. From an evolutionary point of view it is likely that grapevine moth males developed a strategy to use host plant volatiles as additional cues to find females. Firstly, the biosynthesis of secondary plant compounds and pheromones are similar and closely associated. The biosynthetic pathways leading to important plant volatiles have been traced back to intermediates of primary metabolism (Croteau and Karp, 1991). It has been shown carbohydrates, amino acids and fatty acids represent the natural carbon pools for flavor compounds (Schwab et al., 2008). It is therefore possible that the pheromone communication system evolved after the kairomones that were exploited by gravid females to locate suitable oviposition sites. Rather than developing and dedicating an entirely unique set of enzymes for pheromone biosynthesis, lepidopterans appear to have added one or a few enzymes that serve to transform the products of "normal" metabolism to pheromone compounds of high stereochemical specificity. Female-produced sex pheromones derive from fatty acids (Tillman et al., 1999) as do a wide range of plant volatiles. The close relationship between plant volatiles and pheromones is exemplified in some insects that even acquire host plant chemicals to sequester them as sex pheromones or sex pheromone precursors (Reddy and Guerrero, 2004).

It is documented that host plants can serve as a mating sites for insects, with sexual behaviour occurring principally or exclusively on host plants (Visser, 1986), i.e. encounter sites are focused on resources attractive to females (Thornhill and Alcock, 1983). Offspring survival can be maximized when mating occurs directly on a suitable host plant. Grapevine moth females, already using plant volatiles as cues for host location (Masante-Roca et al., 2007; Tasin et al., 2006a), sitting on a host plant would thus release their sex pheromone simultaneously with the volatile emissions from the host plant to be mixed and arrive concurrently at the male grapevine moths olfactory sensory system. Grapevine moth males using plant volatiles for mate-finding would increase mating success in three ways: (1) Males able to exploit additional cues leading them to females would optimize their mating success when pheromone signals are just present at threshold levels; (2) males able to distinguish host from non-host plants, could reduce the number of energetically expensive flights and therefore increase their mating success; (3) furthermore, males that are able to localize host

plants in the absence of pheromone would also increase their competitive chances to find a mate later. Since oviposition sites may also serve as mating sites, mating success is probably highest for males that arrive at such places before the onset of calling by females. The fact that male *L. botrana* emerge before females (protandry) supports this theory. These new insights in the sexual behaviour of *L. botrana* opens new possibilities to affect the behavior of male moths and therefore to improve semiochemical-based control methods.

2.5 References

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CHAPTER 3

Host plant volatiles affect the behavioural response of male grapevine moths, *L. botrana*, to their sex pheromone

Abstract

Host plant volatiles increase the attraction of *L. botrana* males at an underdosed pheromone level as measured by the number of males contacting the odour source. Ten commonly occurring host plant volatiles known to elicit electroantennogram responses in male *L. botrana* were tested in a wind tunnel. Two-component blends of 0.1pg/min pheromone and 100pg/min of either (E)-β-caryophyllene, 1-hexanol, (Z)-3-hexenyl acetate or 1-octen-3-ol had the strongest effects on the attractiveness of the pheromone lifting the activity of the underdosed pheromone release rate up to that of the optimal pheromone release rate of 1.0pg/min. A general pattern concerning dose-response characteristics of male responses was established with an optimal plant volatile release rate at around 100pg/min for a pheromone release rate of 0.1pg/min. In the case of (E)-β-caryophyllene a large window of biological activity was recorded, ranging from 1pg/min to 10'000pg/min combined with the pheromone release rate of 0.1pg/min. (E)-β-caryophyllene proved its importance in the sensory ecology of *L. botrana* also in field experiments where traps dispensing pheromone and (E)-β-caryophyllene captured significantly more males than traps emitting pheromone alone.

Keywords: *Lobesia botrana*, host plant volatiles, sex pheromone, synergism, mate-finding, wind tunnel, field, (E)-β-caryophyllene

3.1 Introduction

Plant volatile – pheromone interactions have already bee investigated for other moth species, such as the European grape berry moth *Eupoecilia ambiguella*, the codling moth *Cydia pomonella* and the beet armyworm *Spodoptera exigua* (Deng et al., 2004; Schmidt-Buesser, 2008; Yang et al., 2004). These studies showed that the attractiveness of the sex pheromone can be increased by presenting it in combination with one or more host plant volatiles. In chapter 2 we established that vine plant volatile emissions enhance the behavioural response of grapevine moth males and identified 12 potential host plant chemostimuli that elicit male antennal receptor cells. However, the sensory code by which host plant volatiles influence grapevine moth male behaviours in recognizing and locating mating sites remains unknown. The challenge is therefore to read the odour bouquet perceived by the males from host plants and to identify the active chemicals via behavioural analysis using plant products. Knowledge of the plant products playing a role and the behavioural mechanisms involved in host recognition is fundamental for the study of plant–insect interactions. Furthermore, the identification of plant volatiles that have additive affects on the attractiveness of the sex pheromone could open new possibilities for the improvement of pheromone-based control methods and must not be underrated. However, the inclusion of plant volatiles in pheromone based control methods such as mating disruption often has to face a major critique concerning the background odours that are already present in the vineyards that could render the applied chemostimuli redundant. It has to be stressed that the moths' sensory organs have evolved to detect minute amounts of chemostimuli and is highly tuned to the detection of contrasts in background levels of odours. Valeur and Löfstedt (1996) carried out an important experiment on male oriental fruit moths, *Grapholita molesta*, demonstrating the moths capabilities to follow an attractive odour plume in the context of background odours. They studied the behaviour of male oriental fruit moths to two overlapping sex pheromone plumes in a wind tunnel, one mimicking the composition and release rate close to that of a female, the other being a suboptimal pheromone mixture in terms of the release rate or blend composition. In all tests males clearly discriminated between different blends and doses in the overlapping plumes of pheromone, for regardless of the lures presented, in series or in parallel, males followed the optimised plume. This demonstrates the capacity of moths to distinguish between overlapping plumes of volatile chemical cues and suggests that plant volatiles combined with the pheromone could affect the behaviour of moths in the field, despite the fact that a vineyard atmosphere may already contain high levels

of plant volatiles. Comparison of the headspace volatiles of different *L. botrana* host plant by GC-EAD identified 15 key chemostimuli for males (see section 2.3.3) but we do not know whether these stimuli play a role in pheromone perception by male grape berry moths. We set out to test 10 plant compounds in combination with an underdosed level of *L. botrana* pheromone for their behavioural activity on males in the wind tunnel and in parallel field tests. The purpose of this study was to identify behaviourally active plant volatiles for grapevine moth males, explore dose response caracteristics of plant volatile release with the sex pheromone and study the effects of a mixture of plant volatiles on the attractiveness of the sex pheromone.

3.2 Material and Methods

Insects

Insects were reared as described in section 2.2.

Synthetic chemicals

The synthetic pheromone compounds used were the same as described in section 2.2. The synthetic plant compounds used in wind tunnel studies were 1-hexanol (>99% purity), (Z)-3-hexenol (>98%), (Z)-3-hexenyl acetate (>98%), R(+)-limonene (>98%), (E)-β-caryophyllene (>98.5%), (+)-terpinen-4-ol (>99%) and methyl salicylate (>99%) supplied by Sigma-Aldrich Chemie (Buchs, Switzerland), 1-octen-3-ol (>97%, from Merck (München, Germany), (E)-β-farnesene (>90%) from Bedoukian Research, Inc. (Danbury, CT, USA), 4,8-dimethyl-1,3(E),7-nonatriene (~94%) from Givaudan (Dübendorf, Switzerland). Synthetic plant volatiles used in field trials were 1-hexanol, (Z)-3-hexenyl acetate, E-β-caryophyllene (>80.0%) methyl salicylate and tea tree essential oil (*Melaleuca alternifolia*) supplied by Sigma-Aldrich Chemie (Buchs, Switzerland) and 1-octen-3-ol from Merck (München, Germany).

The wind tunnel bioassay

All odour mixtures were released using the piezo sprayer (see section 2.2). The ternary pheromone blend of E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac at 100:20:5 was presented at an underdosed level at a release rate of 0.1pg/min. In a first step, plant volatiles were admixed singly to the underdosed pheromone level at a ratio of pheromone to plant volatiles of 1:1000, which correspond to a release rate of 100pg/min of plant volatiles. Plant volatiles were chosen based on results obtained through headspace profile comparisons using *L. botrana* male antennae to detect chemostimuli (see section 2.3.3) and information on behavioural responses of grapevine moth females to plant volatiles from the literature (Gabel et al., 1992; Tasin et al., 2006b). Initially, all plant volatiles were tested at a release rate of 100pg/min which compares to the approximate emission rate of (E)-β-caryophyllene from grape clusters (4.7 ng/h per 100gr berries, Tasin et al., 2006a). The six plant volatiles that had the strongest additive effects on the attractiveness of the underdosed pheromone (1-hexanol, (E)-β-caryophyllene, (Z)-3-hexenyl acetate, 1-octen-3-ol, methyl salicylate and (Z)-3-hexenol) were

chosen to be tested at two further release rates of 1pg/min and 10'000pg/min. Effects of a mixture of (E)-β-caryophyllene and 1-hexanol on the activity of the underdosed pheromone were tested at a ratio of 1:1000:1000. To quantify the responses to the different treatments the behavioural responses of grapevine moth males were recorded as explained in section 2.2.

Field test

In 2007 and 2008 field trials were carried out in a vineyard located in Arnex-sur-Nyon (VD, Switzerland) to establish if plant compounds could affect the activity of the sex pheromone of *L. botrana* using pheromone traps. Tetra traps (Arn et al., 1979) baited with pheromone and plant volatiles were placed in the vineyards, checked twice a week when captured moths were counted and removed. During the second generation of *L. botrana* in 2007, traps were placed in one fully randomized 6x6 (29.06.07 – 17.07.07) and two 2x2 separated latin square designs (06.07.07 – 31.07.07) in the vineyard. Traps baited with pheromone components alone served as control traps. In the 6x6 block the following plant volatiles were combined with the pheromone: 1-hexanol, (E)-β-caryophyllene, (Z)-3-hexenyl acetate, 1-octen-3-ol and tea tree *Melaleuca alternifolia* essential oil. In the two 2x2 blocks methyl salicylate was combined with the ternary pheromone blend (see below).

Odour dispenser

Rubber septa were used to dispense *L. botrana* sex pheromones (Heath et al., 1986). The ternary pheromone blend was formulated after Arn et al. (1988) containing the three main pheromone components (E7Z9-12:Ac, E7Z9-12:OH, and Z9-12:Ac at 100:20:5). An amount of 10µg was applied in 100µl dichloromethane onto the rubber cap by placing the solution in the cavity of the cap and allowing the solvent to evaporate.

Plant volatiles were dispensed from sachets of polyethylene tubing 0.1 mm thick x 50 x 40mm (S. & B. Mariotti, Neuchâtel, Switzerland) sealed using a hot wire sealer. To control the release rate PE-sachets were used singly and with up to four-wall thick. PE-sachets contained one glass bead (Ø 0.8 cm) and contained around 1500µl of pure synthetic compound. The release rate of plant volatiles from PE-sachets with varying numbers of walls was then calculated after measuring the weight loss over several days (AE100 balance, Mettler-Toledo, Switzerland ; Annex F). (E)-β-caryophyllene was released at 32 mg/day (2 walls) and 8mg/day (4 walls), 1-hexanol at 10 mg/day (1 wall) and 5mg/day (2 walls),

(Z)-3-hexenyl acetated at 73mg/day (2 walls) and 31mg/day (4 walls), 1-octen-3-ol at 19 mg/day (1 wall), methyl salicylate at 37mg/day (4 walls) and tea tree essential oil at 116 mg/day (1 wall).

Statistical analysis

The responses of male *L. botrana* in the wind tunnel to different treatments were compared by fitting a generalised linear model (GLM) with a logit link function (logistic regression) to the responses (assumed to be binomially distributed) using the statistical package R (Version 2.4.1). Analysis of deviance based on the asymptotic X^2 distribution was used to test whether the flight responses are significantly dependent on the odour sources ($p<0.05$). When the GLM was significant ($p<0.05$) multiple comparisons (R-package: Multcomp) were made using Tukey-contrasts. Field results were compared using ANOVA with Tukey post-hoc test or and with the Mann-Whitney test. All statistical analyses were performed using the statistical software R (R Development Core Team 2007) with a type I error rate of 0.05.

3.3 Results

3.3.1 Influence of plant volatiles at an underdosed pheromone level

Wind tunnel assays were carried out, exposing *L. botrana* males to mixtures consisting of the ternary pheromone blend (E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac at 100:20:5) in combination with single host plant volatiles, to determine interactions between plant compounds and the sex pheromone. The behaviour of grapevine moth males towards the pheromone was affected by the presence of synthetic plant volatiles and effects on the activity of the sex pheromone can be divided into three classes (Fig. 3. 1, Tab. 3. 1): (1) plant volatiles that significantly increased the response of grapevine moth males of all behavioural elements ((E)-β-caryophyllene, (Z)-3-hexenyl acetate, 1-hexanol, 1-octen-3-ol); (2) plant compounds that significantly enhanced the initial behavioural elements ((E)-4,8-dimethyl-1,3,7-nonatriene, (E)-β-farnesene, (Z)-3-hexenol, methyl salicylate); and (3) plant volatiles that had no effect on the activity of the ternary pheromone blend (R(+)-limonene, (+)-terpinen-4-ol). By adding the four plant volatiles belonging to class (1) to the underdosed pheromone level, the mixtures containing either (E)-β-caryophyllene, 1-hexanol, (Z)-3-hexenyl acetate or 1-octen-3-ol with the pheromone increased the landing rate of *L. botrana* males by 73%, 69%, 58%, and 54%, respectively, compared to sex pheromone alone. With (Z)-3-hexenol admixed to the pheromone, significantly more males undertook upwind flights and reached the midline of the wind tunnel. Additionally, more males were engaged in contact with the odour source, but with the increase of 35% was not significantly different from the pheromone alone. Methyl salicylate and (E)-β-farnesene increased the response levels in all behavioural elements with statistically significant differences to the pheromone alone for the behavioural elements activation up to lock-on to the odour plume. Admixing (E)-4,8-dimethyl-1,3,7-nonatriene to the pheromone blend had only a significant effect on activation of grapevine moth males. Adding (+)-terpinen-4-ol or R(+)-limonene to the pheromone had no significant effect on any of the behavioural elements. R(+)-limonene decreased the attractiveness of the pheromone blend, with only 20% of the tested males contacting the source compared to 33% with the pheromone alone, but this decrease was not statistically significant.

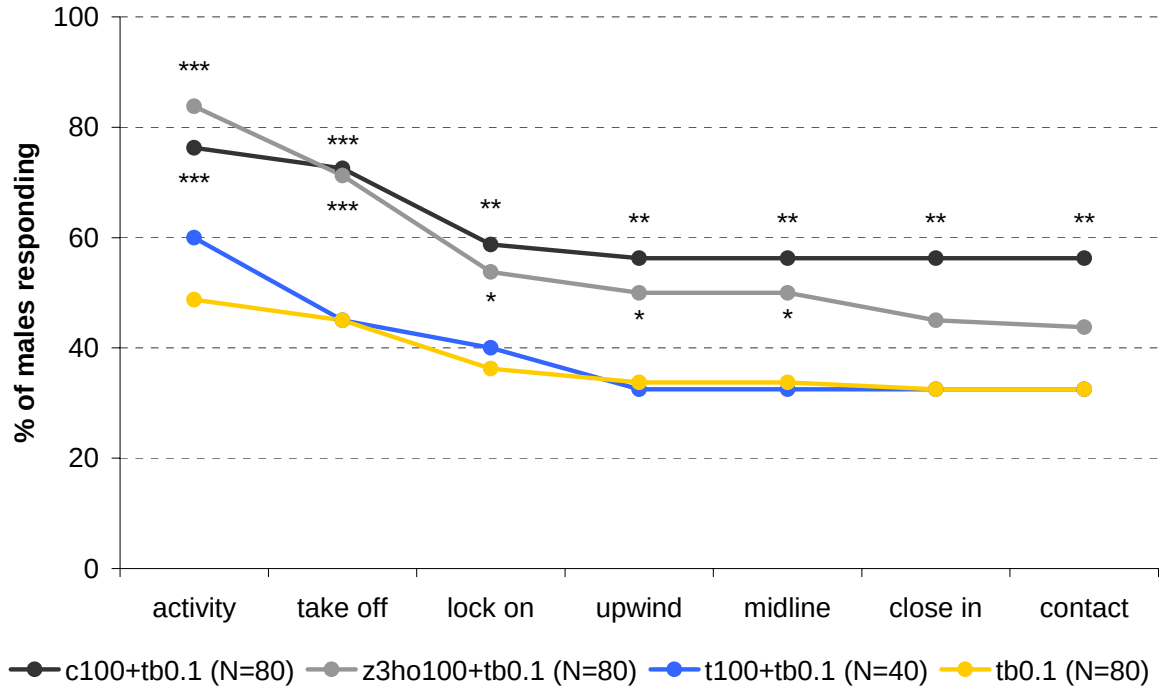

Fig. 3. 1: Behavioural response of grapevine moth males to an underdosed pheromone level (tb, 0.1pg/min) alone and in combination with either (E)-β-caryophyllene (c), (Z)-3-hexenol (z3ho) and terpinen-4-ol (t) released at 100pg/min. The plant volatiles reflect three patterns the way compounds influenced the ternary blend: (1) plant volatiles enhancing male response for all behavioural elements – (E)-β-caryophyllene, (2) plant volatiles enhancing parts of the flight behaviour – (Z)-3-hexenol and (3) plant volatiles that had no significant effect on the attractiveness of the pheromone blend – terpinen-4-ol. Asterisks indicate a significant difference from the pheromone alone (GLM, p-values: *** ≤ 0.001 < ** ≤ 0.01 < * < 0.05)

Tab. 3. 1: Behavioural response of *L. botrana* males flying to different odour mixtures consisting of single plant volatiles and the ternary pheromone blend. Plant volatiles are released each at 100pg/min and the ternary blend at 0.1pg/min. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).Values located above the line dividing the table are significantly different from the values for the ternary pheromone blend.

Synthetic odour blends	activation	take off	lock on	upwind	midline	close in	contact	N
P + 1-hexanol	78 abc	73 ab	63 a	61 a	61 a	59 a	55 a	80
P + (E)-β-caryophyllene	76 bcd	73 ab	59 ab	56 a	56 ab	56 a	56 a	80
P + (Z)-3-hexenyl acetate	85 ab	74 a	63 a	58 a	53 ab	51 ab	51 ab	80
P + 1-octen-3-ol	89 a	79 a	65 a	56 a	54 ab	51 ab	50 ab	80
P + (Z)-3-hexenol	84 ab	71 ab	54 abc	50 ab	50 abc	45 abc	44 abc	80
P + methyl salicylate	78 abc	74 a	58 abc	49 abc	49 abcd	48 abc	46 abc	80
P + (E)-β-farnesene	74 bcd	66 ab	55 abc	49 abc	45 bcde	45 abc	43 abc	80
P + DMNT [a]	70 cd	59 bc	43 cd	38 bc	34 de	33 cd	31 cd	80
P + (+)-terpinen-4-ol	60 de	45 c	40 bcd	33 c	33 cde	33 bcd	33 bcd	40
P + R(+)-limonene	60 de	45 c	30 d	30 c	28 e	25 d	20 d	40
pheromone [b] (P)	49 e	45 c	36 d	34 c	34 de	33 cd	33 cd	80

[a] DMNT=(E)-4,8-dimethyl-1,3,7-nonatriene

[b] pheromone (P) =mixture of E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac 100:20:5 according to Arn et al. (1988)

The optimal release rate of the pheromone ternary blend was found to be 1.0pg/min (see section 2.3.1). Dropping the dose ten times to 0.1pg/min, results in a significant decrease of the behavioural response. Plant volatiles of class (1) are able to restore the activity of the underdosed pheromone (Fig 3. 2). The flight response of *L. botrana* males to mixtures containing one of these four plant volatiles, namely 1-hexanol, (E)-β-caryophyllene, (Z)-3-hexenyl acetate and 1-octen-3-ol, and the underdosed pheromone reaches the same level as the behavioural response obtained with the pheromone alone at the optimal release rate. The four plant volatiles released at 100pg/min compensate for the decrease of the activity of the 0.1pg/min pheromone release rate, with a behavioural response that is not significantly different to the pheromone alone at 1.0pg/min.

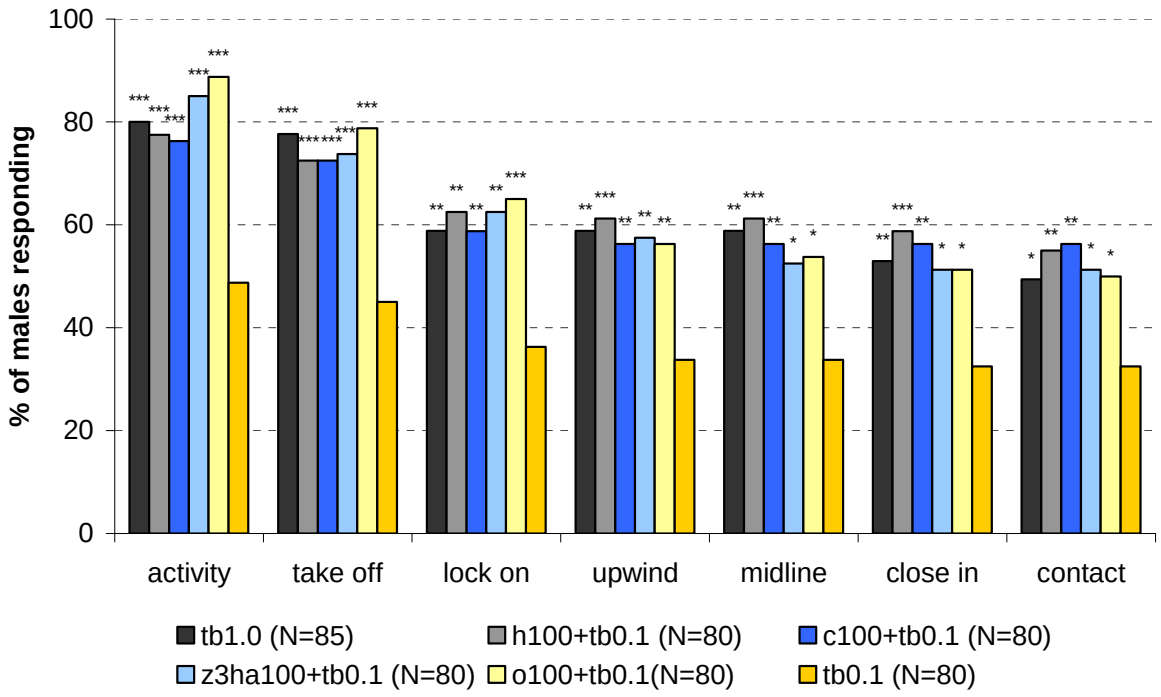

Fig. 3. 2: Percentage of male *L. botrana* responding to the optimal pheromone release level (1.0pg/min; tb1.0) alone and to an underdosed pheromone release level (0.1pg/min; tb0.1) alone and in combination with either 1-hexanol (h), (E)-β-caryophyllene (c), (Z)-3-hexenyl acetate (z3ha) and 1-octen-3-ol (o) released at 100pg/min. Asterisks indicate a significant difference from the pheromone alone released at 0.1pg/min (GLM, p-values: *** ≤ 0.001 < ** ≤ 0.01 < * < 0.05).

Tab. 3. 2: Percentage of *L. botrana* males responding to three different release rates of single plant volatiles (1, 100, 10'000pg/min) admixed with an underdosed release rate of the ternary pheromone blend (0.1pg/min; tb0.1). For each treatment grapevine moth males responded best with the plant compound released at 100pg/min (except for the behavioural element activation) Different letters assigned within a behavioural element for a single plant volatile indicate statistically significant differences (GLM, $p<0.05$).

	plant compound	pg/min	activation	take off	lock on	upwind	midline	close in	contact	N
Ester	(Z)-3-hexenyl acetate+tb0.1	1	61.3 b	55.0 b	40.0 b	33.8 b	32.5 b	31.3 b	31.3 b	80
		100	85.0 a	73.8 a	62.5 a	57.5 a	52.5 a	51.3 a	51.3 a	80
		10'000	62.5 b	57.5 b	42.5 b	33.8 b	33.8 b	26.3 b	25.0 b	80
alcohols	1-hexanol+tb0.1	1	61.3 b	57.5 b	43.8 b	40.0 b	40.0 b	37.5 b	35.0 b	80
		100	77.5 a	72.5 a	62.5 a	61.3 a	61.3 a	58.8 a	55.0 a	80
		10'000	76.7 a	59.2 ab	46.7 b	39.2 b	38.3 b	35.0 b	32.5 b	120
	(Z)-3-hexenol+tb0.1	1	58.8 b	47.5 b	41.3 ab	33.8 b	30.0 b	30.0 a	28.8 b	80
		100	83.8 a	71.3 a	53.8 a	50.0 a	50.0 a	45.0 a	43.8 a	80
		10'000	53.8 b	50.0 b	37.5 b	36.3 ab	36.3 b	30.0 a	30.0 ab	80
	1-octen-3-ol+tb0.1	1	71.3 b	60.0 b	50.0 ab	42.5 a	40.0 a	37.5 ab	36.3 ab	80
		100	88.8 a	78.8 a	65.0 a	56.3 a	53.8 a	51.3 a	50.0 a	80
		10'000	66.3 b	61.3 b	46.3 b	41.3 a	40.0 a	35.0 b	33.8 b	80
aromate	methyl salicylate+tb0.1	1	61.4 b	45.7 b	34.3 b	32.9 b	27.1 b	24.3 b	22.9 b	70
		100	77.5 a	73.8 a	57.5 a	48.8 a	48.8 a	47.5 a	46.3 a	80
		10'000	55.0 b	48.8 b	41.3 b	36.3 ab	33.8 ab	33.8 ab	33.8 ab	80
sesquiterpene	(E)-β-caryophyllene+tb0.1	1	78.8 a	65.0 a	46.3 a	36.3 b	33.8 b	30.0 b	26.3 b	80
		100	76.3 a	72.5 a	58.8 a	56.3 a	56.3 a	56.3 a	56.3 a	80
		10'000	82.3 a	70.9 a	55.7 a	53.2 a	51.9 a	51.9 a	48.1 a	79

The six plant volatiles that increased the activity of the underdosed pheromone at a release rate of 100pg/min were tested at two additional release rates of 1.0 and 10'000pg/min to investigate dose dependent effects. Grapevine moth males responded in a dose-dependent manner to mixtures containing single plant volatiles and the ternary pheromone blend (Tab. 3. 2, Fig. 3. 3). For all compounds a general pattern was found with a peak of attractiveness when the plant volatiles were released at 100pg/min. The additive effect of the plant volatile is lost both by decreasing the release rate to 1.0pg/min and increasing it to 10'000pg/min, except for (E)-β-caryophyllene. Differences between release rates were not always statistically significant for a single product but strong tendencies confirm the general pattern. For the plant compound (E)-β-caryophyllene the behavioural response of *L. botrana* males at the three release rates tested were different to that recorded for the other five plant volatiles: for activation, take off and lock on the same number of males engaged in these three behavioural elements across the release rates tested.

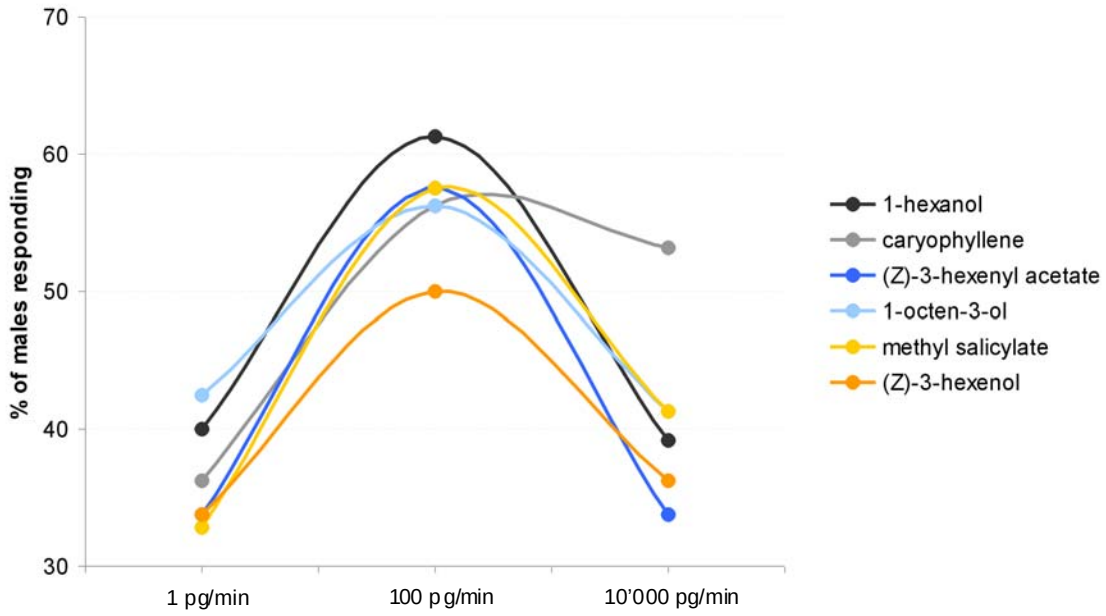

Fig. 3. 3: Percent of males engaging in upwind flight to three different release rates of plant volatiles combined with a release rate of 0.1pg/min of the ternary pheromone blend. Grapevine moth males respond in a dose-dependent manner to plant compounds with the highest response at a release rate of around 100pg/min except for (E)-β-caryophyllene where the behavioural response stayed at the same level at the two higher release rates.

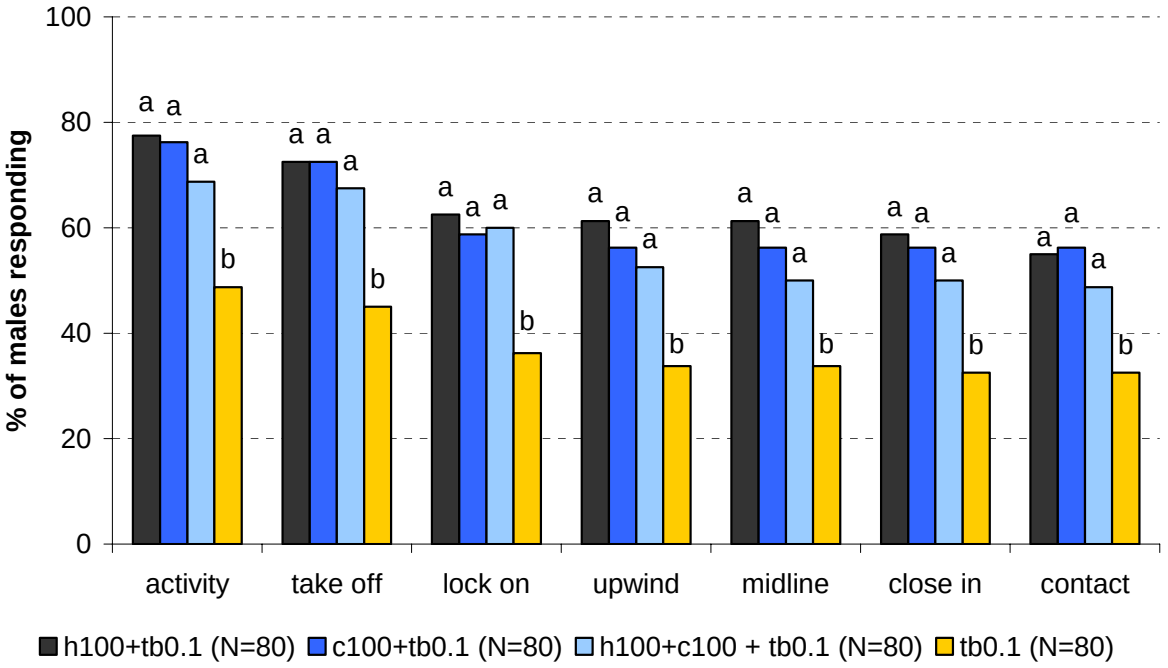

Fig. 3. 4: Behavioural response of grapevine moth males to an underdosed pheromone release rate of 0.1pg/min (tb0.1) alone and in combination with 1-hexanol (h, 100pg/min), (E)-β-caryophyllene (c, 100pg/min) and a mixture of both (1:1, 100pg/min). The behavioural response of grapevine moth males to a plant volatile blend added to the pheromone was not significantly different from the response to the single compounds added to the pheromone. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).

For the behavioural steps from upwind flight up to source contact the response significantly decreased at the low release rate of 1.0pg/min but stayed unchanged between the high release rate of 10'000pg/min and 100pg/min. Exposing grapevine moth males to a mixture of the two plant volatiles 1-hexanol and (E)-β-caryophyllene and the underdosed pheromone did not result in a increase of the attractiveness of the pheromone plus the plant compounds added singly (Fig. 3. 4). *L. botrana* males responded to the same extent to a mixture consisting of (E)-β-caryophyllene and 1-hexanol at a ratio of 1:1 and released at 100pg/min each with the pheromone as to the single compounds with the pheromone. The behavioural response was significantly different from the response to the underdosed pheromone alone but not from the plant volatiles combined singly with the pheromone.

3.3.2 Interaction of plant volatiles and the sex pheromone – a field study

In 2007 two field trials were put in place during the 2nd generation of *L. botrana* flight (June-July) in a vineyard in the Leman-basin of Switzerland to test the effects of six plant volatiles on the attractiveness of the ternary pheromone blend. In a 6x6 randomized latin square design the ternary pheromone blend was presented alone and in combination with the following plant volatiles: (1) (E)-β-caryophyllene, (2) 1-hexanol, (3) 1-octen-3-ol, (4) tea tree essential oil and (5) (Z)-3-hexenyl acetate. The results suggest that the behavioural response of grapevine moth males to their sex pheromone can be affected by plant volatiles in a wind tunnel (see section 3.3.1) but also in a field situation where an odourous background awash with plant volatile compounds was already present. Significant differences were found between the six treatments comparing the number of males caught per trap (Fig. 3. 5). The control traps emitting the ternary pheromone blend alone, had a median catch rate of 6.5 grapevine moths per trap. Adding PE-sachets emanating (Z)-3-hexenyl acetate to the ternary pheromone blend results in a decrease of the number of grapevine moths captured. With a median catch rate of 1.0 *L. botrana* males per trap this treatment was significantly less attractive than the other five treatments. In fact, (Z)-3-hexenyl acetate acted as a repellent with the release rate of 73mg/day. Adding either 1-octen-3-ol or tea tree essential oil to the pheromone also led also to a reduction of the median catch rate from 6.5 to 5.5 and 4.5 grapevine moths per trap, respectively, levels that were not statistically significant. The plant compound 1-hexanol increased the attractiveness of the ternary pheromone blend in the field from 6.5 to 8.0 grapevine moth males per trap, but this increase was not significant. Traps combining the ternary pheromone blend with (E)-β-caryophyllene were the most attractive with a median catch rate of 10.5 grapevine moth males per trap. This treatment captured a significant higher number of grapevine moth males than all the other treatments, except for the 1-hexanol plus pheromone baited traps.

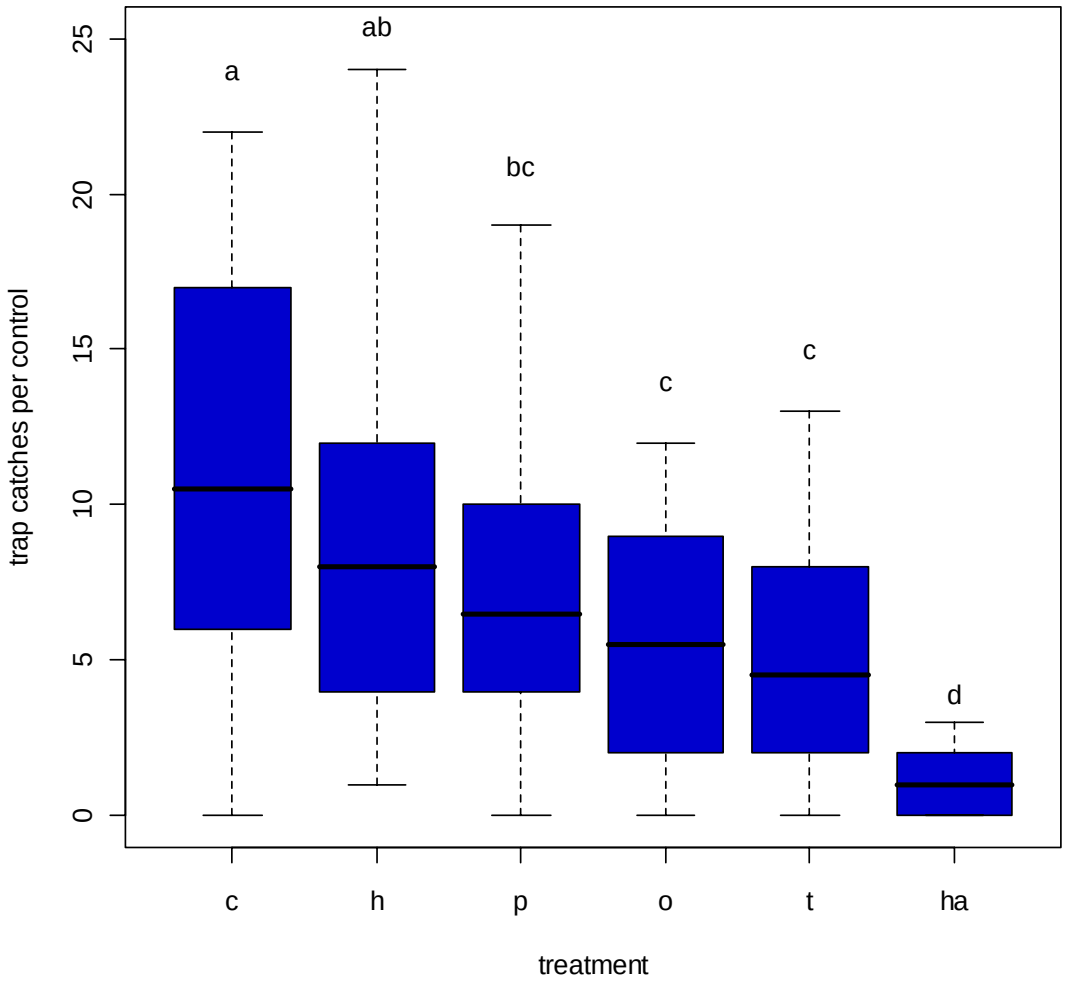

Fig. 3. 5: Number of grapevine moth males trapped during the 2nd generation *L. botrana* flight in a Swiss vineyard in 2007. Six treatments (pheromone+(E)-β-caryophyllene (c), pheromone+1-hexanol (h), pheromone alone (p), pheromone+1-octen-3-ol (o), pheromone+ tea tree essential oil (t), pheromone+(Z)-3-hexenyl acetate (ha)), were put out in a fully randomized 6x6 latin square design. Traps emitting pheromone and (E)-β-caryophyllene captured significantly more grapevine moth males than the pheromone alone. Different letters assigned to the treatments indicate statistically significant differences (ANOVA, Tukey post-hoc, $F_{5,20}= 28.5$, $p<0.05$).

Two weeks after the start of the first field trial in 2007, two 2x2 squares were put in place in the same vineyard, with methyl salicylate in combination with the pheromone and the pheromone alone as odour sources. Significantly less grapevine moth males were captured in traps with methyl salicylate than with the pheromone alone (Fig. 3. 6).

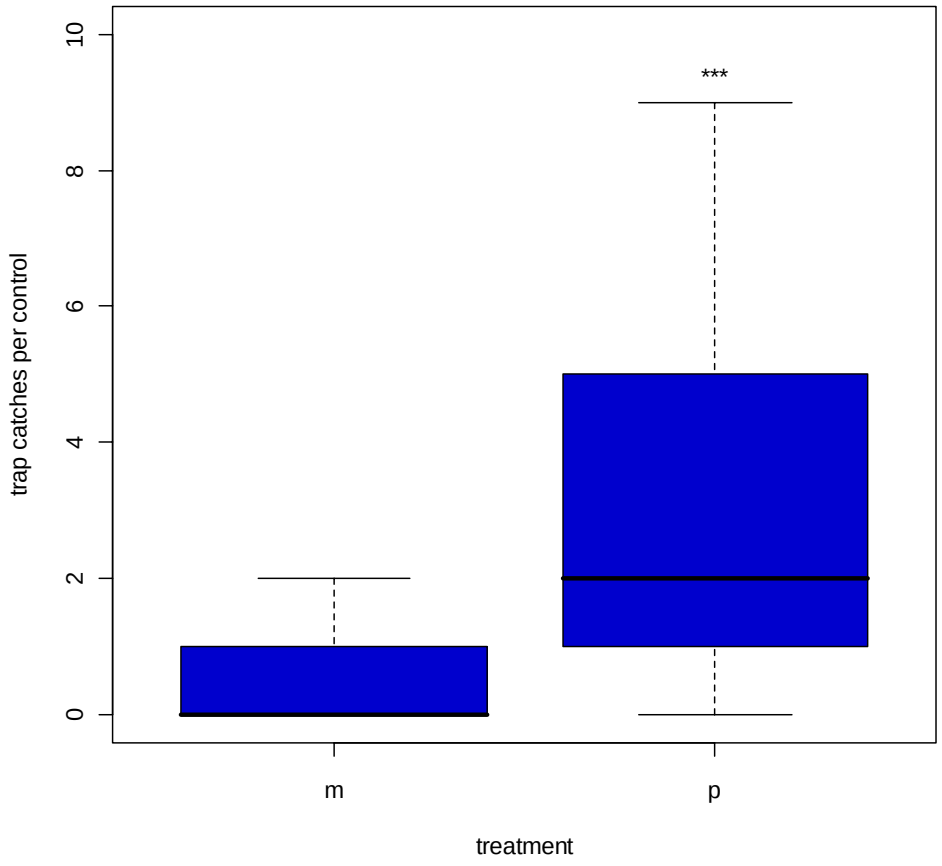

Fig. 3. 6: Number of grapevine moth males trapped during the 2nd generation *L. botrana* flight in a Swiss vineyard in 2007. Two treatments (pheromone+methyl salicylate (m) and pheromone alone (p)), were put out in two 2x2 squares and controlled eight times. Traps emitting pheromone and methyl salicylate captured significantly less grapevine moth males than the pheromone alone. Asterisks indicate a significant difference between treatments (Mann-Whitney test, p-value: *** < 0.001).

After eight controls the total number of captured moths was 21 in traps baited with methyl salicylate plus pheromone versus 116 in traps emitting only the pheromone.

During the 1st generation *L. botrana* flight in 2008 a further field experiment was carried out including the four following treatments arranged in a 4x4 randomized latin square design: (1) the ternary pheromone blend on its own, (2) the ternary blend plus (E)-β-caryophyllene, (3) the ternary blend plus 1-hexanol and (4) the ternary blend plus (Z)-3-hexenyl acetate. The release rates of the plant volatiles were decreased compared to the preceding tests in 2007 by enclosing the plant products in multiple PE-sachets.

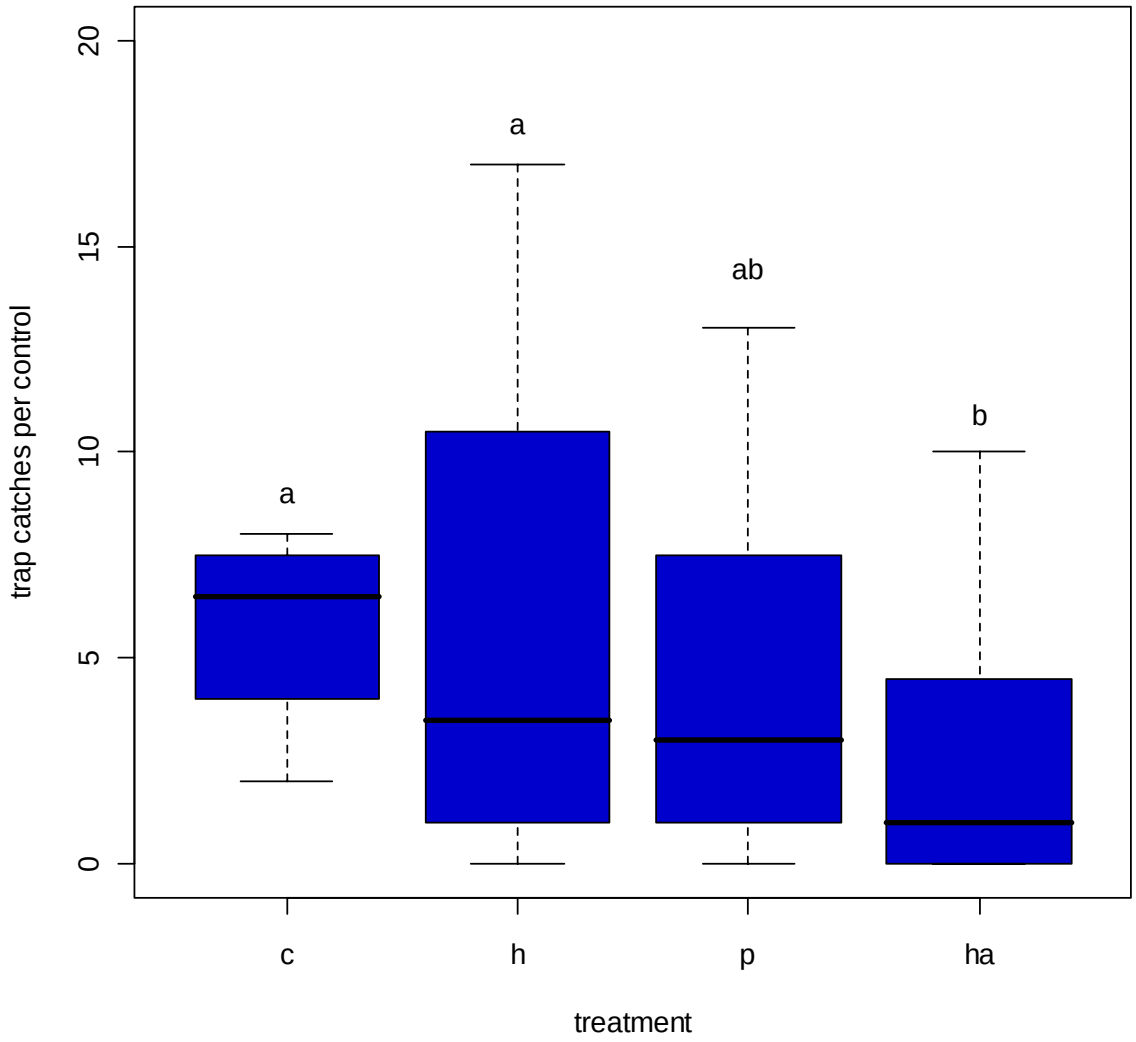

Fig. 3. 7: Number of grapevine moth males trapped during the 1st generation *L. botrana* flight in a Swiss vineyard in 2008. Four treatments, pheromone+(E)-β-caryophyllene (c), pheromone+1-hexanol (h), pheromone alone (p) and pheromone+(Z)-3-hexenyl acetate (ha), were put out in a fully randomized 4x4 latin square design. There was a tendency for traps emitting pheromone plus (E)-β-caryophyllene to capture more grapevine moth males than the pheromone alone ($p = 0.055$). Different letters assigned to the treatments indicate statistically significant differences (ANOVA, Tukey post-hoc, $F_{3,6}=4.3$, $p<0.05$).

The control traps emitting the ternary pheromone blend alone captured 50 grapevine moth males during the test period. As in 2007, adding (Z)-3-hexenyl acetate decreased the attractiveness of the pheromone blend, but not significantly. With 30 grapevine moth males captured, this treatment was significantly less attractive than the other two treatments containing plant volatiles. Again, adding 1-hexanol to the ternary pheromone blend rendered the traps more attractive, with 68 males captured versus 50 males caught with traps containing pheromone only, but the increase was not significant. The combination of

(E)-β-caryophyllene and the ternary pheromone blend was the most attractive with 74 grapevine moth males captured almost statistically different from the control traps baited with pheromone components only (GLM, p-value 0.055; Fig. 3. 7).

3.4 Discussion

3.4.1 Effects of host plant volatiles on the behaviour of grapevine moth males at an underdosed pheromone level

The results presented here demonstrate conclusively that the behaviour of *L. botrana* males towards their sex pheromone is affected by host plant volatiles. Eight commonly occurring host plant volatiles, synthesised in plants through different biosynthetic pathways, significantly enhanced the behavioural response of *L. botrana* males for at least one or more of the behavioural elements recorded in the wind tunnel. It was already shown that grapevine moth males carry receptor cells for commonly occurring plant volatiles on their antennae (see section 2.3.3), and here we provide evidence that volatile plant products influence the behavioural response to the female-produced sex pheromone in *L. botrana* males. Most of the previous studies focusing on the effects of plant volatiles on the behaviour of *L. botrana* concerned gravid *L. botrana* females where plant volatiles were presented in the absence of the sex pheromone. Tasin and his colleagues (2005; 2007; 2006a; 2006b) undertook extensive studies on flight behaviour of gravid *L. botrana* females documenting upwind flights to plant parts, to piezo-sprayed headspace collections and to mixtures of synthetic compounds. Masante-Roca et al. (2007) tested the attraction of *L. botrana* to host and non-host plant parts in the wind tunnel, focusing on effects of plant phenology, moth sex and mating status. These authors found that gravid female grapevine moths were the only adults to exhibit upwind orientation to grape plant parts in a wind tunnel. To our knowledge no study has been carried out so far to investigate additive or synergistic effects of plant volatiles on the pheromone in *L. botrana*. In this study eight compounds proved to render the pheromone more attractive in the wind tunnel: the terpenes (E)-β-caryophyllene, (E)-β-farnesene, and (E)-4,8-dimethyl-1,3,7-nonatriene, the ester with (Z)-3-hexenyl acetate, the aliphatic alcohols 1-hexanol, (Z)-3-hexenol and 1-octen-3-ol and the aromatic compound methyl salicylate. The strongest additive effects on the pheromone were achieved with either (E)-β-caryophyllene, (Z)-3-hexenyl acetate, 1-hexanol or 1-octen-3-ol. The three terpenes, methyl salicylate and 1-octen-3-ol were present in a collection of grape headspace volatiles that elicited upwind flights in gravid females (Tasin et al., 2005). Afterwards, (E)-β-caryophyllene and (E)-4,8-dimethyl-1,3,7-nonatriene were part of a 3-component blend that were considered essential host plant cues for gravid grapevine moth females searching for oviposition sites, in that the 3-componenent blend attracted females as well as piezo-sprayed grape headspace in a

wind tunnel (Tasin et al., 2006b). These insights show a considerable overlap in the types of plant volatiles that are behaviourally active on *L. botrana* males and females. Additionally, an overlap between behaviourally active plant volatiles exists not only across the sexes but also across the different developmental stages. Grapevine moth larvae walked upwind on a locomotion compensator when vapours of either (E)-4,8-dimethyl-1,3,7-nonatriene, (Z)-3-hexenyl acetate, methyl salicylate or 1-hexanol were presented singly (Paul Becher, University of Neuchâtel, personal communication). Evidence that 1-octen-3-ol is behaviourally active for *L. botrana* larvae was documented in work done with grapes infected with *Botrytis cinerea*. Guerche et al. (2006) identified 1-octen-3-ol in headspace extracts of *B. cinerea*-infected grapes and Mondy et al. (1998) observed that infected grapes attracted more grapevine moth larvae that non-infected grapes. These insights document the importance of volatile secondary host plant compounds in the sensory ecology of *L. botrana*.

Effects of plant volatiles on the activity of the sex pheromone have already been studied in detail for two moth species that are close relatives of *L. botrana*, namely the European grape berry moth *Eupoecilia ambiguella* and the codling moth *Cydia pomonella*. For *E. ambiguella* Schmidt-Buesser (2008) showed additive effects by releasing either (E)-β-caryophyllene, (+)-terpinen-4-ol, (Z)-3-hexenol or methyl salicylate in combination with an underdosed ternary pheromone blend (Z9-12:Ac, 12:Ac and 18:AC at 1:1:2 ratio). In wind tunnel studies with *C. pomonella* behavioural activity was shown for linalool, (E)-β-farnesene and (Z)-3-hexenol, in that each of these plant compounds added to the sex pheromone (E,E)-8,10-dodecadien-1-ol (codlemone) attracted significantly more males to the source combined plant volatile – pheromone than the codlemone alone (Yang et al., 2004). Additive effects of plant volatiles on the attractiveness of codlemone were also observed when either the host plant volatiles limonene, pear ester, (E)-β-farnesene or linalool were admixed with codlemone, increasing the proportion of males showing upwind flight and making contact with the odour source compared to the codlemone alone (Denes Schmera, University of Neuchâtel, personal communication). We carried out wind tunnel assays with *L. botrana* males admixing plant volatiles in several ratios with the pheromone in order to find any dose-response relationships. The six plant volatiles that rendered the pheromone the most attractive at 100pg/min were tested at two further release rates of 1.0pg/min and 10'000pg/min. For all compounds, except (E)-β-caryophyllene, we found the same pattern, in that significant additive effects on the flight behaviour achieved by plant volatiles released at 100pg/min got lost by either decreasing or increasing the release rate 100-fold. Our results suggest therefore an optimal

release rate of plant volatiles in the range of 100pg/min at an underdosed pheromone level of 0.1pg/min. This fits well with documentation on plant volatiles released from the vine in nature. Using a calibrated headspace collection system Tasin et al. (2006a) quantified release rates of 10 plant volatiles and estimated a release rate of 242pg/min of product per 100gr green berries. In two behavioural studies focusing on the effects of host volatiles on the sex pheromone of *C. pomonella*, plant compounds were also behaviourally active at picogram levels. In *C. pomonella* positive effects of plant volatiles on the attractiveness of codlemone were recorded when plant volatiles were released at 100pg/min with 10pg/min codlemone (Denes Schmera, University of Neuchâtel, personal communication) or 1pg/min codlemone (Yang et al., 2004). Our results with (E)-β-caryophyllene differed from the general pattern observed for the five other compounds. By releasing (E)-β-caryophyllene at 10'000pg/min in combination with the pheromone at 0.1pg/min the entire male behavioural sequence from activation to source contact was significantly increased, as already observed at a release rate of 100pg/min at the underdosed pheromone level of 0.1pg/min. Furthermore, (E)-β-caryophyllene released at 1.0pg/min with 0.1pg/min pheromone activated more males and induced more take offs than the pheromone on its own at a release rate of 0.1pg/min. These results suggest a wide window of biological activity for (E)-β-caryophyllene since significant effects on the attractiveness of the pheromone persist over a large range of release rates from 1.0pg/min to 10'000pg/min.

Taking into account that plants emit a large variety of secondary plant products simultaneously, *L. botrana* males are confronted rather with complex odour blends that with single compounds in the field. To search for any evidence for synergistic interactions between plant compounds we admixed (E)-β-caryophyllene and 1-hexanol to the underdosed pheromone at a ratio of 1000:1000:1. The 3-component blend was not significantly more attractive than the blend of pheromone and either (E)-β-caryophyllene or 1-hexanol. Yang et al. (2004) also tested effects of plant volatile blends on the attractiveness of codlemone for *C. pomonella*. They mixed codlemone (1 pg/min) with two (linalool and (E)-β-farnesene) or four (linalool, (E)-β-farnesene, (Z)-3-hexenyl acetate and (Z)-3-hexenol) plant compounds at either equal or 100-fold greater amounts (1:1:1; 1:100:100; 1:1:1:1:1; 1:100:100:100:100) but found no significant differences between multicomponent blends and a blend of just codlemone and linalool. Grape headspace contains a multitude of compounds, at least 15 of which elicit antennogram responses in *L. botrana* males (see section 2.3.3). It is clear that for optimal attraction blend ratios could be essential. Tasin et al. (2006b) provided experimental

support for the hypothesis that a ratio-specific blend is employed for host recognition in *L. botrana* in that a 3-component plant volatile blend mimicking the ratio released by green grapes attracted grapevine moth females whereas a blend of the same compounds released at the ratios found in non-host apple was not. Another example where a specific ratio of generally occurring plant volatiles conveys information regarding the host plant with sufficient specificity is documented for the Colorado potato beetle where individual host plant compounds that were added in excess amounts to an otherwise attractive blend abolished the attractiveness of the latter (Visser and Ave, 1978). It is likely that the dosage of our 3-component blend was suboptimal and the major reason for the lack of positive interactions between the two plant volatiles.

The overlap in behaviourally active compounds between larvae and adults could be interesting in order to determine key chemostimuli that trigger fundamental behaviours essential for survival and reproduction in grapevine moths. Adults and larvae are anatomically and behaviorally quite different, reflecting their different lifestyles. For example, adult grapevine moths need to find mates, egg-laying sites and shelter, which requires, among others, sophisticated odor-driven behavior. Grapevine moth larvae, in contrast, live on their food source and hence do not need long-range odor detection to find food and could dispose therefore of a much less complex olfactory system. In the well studied model organism *Drosophila* it was found that larvae possess only 21 olfactory receptor neurons (ORNs) innervating 21 glomeruli in the larval antennal lobe, whereas adults have 1,300 ORNs on the antennae converging to 43 glomeruli in the adult antennal lobe (Heimbeck et al., 1999; Stocker, 2001). It is likely that in *L. botrana* the differences between the larval and adult sensory system is similar to that in Drosophila, but comparative data is lacking. The number of glomeruli in the adult antennal lobe of grapevine moths is 60-64 in males (Masante-Roca et al., 2005). Each ORN expresses one or few of a wide variety of olfactory receptor genes. Olfactory receptors (ORs) are presented in the membrane of ORNs. In holometabolous insects the central nervous system undergoes substantial changes and the sensory organs are lost during metamorphosis. Jefferis et al. (2004) suggest that larval elements do not supply crucial patterning information for the adult antennal lobe. Yet, there are persisting embryonic-born projection neurons (PNs) emerging from the AL that are functionally integrated in both larval and adult olfactory pathways (Jefferis et al., 2004). It is likely that this is also the case for *L. botrana* and therefore former larval PNs play a role in odour coding in the adult central nervous system. It remains unclear if these PNs serve for similar functions in both life stages,

in particular, with respect to response spectra and the kinds of behavioral responses supported. Bichao et al. (2005) characterised ORN types in strawberry blossom weevil *Anthonomus rubi*, specialised in the perception of generally occurring plant volatiles that belong to terpenes, aromatics and aliphatic esters, alcohols and aldehydes. The sensory neurones were characterised by a strong response to one or two primary odorants such as (Z)-3-hexenyl acetate, 1-hexanol, (Z)-3-hexenol 1-octen-3-ol and weaker responses to a few others having similar chemical structure. With one exception, the molecular receptive range of each neurone type was within one chemical group. Wibe et al. (1996) documented ORNs tuned to a narrow range of plant volatiles in the pine weevil *Hylobius abietis,* where the majority of the tested ORNs responded to a limited number of compounds (<5) of the same chemical class with a best response to one of them, whereas additional responses to several other structurally similar products were recorded for some neurons. This suggests that the ORNs for plant products are rather narrowly than broadly tuned and that each neuron is specialized for coding information about one or a few related compounds. It is therefore likely that grapevine moth larvae and adults are equipped with some identical ORN types since behavioural responses were observed both in grapevine moth larvae and adults for the same plant compounds 1-hexanol, (Z)-3-hexenyl acetate, methyl salicylate and (E)-4,8-dimethyl-1,3,7-nonatriene. The ability to perceive a spectrum of plant volatiles by the "elementary" olfactory sensory system in grapevine moth larvae and the preservation of this spectrum in the sophisticated olfactory system of adults suggests that the plant products perceived are key chemostimuli conveying fundamental information about resources and are therefore of high survival value in the sensory ecology of the insect.

3.4.2 Effects of host plant volatiles on the attractiveness of the sex pheromone in the field

With prospect of future integration of plant volatiles in pheromone-based integrated pest management methods, our findings in the wind tunnel needed to be tested in the field. Conditions in the wind tunnel are far from the conditions grapevine moth males encounter in nature such that discrepancies between laboratory and field data on moth could occur. We identified, for the first time, a plant compound that enhanced the response of grapevine moth males to its sex pheromone in the field, confirming partly the results obtained in the wind tunnel. Additive effects of plant volatiles were observed for traps containing pheromone plus either (E)-β-caryophyllene or 1-hexanol that captured more grapevine moth males than traps

emitting only the pheromone in two successive field trials. Adding (E)-β-caryophyllene to the pheromone significantly increased trap captures in 2007, and in 2008 statistical the field tests indicated a strong similar tendency (GLM, p=0.055). Plant volatiles enhancing the behavioural response to sex pheromones in the field have already been identified for some moth species. More adult corn earworm and codling moth males were captured with traps containing sex pheromone and (Z)-3-hexenyl acetate than with the sex pheromone alone (Light et al., 1993). Dickens et al. (1993) proved the same phenomena in the tobacco budworm *Heliothis virescens*, where a mixture of (Z)-3-hexenyl acetate and (E)-2-hexenyl acetate enhanced responses of *H. virescens* males to the sex pheromone in the field. The positive effects of 1-octen-3-ol on the attractiveness of the pheromone found in the wind tunnel for *L. botrana* in this study was not confirmed in the field. Combining pheromone and 1-octen-3-ol dispensers resulted in a lower number of captured grapevine moth males than with the pheromone alone. The same effect, but to a stronger extent, was observed for methyl salicylate and (Z)-3-hexenyl acetate. These two plant compounds shut down trap captures. Hence, 1-octen-3-ol, methyl salicylate and (Z)-3-hexenyl acetate there is a discrepancy between laboratory studies and field trials, in that an enhancement of the attractiveness of the sex pheromone observed in wind tunnel could not be confirmed in a field situation. Coracini et al. (2004) document a similar case in *C. pomonella* where (E,E)-α-farnesene had a synergistic effect on attraction to (E)-β-farnesene in the wind tunnel but not in the field. Knudsen et al. (2008) also observed discrepancies between laboratory and field attraction of the apple fruit moth *Argyresthia conjugella* to host plant volatiles, in that anethole on its own attracted moths in the wind tunnel but not in the field. The authors suggest that the discrepancy between field and wind tunnel findings may be due to the presence of other host plant volatiles in the orchard atmosphere. However, these studies lack dose-dependent tests of the products as plant volatiles were tested only at one release rate. We suggest inappropriate release rates rather than an effect of background odours as a possible reason accounting for the mismatch between laboratory and field data concerning 1-octen-3-ol, (Z)-3-hexenyl acetate and methyl salicylate. Rubber septum provide suitable dispensers for releasing pheromone components over a long time period in monitoring traps (Butler and McDonough, 1979) but are inappropriate for a constant release of plant volatiles (Annex E). For our field trials we therefore used polyethylene sachets, known to release volatile compounds at a constant rate over time (Torr et al., 1997). We gravimetrically assessed the release rate of all the plant volatiles tested in our field trials and obtained constant release rates over time. However, due to the high volatility of the plant compounds the released amounts were very

high ranging from 5 to 73 mg/day (Annex F). In previous wind tunnel assays we showed that the optimal release rate of plant volatiles is around 100pg/min (equivalent to 0.000144mg/day) and that additive effects of plant volatiles on the attractiveness of the pheromone disappeared with an increase of the release rate of the plant volatiles to 10'000pg/min. Hence, release rates from polyethylene sachets in the field were at least 10'000 times higher than the optimal release rate found in the wind tunnel. Interestingly, increased trap catches of males compared to the pheromone alone were obtained with 1-hexanol, which had the lowest release rate among the compounds tested in the field, and with (E)-β-caryophyllene for which we provided evidence of a large window of biological activity in the wind tunnel assays where significant effects persisted even at high release rates. The results with (Z)-3-hexenyl acetate suggest also that dose effects are the major cause for the discrepancies between the wind tunnel and field data. In 2007 (Z)-3-hexenyl acetated was tested at a release rate of 73mg/day and trap catch was significantly decreased compared to the pheromone alone. Subsequently, in 2008 the release rate of (Z)-3-hexenyl acetate was decreased 31mg/day and trap catches were no longer significantly different from the traps with pheromone alone. Our insights suggest excessive release rates to be the major cause for a lack of increased attraction in the field to certain treatments containing the sex pheromone and a host plant volatile. Evidence that positive effects of odour mixtures persist also with background odours are provided by electrophysiological studies on ORNs of *Helicoverpa zea* and behavioural studies on *Bombyx mori* suggest. Ochieng and his colleagues (2002) recorded the spike-frequency of pheromone specific ORNs in response to a stimulation of the antenna with single compounds or with 2-component mixtures of. Interestingly, the firing rate was considerably increased when the pheromone was applied on the antenna in combination with either linalool or (Z)-3-hexenol compared to a stimulation with the pheromone alone. On the other hand, the two plant volatiles applied on its own had no effect on the firing rate of the pheromone-specific ORNs. Namiki and his colleagues (2008) carried out behavioural studies with *B. mori* and provided support for the findings of Ochieng et al. (2002). They provided evidence that pheromone compounds and plant products have to arrive at the same location on the antenna simultaneously to have synergistic effects. Their results indicate that moths become more sensitive to bombykol when they detect bombykol and *cis*- 3-hexen-1-ol at the same spatial location on the antennae. In the field, where a complex background of various plant volatiles is present, one could imagine that odour packets consisting of pheromone components and plant volatiles will arrive at the same time and at the same location on the insects antenna and therefore would not be masked by other plant volatiles.

In summary, our experiments demonstrate conclusively that plant volatiles positively affect the attractiveness of the sex pheromone of *L. botrana* and provide evidence for our hypothesis that grapevine moth males use host plant volatiles as a strategy to optimise reproduction. In the wind tunnel, plant volatiles presented singly were shown to increase the attractiveness of the sex pheromone presented at an underdosed level. In some cases, release rates in the range of 1pg/min of plant volatiles were enough to induce a considerable effect on the attractiveness of the sex pheromone. This underlines the moth's sensory capabilities and the importance of plant volatiles in the biology of male *L. botrana*. The transfer of insights obtained from the wind tunnel experiments to the field proved to be difficult in the sense that we did not have dispensers to release plant volatiles at biologically meaningful levels. Nevertheless, our field trials indicate that plant volatiles affect the behavioural response of grapevine moth males to their sex pheromones, even in the presence of a complex odour background from host plants. This raises the question as to what would be possible to achieve should appropriate dispensers for the release of plant volatiles be available.

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CHAPTER 4

Attraction of *Lobesia botrana* males to vine plant volatiles

Abstract

A wind tunnel study was made to investigate the behavioural effect of four vine plant headspace volatiles on *L. botrana* males. (E)-β-caryopyllene, 1-hexanol, (Z)-3-hexenyl acetate and 1-octen-3-ol elicited oriented upwind flights when presented singly in the wind tunnel, and behavioural responses persisted down to release rates at biological relevant picogram levels. Blending these four compounds had a strong synergistic effect on male response. However, some of the blend components are redundant as attraction to the 2-component mixture of (E)-β-caryophyllene and 1-hexanol was not significantly different from the 4-component mixture. In the wind tunnel, male attraction to plant volatiles was achieved at levels equivalent to sex pheromone components. However, field data could not confirm the wind tunnel data probably due to methodical drawbacks.

Keywords: *Lobesia botrana, Vitis vinifera*, plant volatiles, upwind flight attraction, host location, wind tunnel, field

4.1 Introduction

Behavioural studies investigating the effects of host plant volatiles on moth behaviour have primarily focused on females (Fraser et al., 2003; Hern and Dorn, 2002; Knudsen et al., 2008; Pinero and Dorn, 2007; Yan et al., 2003) with male moths often overlooked. This is also the case for *L. botrana* where quite a lot of plant compounds that attract grapevine moth females have been identified (Gabel et al., 1992; Katerinopoulos et al., 2005; Tasin et al., 2006a), but data is lacking concerning behavioural responses of grapevine moth males to any host plant products. Knowledge of the chemostimuli and the behavioural mechanisms involved in host recognition is crucial for the development of novel semiochemical-based insect control techniques and should be investigated for both females and males. Light et al. (2001) showed the potential of a host plant compound in pest management when they tested ethyl (2E, 4Z)-2,4-decadienoate (pear ester), a pear-derived volatile on *C. pomonella*. They found that this kairomone was highly attractive to *C. pomonella* males in the field. We demonstrated in the previous chapters that grapevine moth males selectively perceive at least 15 host plant volatiles and identified eight behaviourally active compounds that positively affected the attractiveness of the *L. botrana* sex pheromone in the wind tunnel. The purpose of the work described in this chapter was to further explore the olfactory perceptual space in the absence of sex pheromones that accommodates host finding in grapevine moth males and to gain further insights into host attraction through tests with plant volatile blends. Effects of single host plant products and their mixtures on the flight behaviour of *L. botrana* males were investigated in the wind tunnel, and subsequently in the field for confirmation of wind tunnel data.

4.2 Material and Methods

Insects

Insects were reared as described in section 2.2.

Synthetic chemicals

The synthetic pheromone compounds used in wind tunnel and field assays were the same as described in section 2.2. The synthetic plant compounds used in wind tunnel studies were 1-hexanol, (Z)-3-hexenyl acetate, (E)-β-caryophyllene and 1-octen-3-ol also described in section 2.2. (E)-β-caryophyllene used in field trials was the same as described in section 3.2.

Headspace collection and analysis

Solventless headspace collections were made from entire intact vine plants (*V. vinifera* cv. Solaris) using the same protocol described in section 2.2.

Wind tunnel bioassay

At the beginning of the scotophase, three day-old males were individually exposed to the test odour source. Moth behaviour was quantified as described in section 2.2. (E)-β-caryophyllene, 1-hexanol, (Z)-3-hexenyl acetate and 1-octen-3-ol were released singly using the piezo sprayer at a release rate of 10'000pg/min. In following tests the release rate was decreased 100 times to 100pg/min to study the attraction of single plant compounds to *L. botrana* males at biologically meaningful release rates. To investigate the effects of a mixture, (E)-β-caryophyllene, 1-hexanol, (Z)-3-hexenyl acetate and 1-octen-3-ol were admixed at a ratio of 1000:200:100:50, based on gas chromatography linked mass spectrometry analyses of solventless headspace collections from vine plants (*V. vinifera* cv. Solaris). Male response to the 4-component mixture, released at 10'000pg/min and 100pg/min (release rates refer to the (E)-β-caryophyllene content of the mixture) was recorded. To investigate possible synergism and redundancy in the 4-component plant volatile blend for attracting grapevine moth males a subtractive bioassay was carried out. The 4-component blend was split into three blends: (1) 1-hexanol, (Z)-3-hexenyl acetate plus 1-octen-3-ol at 200:100:50 released at 2000pg/min, (2) (E)-β-caryophyllene released at 10'000pg/min and (3) (E)-β-caryophyllene and 1-hexanol at 1000:200 released at 10'000pg/min.

Field test

In May 2008 (4.5.-20.5.) a field trial was carried out in a vineyard located in Arnex-sur-Nyon (VD, Switzerland). Three treatments were arranged in a 3x3 block design, with empty traps, traps containing rubber septa loaded with 10µg of the ternary pheromone blend (Arn et al., 1988) and traps containing polyethylene-sachets (4 walls; see Annex F) filled with (E)-β-caryophyllene (purity>80%). For more detailed information about traps and odour dispensers see section 3.3. The block design was completely randomized two times (N=6 counts per treatment).

Statistics

The responses, assumed to be binomially distributed, of male *L. botrana* to different treatments in the wind tunnel were compared by fitting a generalised linear model (GLM) with a logit link function (logistic regression) using the statistical package R (Version 2.4.1). Analysis of deviance based on the asymptotic X^2 distribution was used to test whether the flight responses were significantly dependent on the odour sources ($p<0.05$). When the GLM was significant ($p<0.05$) multiple comparisons (R-package: Multcomp) were made using Tukey-contrasts.

4.3 Results

4.3.1 Behavioural response to single presentation of plant volatiles

Since plant volatiles were shown to influence the flight behaviour of grapevine moth males to their sex pheromones (see section 3.3) this begged the question if plant volatiles could also affect the behaviour of *L. botrana* males on their own in the absence of the sex pheromone. Wind tunnel tests were carried out exposing grapevine moth males to the following plant volatiles presented singly: (E)-β-caryophyllene, 1-hexanol, (Z)-3-hexenyl acetate and 1-octen-3-ol, each released at 10'000pg/min. The behaviour of *L. botrana* males was strongly affected by all of the tested plant compounds (Fig. 4. 1). Compared with the response of grapevine moth males to ethanol alone (pro Analysis, Merck, Germany) significantly more males were observed showing the behavioural elements from activation to upwind flight in the presence of any of the plant volatiles presented singly.

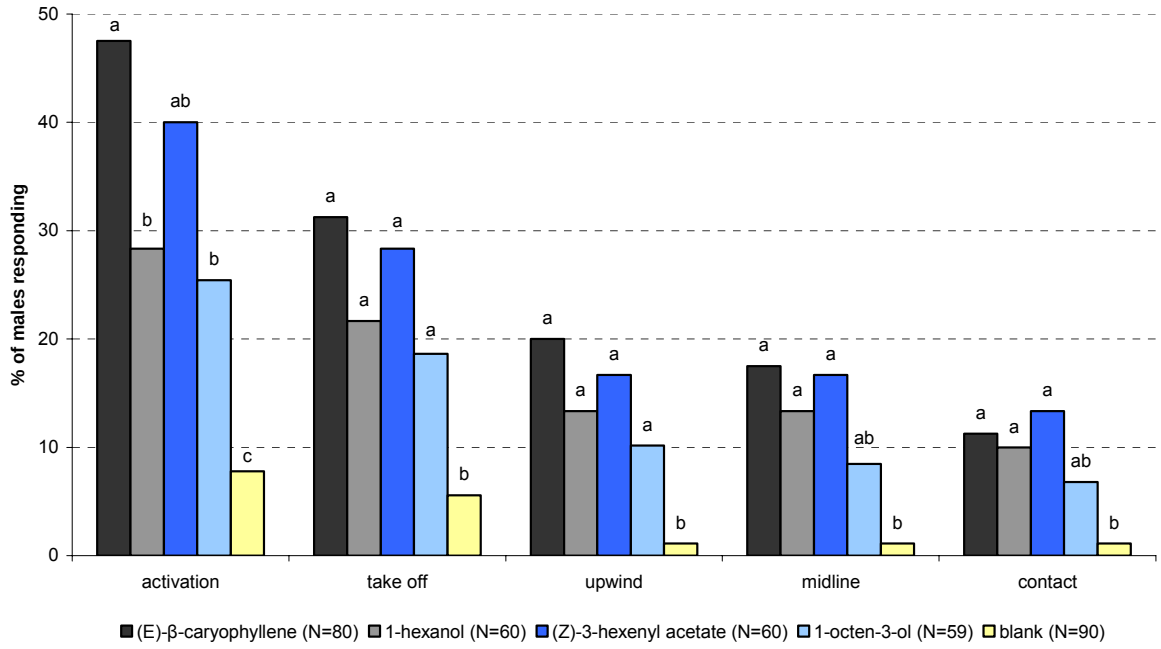


Fig. 4. 1: Behavioural response of grapevine moth males to four plant volatiles released singly at 10'000pg/min and to ethanol (blank) alone. All treatments are significantly different from the control (except the criterions midline and contact for 1-octen-3-ol). The blank stimulus was ethanol on its own released at 10µl/min. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).

As for (E)-β-caryophyllene, 1-hexanol and (Z)-3-hexenyl acetate, 1-octen-3-ol caused a higher number of males to pass the middle of the wind tunnel (9%) and to contact the source (7%) than ethanol alone, but in contrast to the other plant volatiles 1-octen-3-ol was not significantly different from the latter. (E)-β-caryophyllene and (Z)-3-hexenyl acetate were strongly attractive on their own with, respectively, 48% and 40% of the tested males activated, 20% and 17% engaging an upwind flight and 11% and 13% contacting the odour source, compared to levels of 8% for activation, 1% of upwind flight and 1% of contact for ethanol alone. Overall, the behavioural response of *L. botrana* males was comparable across the four plant volatiles as significant differences between effects of the different plant volatiles were only found for the behavioural element activation with (E)-β-caryophyllene being significantly different (48%) from 1-hexanol (28%) and 1-octen-3-ol (25%). In a second step the release rate of the four plant volatiles was decreased 100fold to 100pg/min to investigate possible effects of single plant volatiles on grapevine moth behaviour at biological relevant release rates.

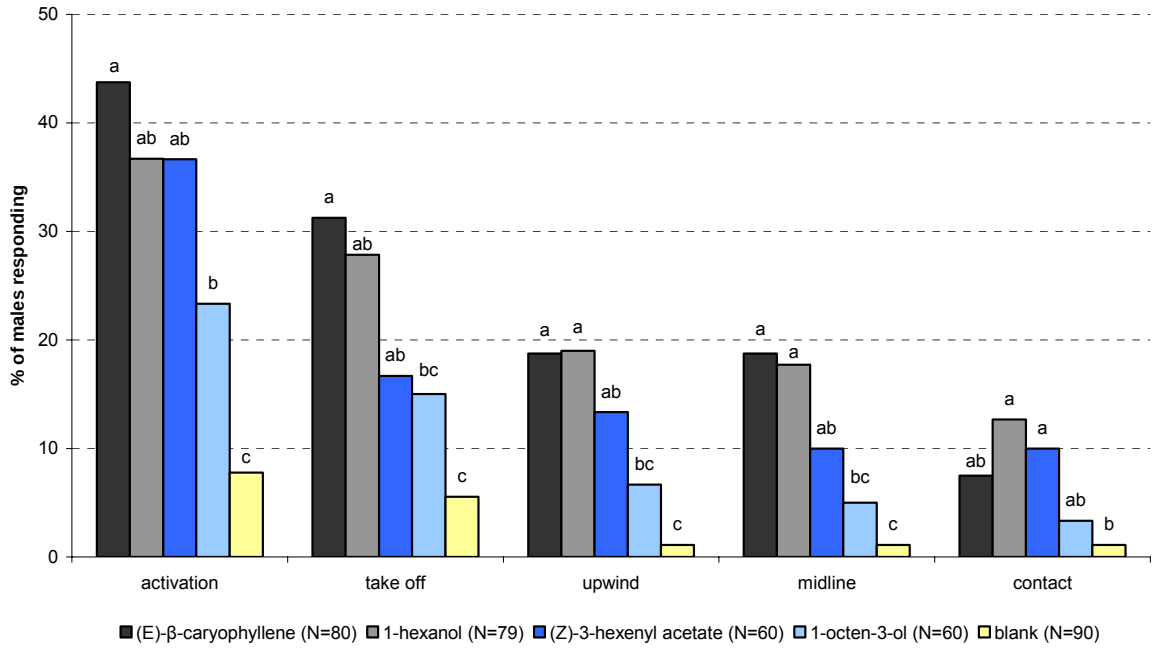


Fig. 4. 2: Behavioural response of grapevine moth males to four plant volatiles each released singly at 100pg/min and to ethanol (blank) alone. Significantly more males take off compared to the control in the presence of any of the plant volatiles. 1-hexanol and (Z)-3-hexenyl acetate engaged more male moths to contact the odour source. Ethanol was released at 10µl/min. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).

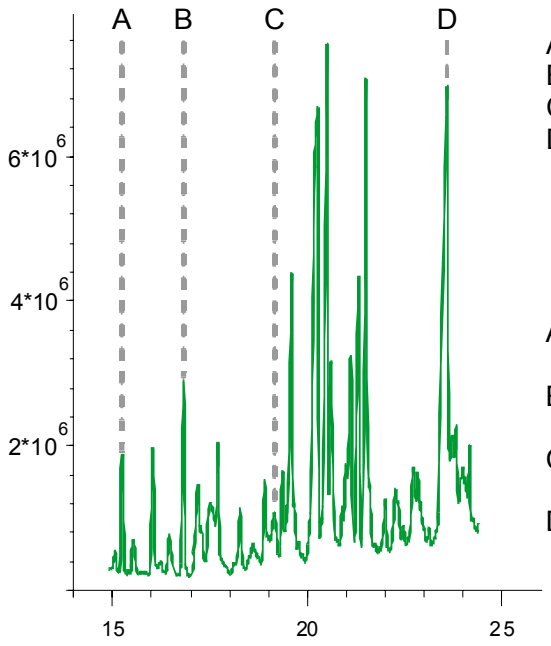
It was found that plant volatiles released at 100pg/min significantly influenced the flight behaviour of male *L. botrana* in the wind tunnel (Fig. 4. 2). 1-hexanol and (Z)-3-hexenyl acetate were significantly different from ethanol alone for the entire succession of behavioural elements, with, respectively, 13% and 10% of the tested males contacting the odour source compared to 1% for the control. (E)-β-caryophyllene was also a strong attractant at a release rate of 100pg/min inducing the highest percentage of activation (44%), take off (31%) and passing through the midline of the wind tunnel (19%) among the four plant volatiles. The contact rate with 8%, by contrast, was no longer significantly different from the blank control. Further, 1-octen-3-ol evoked higher behavioural responses than the blank with a statistically significant difference for the behavioural element activation. Comparing the data for the 10'000pg/min with the 100pg/min release rate of the four host plant compounds revealed no dose-dependency between the two release rates on the flight response of *L. botrana* males for any behavioural element (Tab. 4. 1). Nevertheless, (E)-β-caryophyllene, (Z)-3-hexenyl acetate and 1-octen-3-ol tended to be slightly more attractive when released at 10'000pg/min. In contrast the attractiveness of 1-hexanol decreased by augmenting its release rate from 100pg/min to 10'000pg/min.

Tab. 4. 1: Percent of *L. botrana* males engaging in different behavioural elements towards two different release rates of four single plant volatiles. The blank control (ethanol) was released at 10µl/min. Statistical tests revealed no significant differences between the responses to the two release rates of a plant compound (GLM $p<0.05$). Asterisks indicate a significant difference from the blank (GLM, p-values: *** ≤ 0.001 < ** ≤ 0.01 < * < 0.05).

	pg/min	activation	take off	upwind	crossing midline	contact	N
(E)-β-caryophyllene	10'000	47.5***	31.3***	20.0**	17.5**	11.3*	80
	100	43.8***	31.3***	18.8**	18.8**	7.5	80
1-hexanol	10'000	28.3**	21.7**	13.3*	13.3*	10.0*	60
	100	36.7***	27.8***	19.0**	17.7**	12.7*	79
(Z)-3-hexenyl acetate	10'000	40.0***	28.3***	16.7**	16.7**	13.3*	60
	100	36.7***	16.7*	13.3*	10.0*	10.0*	60
1-octen-3-ol	10'000	25.4**	18.6*	10.2*	8.5	6.8	59
	100	23.3**	15.0	6.7	5.0	3.3	60
solvent blank	-	7.8	5.6	1.1	1.1	1.1	90

4.3.2 Behavioural response to mixtures of plant volatiles

Having shown that single host plant volatiles can elicit oriented upwind flights in *L. botrana* males the question arose if a mixture of plant volatiles could have a stronger effect on male behaviour that the single host plant compounds. We set out to test the effects of a 4-component mixture, consisting of (E)-β-caryophyllene, 1-hexanol (Z)-3-hexenyl acetate and 1-octen-3-ol, on the behaviour of *L. botrana* males in a wind tunnel. To provide a biological basis for the choice of the release ratios in the mixture, gas chromatography linked mass spectrometry analyses of thermally desorbed headspace collections obtained from vine plants (*Vitis vinifera* cv. Solaris; Fig. 4. 3) were analysed. A comparison of the mass selective detector-areas showed that (E)-β-caryophyllene, 1-hexanol (Z)-3-hexenyl acetate and 1-octen-3-ol were represented at a ratio of 1000:179:118:51 in the headspace, suggesting a release ratio for the 4-component mixture of 1000:200:100:50 after rounding off the values.



A (Z)-3-hexenyl acetate
B 1-hexanol
C 1-octen-3-ol
D (E)-β-caryophyllene

	Proportion in headspace	Proportion in 4mix
A	118	100
B	179	200
C	51	50
D	1000	1000

Fig. 4. 3: Gas chromatograph linked mass spectrometry analysis (GC-MS) of a headspace sample (thermally desorbed from the porous polymer) of grapevine plants (*V. vinifera* cv. Solaris). The four plant volatiles that enhanced male responses for all the recorded behavioural elements at an underdosed pheromone level (see Tab. 3.1) and attracted grapevine moth males on their own in the wind tunnel were present in the vine headspace. The formulation of a 4-component mixture (4mix) was based on the amounts detected by GC-MS in the headspace sample.

The 4-component mixture released at 10'000pg/min proved to be more attractive on grapevine moth males than any of its components presented on their own at the same release rate. Comparing the behavioural response of grapevine moth males to the 4-component mixture released at 10'000pg/min with the response towards its main component (E)-β-caryophyllene alone at the same release rate, it is evident that adding 1-hexenol, (Z)-3-hexenyl acetate and 1-octen-3-ol rendered the main component (E)-β-caryophyllene significantly more attractive for every behavioural element with for example 38% instead of 26% of male moths flying upwind and 29% instead of 18% contacting the source (GLM $p<0.05$; Fig. 4. 4). A 100-fold decrease of the release rate of the 4-component mixture led to a significant decrease in the number of activated moths and in the number of males that took off (GLM, $p<0.05$). The level of the remaining successive behavioural elements was also reduced but not significantly different. All in all it can be stated that grapevine moth males respond in a dose-dependent manner to the 4-component mixture of host plant volatiles with a higher

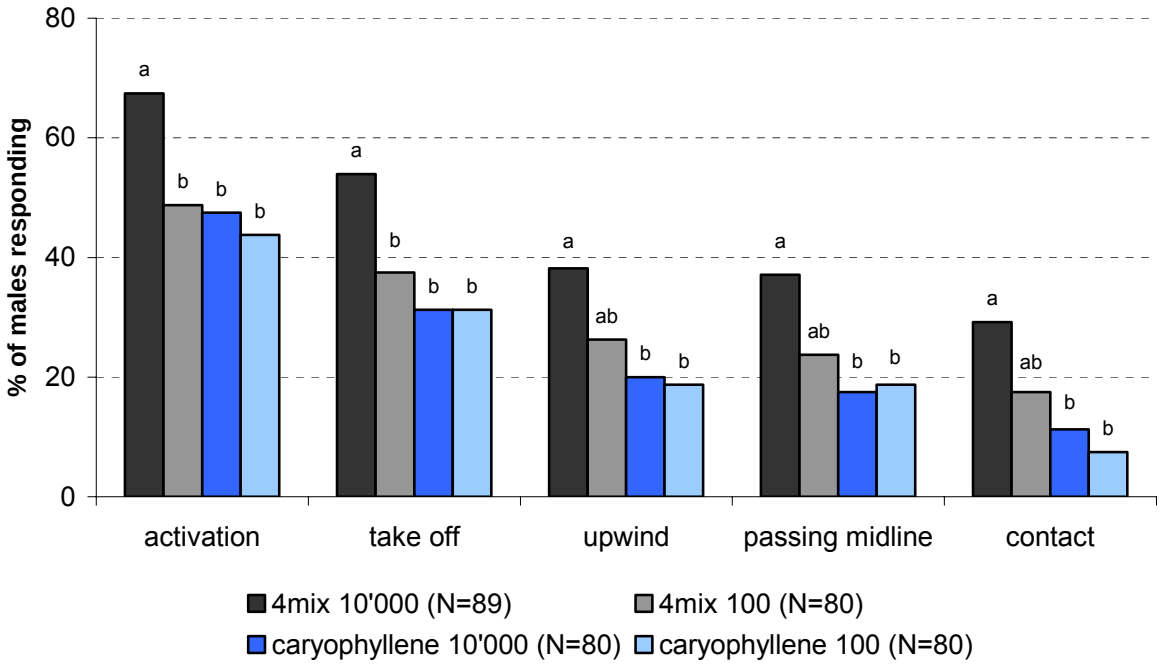


Fig. 4. 4: Percentage of *L. botrana* males responding to a 4-component mixture (4mix, (E)-β-caryophyllene, 1-hexanol, (Z)-3-hexenyl acetate and 1-octen-3-ol at 1000:200:100:50) and to (E)-β-caryophyllene alone released at 10'000 and 100pg/min (release rates of the 4.component mixture refer to the (E)-β-caryophyllene content). The 4-component mixture released at 10'000pg/min is significantly more attractive than the single plant volatile at 10'000pg/min. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).

behavioural response at a higher release rate. Additive effects of the three secondary host plant compounds to (E)-β-caryophyllene released at 100pg/min are less pronounced than at a release rate of 10'000pg/min. Comparing the behavioural response of grapevine moth males to the 4-component mixture released at 100pg/min with the response towards its main component (E)-β-caryophyllene alone at the same release rate showed still an enhancement. Adding 1-hexanol, (Z)-3-hexenyl acetate and 1-octen-3-ol to (E)-β-caryophyllene released at 100pg/min resulted in 26% instead of 19% of the tested males to engage in upwind flights and 18% instead of 8% to contact the source, although the differences are not statistically significant different (Fig. 4. 4).

Comparing the level of the behavioural response to the 4-component mixture released at 10'000pg/min with the response to the pheromone ternary blend at different release rates indicates interesting insights (Fig. 4. 5). The behavioural response of grapevine moth males to the 4-component mixture reaches the level of the behavioural response to the pheromone

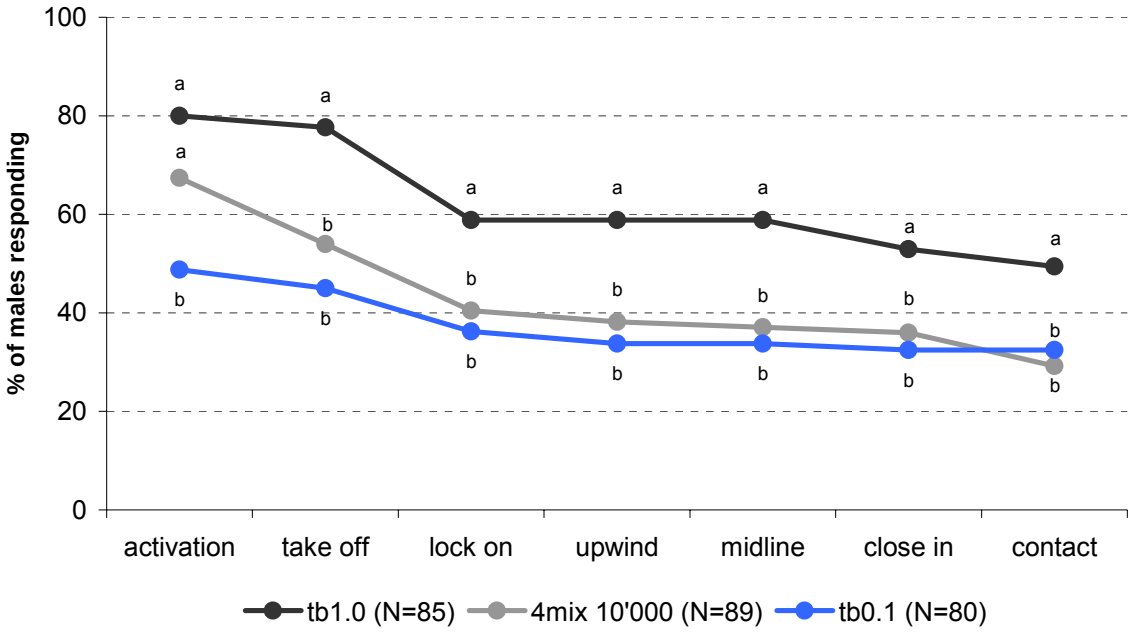


Fig. 4. 5: Behavioural response of grapevine moth males to a 4-component mixture (10'000pg/min), a suboptimal level (0.1pg/min) and the optimal level (1.0pg/min) of the ternary pheromone blend. In the absence of pheromone components similar behavioural responses can be evoked exposing males to plant volatiles only. The level of activation with plant volatiles is not significantly different from the one at the optimal pheromone release rate. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).

ternary blend released at 0.1pg/min. The number of activated males of 67% in the presence of the 4-component mixture is even significantly higher than the 49% of activated males obtained at an underdosed pheromone release rate of 0.1pg/min and reaches the level of 80% of activation obtained at the optimal pheromone release rate of 1.0pg/min. For the following successive behavioural elements, the behavioural response of grapevine moth males to the 4-component mixture was not significantly different from the response to the underdosed pheromone level.

4.3.3 Identifying key stimuli - a subtractive bioassay

Further wind tunnel tests were done to decipher the behavioural effect of grape volatiles on the grapevine moth. Our goal was to identify key stimuli that are essential for grapevine moth attraction and any redundant signals in the 4-component host plant volatile mixture. The starting point of this wind tunnel study was a blend of four grape volatiles which had been

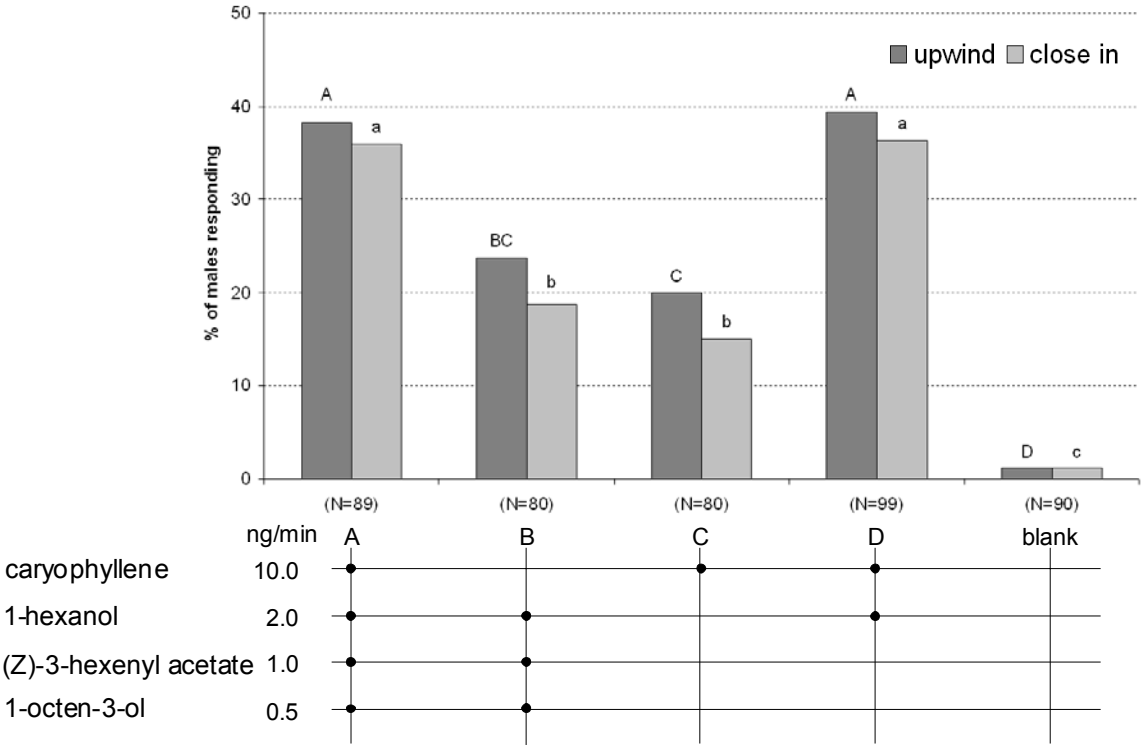


Fig. 4. 6: Behavioral response of *L. botrana* males to mixtures of four compounds in grapevine headspace eliciting an antennal response. Percentages of males flying upwind towards (dark bars) and approaching a source of grape volatiles (light bars). Ratios based on GC-MS analyses of grapevine headspace (see Fig. 4. 3). Columns with the same letter are not significantly different (GLM, $p<0.05$).

shown to attract grapevine moth males (see section 4.3.2). The blend consists of the plant volatiles (E)-β-caryophyllene, 1-hexanol, (Z)-3-hexenyl acetate and 1-octen-3-ol at a ratio of 1000:200:100:50. This 4-component blend (A) released at 10'000pg/min elicited upwind flights towards the source in 38% of the test males and 36% of the males approached the odour source at the upwind end of the wind tunnel (A in Fig. 4. 6). The grapevine moth males did respond weakly to the solvent blank, consisting of ethanol, with 1% of the tested males starting upwind flights and close-in within 10cm of the odour source. A series of tests was done to identify the compounds which were essential for male attraction. Blend A was arbitrarily split into blends B through D. Omission of (E)-β-caryophyllene (B) resulted in a significant reduction in the number of grapevine moth males engaging in upwind flight and flying close to the source (Fig. 4. 6).Presenting the main host plant volatile component (E)-β-caryophyllene alone (C) also led to a significant decrease in both of these behavioural elements. A 2-component blend (D) of (E)-β-caryophyllene and 1-hexanol, in a 1000:200 ratio, was not significantly different from the response to the 4-component grape mimic A. These results confirm the important role of (E)-β-caryophyllene and 1-hexanol in grapevine moth male upwind attraction, and that grape volatiles contained in the 4-component mixture are in part behaviourally redundant, since attraction to the 2-component blend, consisting of (E)-β-caryophyllene and 1-hexanol, is not significantly different from the 4-component blend.

4.3.4 Attraction of grapevine moth males to (E)-β-caryophyllene in a vineyard

In 2008 a field trial was carried out during May to investigate the attraction of grapevine moth males to (E)-β-caryophyllene in a natural environment, since this plant volatile attracted grapevine moth males on its own in a wind tunnel. Three treatments were arranged in 3x3 randomized design: (1) 10µl of the ternary pheromone blend on a rubber septum, (2) 4-wall PE-sachets filled with (E)-β-caryophyllene (estimated release rate of 8mg/day) and (3) empty control traps. No grapevine moth males were captured either with traps containing (E)-β-caryophyllene dispensers or with the empty control traps. Pheromone traps captured an average of 10.6 males per trap per control

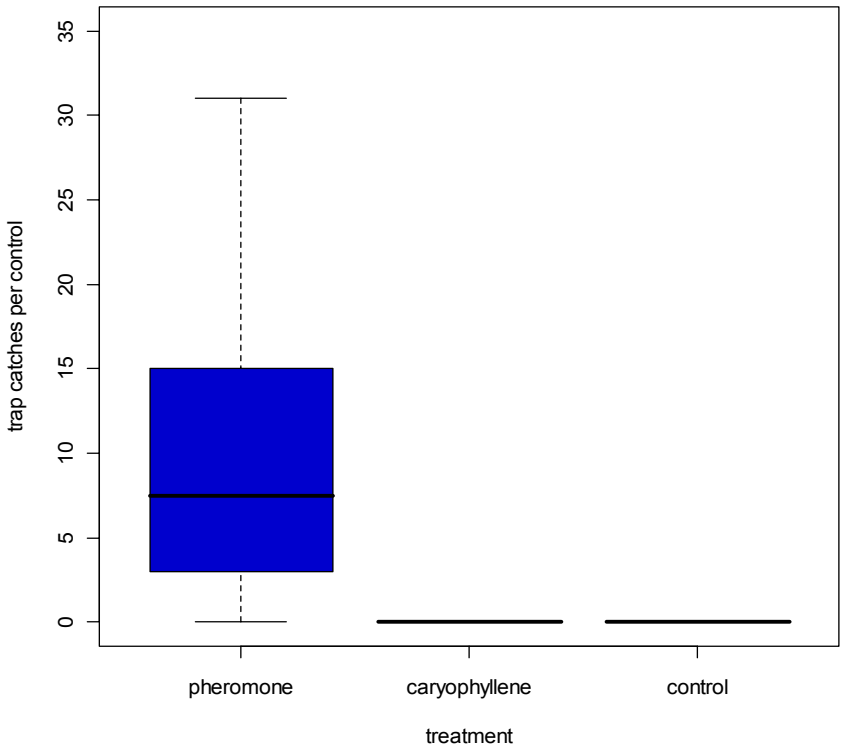


Fig. 4. 7: Number of grapevine moth males trapped during the 1st generation flight of *L. botrana* in a Swiss vineyard in 2008. Three treatments (10µg of the ternary pheromone blend at 100:20:5 on a rubber septum (pheromone), (E)-β-caryophyllene in 4-wall PE-sachets (caryophyllene) and empty control traps (control)), were put out in a randomized 3x3 design and controlled 6 times (N=18 per treatment). No males were captured in caryophyllene-baited and control traps.

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4.4 Discussion

Field observations of the pea moth, *C. nigricana* and the codling moth have shown that females are mated within a few minutes after they start to release pheromone (Witzgall et al., 1999; Witzgall et al., 1996). Pea moth and codling moth females are typically found on fruitbearing host plants suitable for oviposition, and mating success is supposedly highest for the males which arrive at such host plants before the onset of female calling. Such males are likely to be able to exploit information contained in the host plant volatile blend. Olfactory cues from the host plant are known to provide an essential ingredient to the mate-finding process in phytophagous insect (Landolt and Phillips, 1997). Evidence is provided in this thesis that this is also the case in *L. botrana* in that we showed additive effects of single plant volatiles on the attractiveness of the sex pheromone (see section 2.3 and 3.3). In addition data is presented here to show for the first time that grapevine moth males engage in oriented upwind flight in a wind tunnel to mixtures as well as to single plant compounds identified from vine plants. The key compounds that elicited grapevine moth males to undertake oriented upwind flight were: (E)-β-caryophyllene, 1-hexanol, (Z)-3-hexenyl acetate and 1-octen-3-ol. Single plant volatiles attracted grapevine moth males not only at high release rates of 10'000pg/min but also at release rates at the biologically meaningful level of 100pg/min which males could encounter in nature. That demonstrates that male moths are highly sensitive to plant volatiles. All in all, better attraction of grapevine moth males was achieved when plant compounds were released at 10'000pg/min but the levels of attraction were not significantly different from the responses obtained with the same plant volatiles released at 100pg/min. The number of plant volatiles found necessary to induce upwind flights in *L. botrana* is at variance with wind tunnel studies with gravid *L. botrana* females where it was found that females do not undertake oriented upwind flights to single host plant volatiles (Tasin et al., 2007). These authors found that at least a 3-component blend was necessary to elicit significantly more upwind flights in grapevine moth females. Upwind flights of male moths to single host plant compounds have been documented in a few other moth species but only at high release rates: *Ephestia cautella* and *Plodia interpunctella* males engaged in upwind flights when 50'000pg/min of phenylacetaldehyde was released into a wind tunnel (Olsson et al., 2005). By presenting either (E)-β-farnesene, (E,E)-α-farnesene, (E,E)-farnesol or (Z)-jasmone at 10'000pg/min a higher number of codling moth males engaged in upwind flights than with the solvent blank (Coracini et al., 2004; Yang et al., 2004). These studies lack data at low and biological meaningful release rates of the host plant

volatiles and, to our knowledge, no example is documented where male moths engage in upwind flights to single compounds at release rates in the range of 100pg/min.

To investigate the effects of mixtures of host plant volatiles on the behaviour of grapevine moth males a 4-component mixture of (E)-β-caryophyllene, 1-hexanol, (Z)-3-hexenyl acetate and 1-octen-3-ol mixed at a ratio of 1000:200:100:50 according to proportions found in vine plant headspace samples. This 4-component mixture released at 10'000pg/min had a clear effect on the entire male behavioural sequence from activation to source contact compared with the male response to (E)-β-caryophyllene released singly at 10'000pg/min. The male response to the 4-component mixture was significantly higher than any of the responses recorded with any single plant volatile. Adding the three secondary plant volatiles to (E)-β-caryophyllene released at 100pg/min also resulted in an increase in the entire behavioural sequence with 17.5% of the tested males contacting the source compared to 7.5% with (E)-β-caryophyllene alone, but the positive effects were no longer statistically different to (E)-β-caryophyllene alone as observed at the higher release rate. Coracini et al. (2003) also documented synergistic effects of plant volatiles on the attraction of codling moth males at a release rate of 10'000pg/min by mixing (E,E)-α-farnesene and (E)-β-farnesene which attracted more codling moth males than (E,E)-α-farnesene alone. When the attractiveness of the 4-component plant volatile blend released at 10'000pg/min was compared with the attractiveness of the ternary pheromone blend released at the optimal level of 1.0pg/min no significant difference in the number of activated males was recorded. Plant volatiles even induced wing-fanning in grapevine moth males (data not shown), which is considered typical for pheromone mediated courtship behaviour. Coracini et al. (2004) also reported a very similar behavioural sequence towards plant volatiles and sex pheromone in the wind tunnel for *C. pomonella* males, in that upwind orientation flights towards a 2-component blend of (E)-β-farnesene and farnesol are indistinguishable from flights to codlemone. This poses the question as to whether the sex pheromone and plant volatiles can elicit similar motor output patterns as for example, upwind orientation flight and if the sensory channels for sex pheromones and plant volatiles are rather cross-linked than isolated one from the other.

Pheromone tuned olfactory receptor neurons (ORNs) project into the macroglomerular complex (MGC) in the antennal lobe (AL) of male moths. ORNs tuned to plant volatiles also project into isomorphic glomeruli in to AL. In the AL a network of local interneurons (LNs) arborizing into different glomeruli and projection neurons (PNs) leading to higher brain centres are present. Anton and Hansson (1995) proved evidence that sensory input from plant

volatiles affects pheromone sensory channels when they identified in *Spodoptera littoralis* LNs that arborize equally in the MGC and in the isomporhic glomeruli, and PNs that respond to both pheromone components and plant volatiles. Neuronal structures like these could explain the similar behavioural sequences evoked in grapevine moth males by host plant compounds and sex pheromones.

Further wind tunnel experiments were made focusing on the 4-component plant volatile blend to determine which component parts are essential to evoke upwind flight and landing behaviour, and if the 4-component mixture could contain redundant signals. In a subtractive bioassay the 4-component blend was reduced arbitrarily into 3-, 2- and in a 1-component treatments. Omitting (E)-β-caryophyllene from the 4-component blend revealed the importance of the latter in the host finding process, since significantly fewer males engaged in upwind flights and flew close to the source when they were exposed to the 3-component blend compared to the 4-componenent blend containing (E)-β-caryophyllene. However, (E)-β-caryophyllene presented on its own was not sufficient to attract as many grapevine moth males as the 4-component blend. Again a significantly lower number of males engaged in upwind flight and closed-in on the odour source, suggesting that at least a 2-component mix is necessary to reach the attraction level of the 4-component blend. Indeed, by mixing (E)-β-caryophyllene with 1-hexanol a similarly high proportion of males flew upwind (39.4%) and closed-in on the source (36.4%) as with the 4-component blend (38.2% and 36%, respectively). Our data suggest that (E)-β-caryophyllene and 1-hexanol are semiochemicals that play a key role in host finding behaviour of *L. botrana*. Furthermore, our data indicate that the constituents of the 4-component blend are in part behaviourally redundant, since attraction to a 2-component blend of (E)-β-caryophyllene and 1-hexanol was not significantly different from the 4-component blend. However, we cannot rule out the possibility that (E)-β-caryophyllene or 1-hexanol could be substituted by the behaviourally active compounds (Z)-3-hexenyl acetate or 1-octen-3-ol and restore attraction. To make definitive statements further tests are necessary, since we tested only a few blend combinations. However, field trials with (E)-β-caryophyllene do not confirm the promising results from the wind tunnel. Traps equipped with (E)-β-caryophyllene dispensers captured no males at all. In contrast sex pheromone traps captured an average of 10.6 males per trap per control. The differences between field and wind tunnel results concerning (E)-β-caryophyllene presented alone, can be due to the interaction of the test stimulus with the background odour in the natural environment as it has been reported for the apple fruit moth, *Argyresthia conjugella* (Knudsen

et al., 2008). The biological activity of anethole was shown in a wind tunnel in the absence of an odorous background, but could not be confirmed in the natural environment. Knudsen and his colleagues (2008) suggest that the lack of field attraction to anethole indicates that more active or more abundant host plant volatiles mask or interfere with anethole. Schroeder and Hilker (2008) discuss three different types of background odour: (a) irrelevant background odor, (b) background odor that may mask the resource-indicating signals, and (c) background odorants that may "sharpen the view" for resource-indicating odour and enhance the response to these. Thiery and Visser (1986) demonstrated an example for background odours that masked an attractive signal in that the presence of nonhost-plant odours masked the odour blend of the host plant *Solanum tuberosum* attractive to the Colorado potato beetle. Accordingly, (E)-β-caryophyllene emitted from polyethylene bags in the vineyard, could have been masked by a multi-component background odour, in our case emitted from host plants. It is possible that more active or more abundant grapevine volatiles mask or interfere with (E)-β-caryophyllene, demonstrating that in a complex environment a complete volatile blend is necessary to efficiently attract grapevine moth males, rather than a single compound. Furthermore, excessive release rates of (E)-β-caryophyllene could contribute to the shut down of trap catches in the field (see section 3.4.3).

Our wind tunnel data partly confirms the results of Tasin et al. (2007) who identified three key semiochemicals out of ten plant volatiles occurring in grape headspace, with (E)-β-caryophyllene as the major constituent of a 3-component blend that attracted females as well as the complete 10-component blend. Olfactory receptor neurons sensitive to (E)-β-caryophyllene seem to be widespread among insects. For example, specific receptor neurones tuned to (E)-β-caryophyllene are documented for the strawberry blossom weevil *Anthonomus rubi*, for the beet armyworm *Spodoptera littoralis* and for the tobacco budworm *Heliothis virescens* (Anderson et al., 1995; Bichao et al., 2005; Hillier and Vickers, 2007; Jönsson and Anderson, 1999). (E)-β-caryophyllene is used as a semiochemical beyond the Arthropoda by attracting nematodes to insect infested maize roots (Rasmann et al., 2005). This suggests that this commonly occurring plant volatile may play an important role in the sensory ecology of several organisms.

1-hexanol is a rather minor constituent in vine plant headspace but seems to be important for the biology of *L. botrana.* Pinero et. al. (2007) documented an example in the oriental fruit moth *Cydia molesta*, where the minor headspace component benzonitrile proved essential to induce oriented upwind flights in females. Furthermore, these authors showed by calcium

imaging in the female antennal lobes that the 5-component benzonitrile-containing host volatile mixture elicited strong activation of one additional glomerulus type that showed specific responses for this mixture. This provides strong evidence that minor components of host plant volatiles can have important effects on the behavioural response to odours (Pinero et al., 2008). Despite the use of various collection techniques, Tasin and his colleagues (2005) did not identify 1-hexanol in headspace samples from different vine plant parts of different phenolocigal stages. We detected 1-hexanol with the sensitive thermal desorption headspace sampling method but in our headspace extracts from grape plants using solvent desorption 1-hexanol was absent (see section 2.3). Our results show that care should be exercised when analysing headspace collections from plants. The assumption that the most abundant volatiles released from plants are also behaviourally the most relevant can be challenged. A holistic approach is needed to trace plant volatiles released at low quantities from plants that may be important in insect-plant interactions. This implies the analysis of as many headspace samples as possible and the application of different headspace sampling techniques. While minor constituents of mixtures are often overlooked, they may, as we document here for 1-hexanol, be important for discrimination of suitable host plants by herbivore insects.

Concerning host-plant recognition, there are currently two accepted models: (1) species-specific odour recognition and (2) ratio-specific odour recognition. The first model is based on the theory of Gottfried Fränkel (1959), which proposes that specific compounds are used for host detection by herbivorous insects. The other model argues that plant odour specificity is achieved by a particular ratio between constituent volatiles distributed generally among plant species (Bruce et al., 2005). Response to widespread rather than host-specific chemicals could have an important consequence as it may facilitate the colonization of new host plants (Linn et al., 2003). Attraction to commonly occurring plant volatiles may thus lead to a generalist feeding habit on a range of plants which differ in their nutritional value. If this would be the case, polyphagous insects should rely on blend specificity for host plant recognition. Tasin et al. (2006b) provides experimental support that blend ratio of commonly occurring plant volatiles is essential in *L. botrana,* as a 3-component blend mimicking the ratio released by green grapes was attractive, while a blend ratio released by the non-host apple was not. Our results tend to support the ratio-specific odour recognition since a 4-component blend of commonly occurring plant volatiles admixed at the ratios found in headspace samples attracted a high number of grapevine moth males but further experiments testing off-blends are necessary to make more clear-cut conclusions.

We found that a blend of (E)-β-caryophyllene and 1-hexanol attracted grapevine moth males to the same extent as the sex pheromone released at the underdosed level of 0.1pg/min in the wind tunnel. It has been shown that grapevine moth females also use (E)-β-caryophyllene for location of oviposition sites (Tasin et al., 2006a). Is it possible that (E)-β-caryophyllene and 1-hexanol indicate resource quality to moths? There is tremendous variety in both the source and structure of natural odorants that can be emitted from nearly any plant tissue. A lot of plant volatiles are secondary products, such as phenylpropanoids and monoterpenes, which appear to be not essential to the metabolism of the producing organism, but which may have ecological functions like for example attractants for pollinators or phytoalexins to fight pathogens. These compounds are formed by extensions and offshoots of primary metabolism, and often employ minor pathways of limited distribution in the plant kingdom (Dudareva et al., 2004; Knudsen et al., 1993). Flavour volatiles are derived from an array of nutrients, including amino acids, fatty acids, and carotenoids. For example in tomato, almost all of the important flavour-related volatiles are derived from essential nutrients (Goff and Klee, 2006). The predominance of volatiles derived from essential nutrients and health-promoting compounds suggests that these volatiles provide important information about the nutritional makeup of foods and it is likely that volatile emissions have evolved in part to provide positive information to pollinating or seed-dispersing organisms. Therefore, phytophagous insects could on their part have evolved to also exploit olfactory cues that signal essential nutrients and thereby improve their fitness by locating appropriate hosts for their progeny. Thiery and Moreau (2005) illustrated in *L. botrana* that the plant fed on by larvae affects adult reproductive success in that the larval diet strongly influenced female fecundity via pupal weight and the duration of egg laying. They showed that grapevine moth females reared on suitable host plants laid more eggs (around 23% more) and lived longer (around 1 day more) than females reared on a less suitable host plant. The influence of food quality available during the larval stadium and its effects on the fitness of adults was demonstrated in *Manduca sexta* in that moths that were reared with a diet deficient in β-carotene were significantly less efficient in locating flowers in a wind tunnel (Raguso et al., 2007). (E)-β-caryophyllene is a sesquiterpene and originates through the condensation of isopentenyl diphosphate (IPP), which is itself a precursor for a vast number of other molecules like for example carotenoids. In higher plants, IPP is derived from the classic mevalonic acid pathway in the cytosol which has Acetyl CoA at its beginning (Schwab et al., 2008). Acetyl CoA is produced during the second step of aerobic cellular respiration during the decarboxylation of pyruvate that originates from glucose. 1-hexanol derives from fatty acids and is formed through the

lipoxygenase pathway. The two key compounds that elicit upwind flights in grapevine moth males originate therefore from two different pathways and could transmit information about the presence of, respectively, glucose and lipids present in a plant, which are essential for larval development.

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Chapter 5

General Discussion

The outcome of this thesis provides strong experimental support for our hypothesis that the grapevine moth males use volatile host plant cues in mate-finding behaviour and that the perception of these plant products contributes to an optimised strategy for reproduction. Plant volatiles rendered the sex pheromone more attractive both at the optimal and an underdosed pheromone level. In addition, we demonstrated that grapevine moth males are almost as sensitive to plant volatiles as to their sex pheromone. The behaviour of *L. botrana* males was affected by minute amounts of plant volatiles down to release rates of 1.0pg/min which is comparable to sex pheromone release rates necessary to induce behavioural responses in grapevine moth males. Moreover, we demonstrated that single plant compounds, in the absence of the sex pheromone, are able to induce oriented upwind flights in grapevine moth males in the wind tunnel suggesting that plant volatiles play an essential part in the behavioural ecology of *L. botrana* males. Concerning the fundamental question how phytophagous insects filter information concerning complex volatile signals in order to locate a host plant, we provide support for the hypothesis that a ratio-specific blend of commonly occurring plant volatiles rather than plant-specific host plant compounds encodes host recognition. Last but not least, we pointed out the considerable potential of integrating plant compounds into semiochemical-based pest control methods in that biological activity of plant compounds shown in the laboratory was partly confirmed under natural conditions in the field.

Plant volatiles could affect the efficacy of mating disruption methods in different manners but before making any suggestions, an explanation about the mechanisms of mating disruption is needed. Until now no general agreement has been reached in how mating disruption affects

the behaviour of male and female moths. Researchers have proposed different modes of action for mating disruption ranging from false-trail-following, camouflage, desensitization, sensory imbalance and finally to combinations thereof (for precise definitions see: Miller et al., 2006a, b). However, a majority of the scientific community agrees on false-trail-following to be the primary cause of mating disruption, and so do I. Therefore, all assumptions made hereafter regarding the role plant volatiles could play in new mating disruption techniques are based on the false-trail-following model which is explained shortly. One has to remember that in moth species that rely on a mating system using sex attractants for long-distance mate finding the males compete heavily during limited windows of time for a share of calling females. Competition, although the sex ratio is usually near 1, between males is high and the first-arriving male usually mates with the calling female. False-trail-following can be described as competitive attraction. By deploying pheromone dispenser, working as point sources, the competition is intensified for males by the presence of "false females". The frequency with which male moths find calling females in a vineyard under a disruption regime is reduced because males are diverted from orienting to females due to occupation with nearby attractive plumes from dispensers of synthetic pheromone. Hence, rendering pheromones dispensers more attractive by adding plant products will intensify competition between calling females and synthetic odour sources. In chapter 3 we demonstrated that plant volatiles rendered the pheromone more attractive at underdosed pheromone levels. Hence, if at a distance from the pheromone dispenser the pheromone concentration reaches threshold-levels, grapevine moth males nevertheless could engage in upwind flights due to the presence of behaviourally active plant volatiles released in combination with the pheromone, suggesting that the dispensers emitting pheromone and plant products have a higher action range than traditional dispensers containing pheromone alone. Intensifying the competition between calling females and synthetic odour sources has implications for the physiology of grapevine moth males with a faster depletion of their energy resources since they will be engaged more likely in energetically costly upwind flights to synthetic odour sources. Furthermore, intensifying the competition could implicate a reduction in the probability of males contacting a female, since males respond to pheromone only during a restricted time window and any time spent in upwind flights to synthetic odour sources reduces the chance of encountering a calling female. All these factors contribute furthermore to delayed mating in the crop which results in a reduction of the female fertility in *L*. botrana (Torres-Vila et al., 2002), providing an additional factor to combat the insect pest. Pheromone – plant volatile

mixtures are likely to be especially powerful during the 1st generation flight of *L. botrana* when nearly no foliage is present on vine plants.

The remarkable overlap of behaviourally active compounds across sexes and life-stages in *L. botrana* points to another interesting aspect in which the integration of plant volatiles in mating disruption could bring about, namely that future traps could affect not only male behaviour but also the behaviour of females or of larvae. We identified two promising plant volatiles that could be interesting from this point of view: (1) (E)-β-caryophyllene which was the most interesting compound throughout all my experiments and was also found to be the main constituent of a 3-component mixture that was highly attractive to grapevine moth females (Tasin et al., 2007). The key distinction of (E)-β-caryophyllene is that the kairomone attracts on the one hand both sexes and on the other hand is behaviourally active on the two sexes at the same picogram levels (Tasin et al., 2006). These insights give (E)-β-caryophyllene special status similarly to pear ester for *C. pomonella* where the ester was found to be an effective attractant for both male and female codling moth in the field (Light et al., 2001). (2) 1-hexanol, a minor constituent in vine plant volatile emissions, was identified (see section 4.3.3) as an essential host plant volatile for *L. botrana* males and was found to elicit upwind orientation in grapevine moth larvae (personal communication, P. Becher, University of Neuchâtel). Again, this shows certain parallels with pear ester, that was found to attract both codling moth adults and larvae (Knight and Light, 2001).

In future studies more knowledge is needed regarding mixtures of plant volatiles to further improve the attractiveness of the pheromone in the field. Until know, I have only discussed the effects of single plant volatiles on the efficacy of pheromone dispensers, but the ultimate goal would be to incorporate optimized mixtures of host plant volatiles into new dispensers. In chapter 4 we showed that a 4-component mixture, formulated at ratios found in vine plant headspace, was highly attractive to grapevine moth males compared to single plant compounds, already behaviourally active on their own. To date, field tests confirming the activity of the 4-component blend in the field are lacking. However, if a transfer of the findings from the wind tunnel to the field would be successful, it would be possible to develop odour dispensers which would be as attractive or more than calling females sitting on a vine plant. This would already revolutionise semiochemical based control techniques for *L. botrana*, but I suggest even greater impact by the use of a plant volatile mixture corresponding to hosts other that *V. vinifera*. I have to stress that vine is not the optimal host plant for *L. botrana*. Grapevine moth females are attracted to and can oviposit on alternative

plants like the flax-leaved *Daphne gnidium* (Maher, 2002) and larvae are able to complete their development on more than 20 plant species (Stoeva, 1982). In addition, natural populations of *L. botrana* were observed on several different plants of Mediterranean origin, such as flowering olive trees (Savopoulou-Soultani et al., 1990). *Daphne gnidium*, is the presumed native host plant of *L. botrana* (Balachowsky and Mesnil, 1935; Bovey, 1966; Stoeva, 1982). In feeding experiments, Thiery and Moreau (2005) clearly showed that alternative host plants can be more suitable than Vitaceae for the individual performance of *L. botrana,* in that larval mortality was reduced and the development time of larvae was shortened, while pupal weight, growth rate, female longevity, female fecundity, egg-laying duration and mating success were increased. I identified numerous compounds in headspace volatile emissions of alternative host plants that elicited antennal receptor cell responses in *L. botrana* males (see section 2.3.3). It would be interesting to conduct further wind tunnel and field tests with plant volatiles blends mimicking plants that are optimal hosts for *L. botrana* development. I would expect pheromone dispensers combined with a multicomponent mixture mimicking an optimal host plant to be highly attractive, meaning that such dispensers might be more attractive to males than a calling female sitting on Vitaceae, which represents a suboptimal host plant. Such an enhancer effect of plant volatiles indicating an optimal host might be useful, since mating with a female present on the optimal host plant pays off when the offspring finds better resources for feeding and therefore for successful completion of larval life-stages. Furthermore, by simulating an abundant presence of "optimal" host plants in vineyards I suggest that *L. botrana* females could afford to be selective in host plant choice and therefore try to settle on the artificially created "optimal" host plants and not on Vitaceae. This would additionally augment the success of new mating disruption techniques based on pheromone and plant products. To find evidence for the mentioned hypotheses much more knowledge is needed concerning the effects of mixtures of plant volatiles on the attractiveness of the pheromone and on the behaviour of grapevine moth females under field conditions. Unfortunately, investigating such questions is at the moment impossible, since no appropriate dispenser for controlled release of plant volatile at low biologically relevant levels, is available.

Critique can be addressed concerning the integration of plant volatiles in pheromone dispenser in the sense that the combination of commonly occurring host plant volatiles like (E)-β-caryophyllene, 1-hexanol or multicomponent plant volatile blends will have no effect on the behaviour of male moths in monocultures that are today covering large geographic

areas all emitting the same products. Therefore one could argue that once a male moth finds itself in a monoculture detecting females sitting on a host or non-host plant is no longer a limiting factor and the behavioural response of male moths will no longer be affected by plant volatiles, or that natural plant volatiles released from plants interfere with the synthetic plant volatiles deployed. However, the olfactory system of male moths evolved long before monocultures existed and is capable for filtering behaviourally relevant signals, such as packets of odour consisting of pheromone and host plant molecules out of a highly diverse odour background. Even in monocultures it is likely that the odour background is not homogenous since different molecules are given off from different plant parts over the day and night and from different Vitaceae varieties. Finally, females use plant volatiles to locate flower buds or grapes for oviposition, despite the role vision is presumed to play at close range (Masante-Roca et al., 2007). This suggests that even in monocultures moths can distinguish and follow odour plumes emitted from different point sources. Furthermore, we have experimental support from our field tests that the behaviour of grapevine moth males is affected by synthetic compounds under natural conditions.

We demonstrated that host plant volatiles play an important role in the biology of *L. botrana*. We showed that single plant volatiles increase the attractiveness of the sex pheromone in a wind tunnel. We found evidence that plant volatiles affect the attractiveness of the sex pheromone also in the field, in that we could augment the attractiveness of the sex pheromone by adding (E)-β-caryophyllene. Finally, we found that single plant volatiles presented in minute amounts are enough to elicit oriented upwind flights in grapevine moth males in a wind tunnel, underlining the olfactory and behavioural capacities of grapevine moth males concerning the perception of host plant compounds. Overall, our findings suggest that plant volatiles hold promise for a possible integration of such products in semiochemical based control techniques, such as mating disruption, for *L. botrana*.

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Annex A - Composition of semiartificial medium for *Lobesia botrana* rearing

water	800g
pulverised agar	25.0g
sugar	30.0g
wheat germ	90.0g
alfa alfa meal	25.0g
casein	40.0g
brewer's yeast	18.0g
Wessen salt	12.5g
sun flower oil	2.5g
cholesterol	1.25g
sorbic acid	2.0g
ascorbic acid	10.0g
Vanderzant vitamin mix	7.5g
Tetramycine (95%)	1.25g
propionic acid	2.5g
linolenic acid	1.0g

Annex B - Behavioural response of *L. botrana* males to different release rates of synthetic pheromone and a calling female

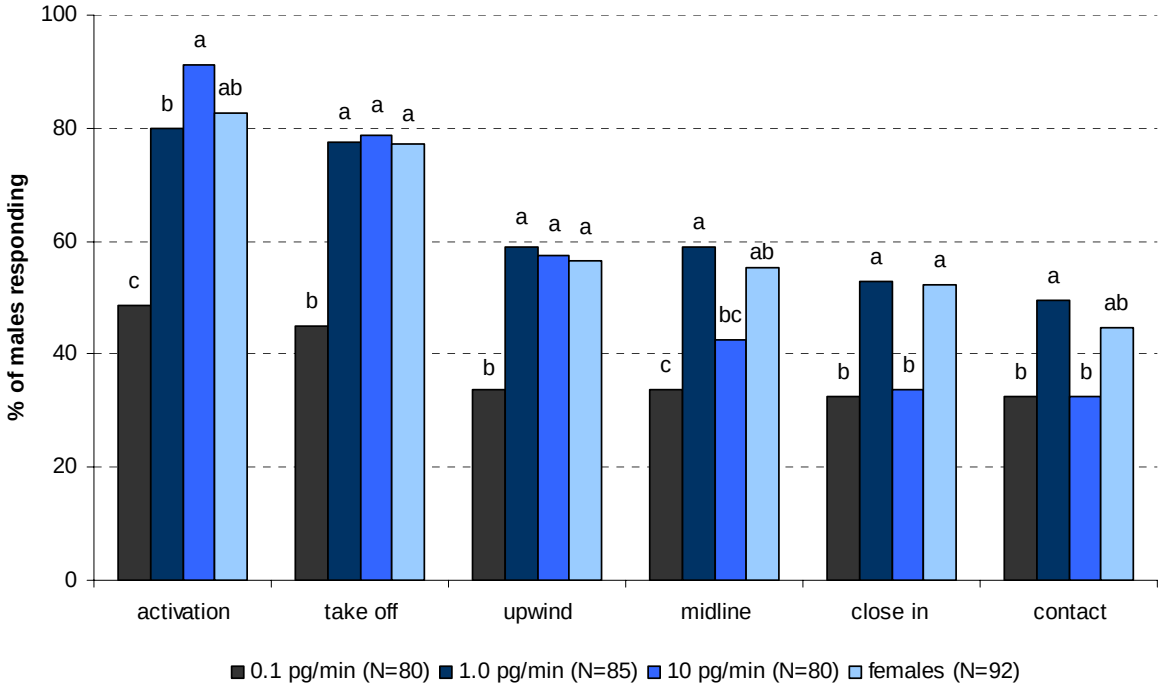


Fig. B. 1: Comparison of upwind flight response of *L. botrana* males to synthetic pheromone and to three calling females. The pheromone (E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac at 100:20:5) was released with the piezo sprayer at 0.1, 1.0 and 10pg/min. Calling females were presented in a glass container closed with a honeycomb grid and connected to an external air supply. Highest male response was observed when synthetic pheromone was released at 1.0pg/min and reached the level of calling females. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).

Annex C - Effects of CO_2 on the attractiveness of the sex pheromone

Experimental design

The climatized wind tunnel (working area: 250cm long 100×100cm) is made of non-reflecting glass. A centrifugal ventilator moves the humid- and temperature-controlled air (85±1% RH, 18±0.1°C) across the tunnel at 30cm/s through active charcoal cartridges placed at either end of the working area. A semi-laminar air flow was produced through two perforated steel screens (1mm thick, 3mm round holes, 51% of air passage) placed upwind and a laminar flow screen at the downwind end of the wind tunnel. Overhead illumination was provided by high frequency fluorescent lighting (36W, >1kHz, with 8 Philips TL-D) running the length of the tunnel in two groups of 4 tubes 20cm apart in a housing 120cm wide. On the roof of the wind tunnel two layers of crepe paper provided ~10lux on the floor. A rubber septum was loaded with 0.1µg of E7Z9-12:Ac and placed in a platform, held be a hollow metal tube, at the upwind end of the wind tunnel at the same height as the release point of adult males. CO_2 was introduced through the hollow metal tube (Fig. C. 1). Batches of ten males were alternately tested with combinations of pheromone and CO_2 or with pheromone alone as stimulus. As a control pure air was introduced at 10ml/min through the hollow metal tube into the wind tunnel instead of CO_2 (Fig. C. 4). In behavioural tests 2-3 day old grapevine moth males were placed singly in a glass tube at the downwind end of the wind tunnel. Moth behaviour was scored for activation, take off, lock on to the odour plume, engaging an oriented upwind flight, passing ¼ of the wind tunnel, passing ½ of the wind tunnel, passing ¾ of the wind tunnel, close in on the odour source and contacting the odour source.

In preliminary test the increase of CO_2 levels were measured in the wind tunnel for release rates of 5ml/min and 10ml/min. Measurements were made at 5cm, 10cm, 20cm, 40cm, 60cm and 80cm downwind from the of the CO_2 source (Fig. C. 3). The test protocol consisted of 1 min blank followed by a CO_2 release of 2min and again a blank of 1min. This procedure was repeated three times for each distance (Fig. C. 2). During behavioural tests CO_2 background levels were measured continuously.

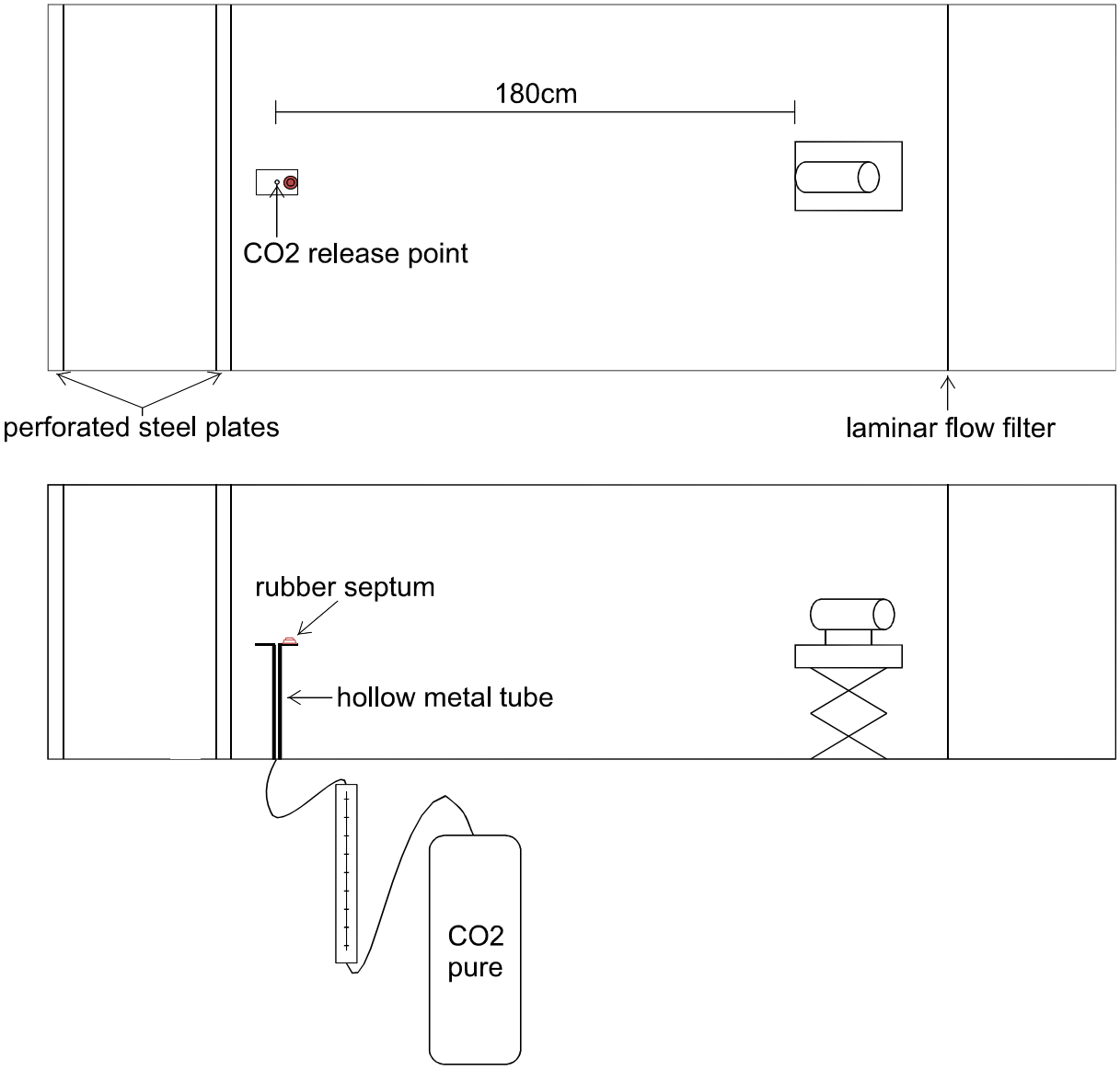


Fig. C. 1: Experimental setup of wind tunnel experiments. Top view (above) and lateral view (below) of the wind tunnel and the odour presentation setup.

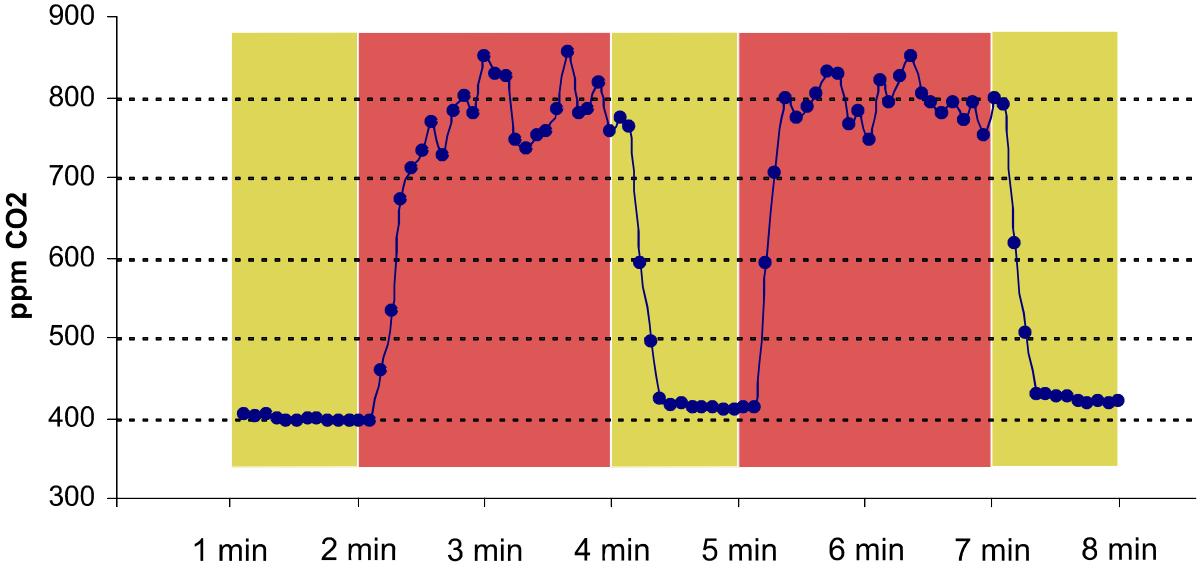


Fig. C. 2: Evaluation of CO_2 increase measured 10cm the CO_2 release point at a release rate of 5ml/min of pure CO_2. Yellow bands signify time segments of 1 min with no additional CO_2. Red bands signifiy time segments of 2min where CO_2 was introduced into the wind tunnel at 5ml/min. The CO_2 level was considerably increased and remained stable during test period.

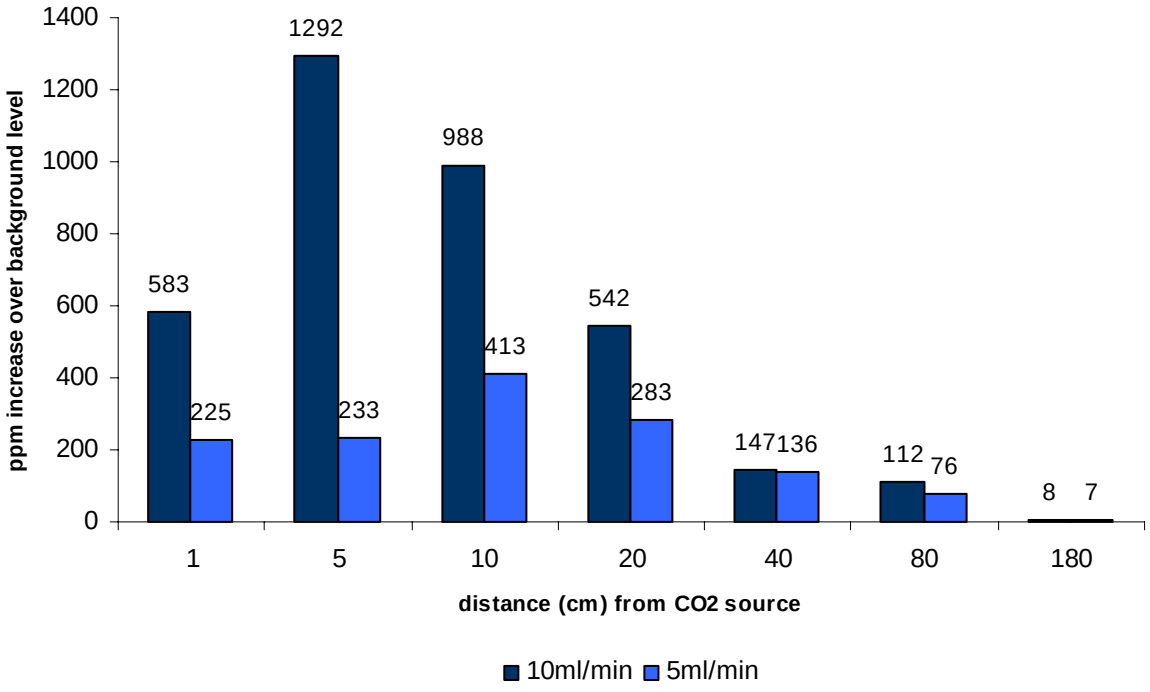


Fig. C. 3: Average CO_2 increase in ppm by introducing CO_2 at 5ml/min and 10ml/min into the wind tunnel.

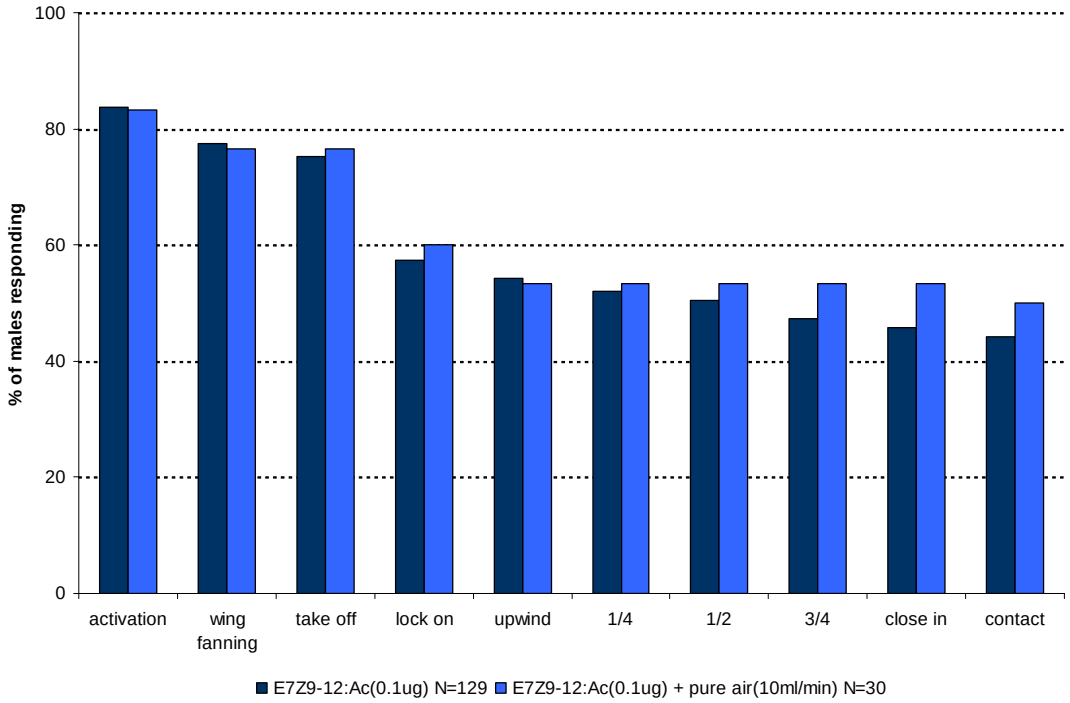


Fig. C. 4: Behavioural response of grapevine moth males to a rubber septum loaded with 0.1µg of E7Z9-12:Ac and to a rubber septum loaded with 0.1µg of E7Z9-12:Ac combined with 10ml/min pure air. No significant differences between the two treatments were found (GLM, $p<0.05$).

Additional CO_2 decreased the attractiveness of the main pheromone component E7Z9-12:Ac in the wind tunnel. Releasing 10ml/min of CO_2 in combination with the pheromone significantly decreased the entire sequence of the male flight behaviour from take off to contacting the source compared to the flight behaviour elicited by presenting the pheromone without additional CO_2 (Fig. C. 5). When 5ml/min of CO_2 were added to the pheromone the decreasing effect on the behavioural response of grapevine moth males was less pronounced than with 10ml/min, since a comparison with the behavioural response to the pheromone was no longer significantly different. Our data suggest that grapevine moth males respond in a dose-dependent manner to CO_2. We provide experimental support that *L. botrana* males perceive CO_2 and are probably highly sensitive to minute changes in CO_2 background levels.

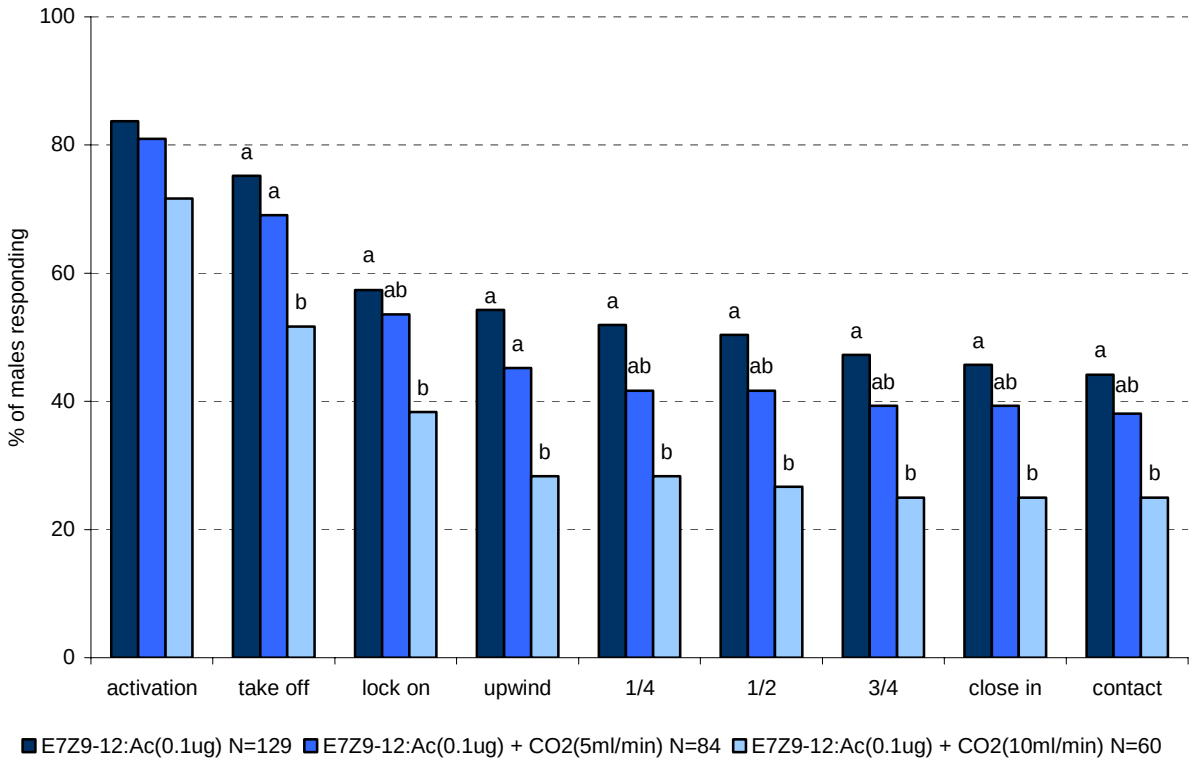


Fig. C. 5: Behavioural response of grapevine moth males to the pheromone alone (0.1µg of E7Z9-12:Ac on a rubber septum), and to the pheromone in combination with either 5ml/min or 10ml/min of additional CO_2 introduce into the wind tunnel. A release rate of 10ml/min of CO_2 significantly decreased the attractiveness of the pheromone (GLM, $p<0.05$).

Annex D - Effects of (+)-terpinen-4-ol on the attractiveness of the sex pheromone presented on rubber septa

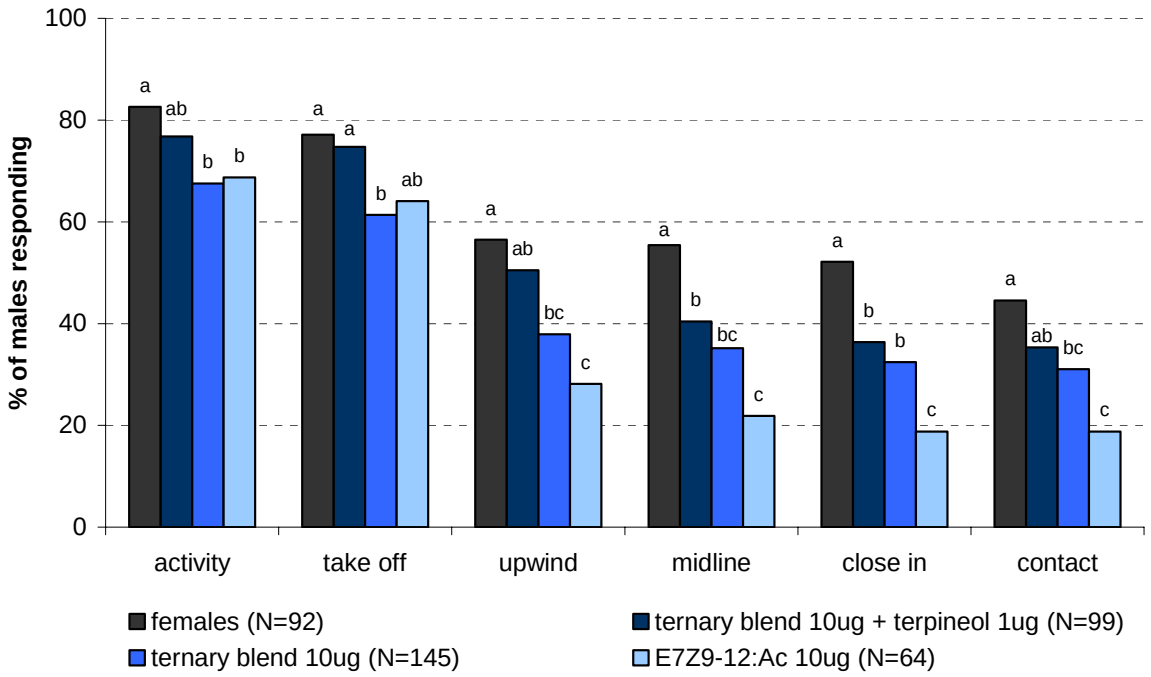


Fig. D. 1: Behavioural response of grapevine moth males to three calling females, a rubber septum loaded with the ternary pheromone blend (E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac at 10:2.0:0.5µg) a rubber septa loaded with the ternary blend plus the plant volatile (+)-terpinen-4-ol (E7Z9-12:Ac, E7Z9-12:OH, Z9-12:Ac and terpineol at 10:2.0:0.5:1.0µg) and a rubber septum loaded with 10µg of E7Z9-12:Ac. All odour sources were presented in glass container closed with a honeycomb grid. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).

Annex E - Is a rubber septum a suitable dispenser for plant volatiles? Estimation of volatile emissions from rubber septa using headspace collection analysis

Material and methods

Headspace collections were obtained from rubber septa impregnated with the main pheromone component E7Z9-12:Ac and three different plant volatiles, (Z)-3-hexen-1-ol, (+)-terpinen-4-ol and methyl salicylate. To determine changes in release ratios over time two times six rubber septa were formulated applying 20µg of E7Z9-12:Ac, 20µg of (Z)-3-hexenol, 20µg of (+)-terpinen-4-ol and 1.0µg of methyl salicylate. One set of 6 rubber septa was sampled 4 hours after formulation, the other set after being placed in the field for 35 days during May-June 2006. Six rubber septa were introduced into a gash wash bottle (100ml) placed into a water bath with a temperature of 45°C and was closed with a T-glass connector. The inlet of the glass connector was coupled to a charcoal filter. The outlet was connected in series with a commercial Tenax™ GR cartridge (6 mm OD, Gerstel®, Switzerland; conditioned under N_2 at 200°C for 90min before collection procedure) a flow meter and a vacuum pump. Air was pulled at 500ml/min through the system for 2h. Tenax cartridges were submitted to gas chromatography coupled mass spectrometry instantly after volatile collection. Further headspace collections were carried out to investigate relative release ratios of plant volatiles compared with E7Z9-12:Ac. Three rubber septa were formulated with 10µg E7Z9-12:Ac, 10µg of (Z)-3-hexenol, 10µg of (+)-terpinen-4-ol and 10µg of methyl salicylate. Headspace collections were carried out as described above but at room temperature (±23°C). Gas chromatography linked mass spectrometry was done as described in section 2.2.

Results

Analytical tests have been carried out to estimate release ratios of plant volatiles and the main pheromone component E7Z9-12:Ac from rubber septa and evaluate the suitability of such dispensers for wind tunnel and field studies. In a first step, headspace profile comparisons from 1 day- and 35 day-old rubber septa, treated with a blend of E7Z9-12:Ac, (Z)-3-hexenol, terpinen-4-ol and methyl salicylate (20:20:20:1.0µg) were performed to investigate a possible distortion of the release ratios of the emitted volatile blend over time (Tab. E. 1).

Tab. E. 1: Comparison of headspace samples obtained from 1 day- and 35 day-old rubber septa loaded with E7Z9-12:Ac, (Z)-3-hexenol, terpinen-4-ol and methl salicylate at 20:20:20:1.0µg. A change in the composition of the emitted volatile blend over time is observed. Due to very low release rate E7Z9-12:Ac could not be identified in headspace samples from 1 day- and 35 day-old rubber septa and was excluded from the analysis.

		(Z)-3-hexenol	terpinen-4-ol	methyl salicylate
1 day	amount applied (µg/rs)[a]	20	20	1.0
	amount collected (ng/rs)[b]	101	62	4
	proportion [c]	26	16	1
35 days	amount applied (µg/rs)	20	20	1.0
	amount collected (ng/rs)	1.3	3.8	1.5
	proportion	0.8	2.5	1

[a] (µg/rs)=µg per rubber septum
[b] (ng/rs)=ng per rubber septum
[c] proportion = amount collected (plant compound) / amount collected (methyl salicylate)

Using a rubber septum as a dispenser for plant volatiles implicates a considerable change of the release ratios of the volatile blend over time. In the headspace analysis of 1 day-old rubber septa the release ratio for (Z)-3-hexenol, terpinen-4-ol and methyl salicylate was found to be 26:16:1 with an estimated amount of 101ng per rubber septum for (Z)-3-hexenol. GC-MS analysis of headspace collections obtained from 35 day-old rubber septa suggest an important change in the release ratio of the three plant volatiles. This time the release ratio of the volatile blend was 0.8:2.5:1 with an estimated amount of (Z)-3-hexenol of 1.3ng per rubber septum. The volatile emission from the rubber septum decreased for all plant volatiles over time, but with different intensity. 35 days after the formulation of the rubber septa the (Z)-3-hexenol emission was 80 times, terpinen-4-ol 16 times and methyl salicylate 3 times lower. Since E7Z9-12:Ac could not be identified in the above-mentioned headspace collections further GC-MS analyses were carried out with the TDS and the CIS running in splitless mode to be able to detect smaller amounts in headspace collections and therefore to be able to compare release ratios of plant volatiles relative to E7Z9-12:Ac emissions. This time GC-MS detected all the constituents of the volatile blend emitted from rubber septa treated with a 4-component mixture ((Z)-3-hexenol, terpinen-4-ol, methyl salicylate and E7Z9-12:Ac, 10µg each, Tab. E. 2). The E7Z9-12:Ac was emitted at low release rates with en estimated amount

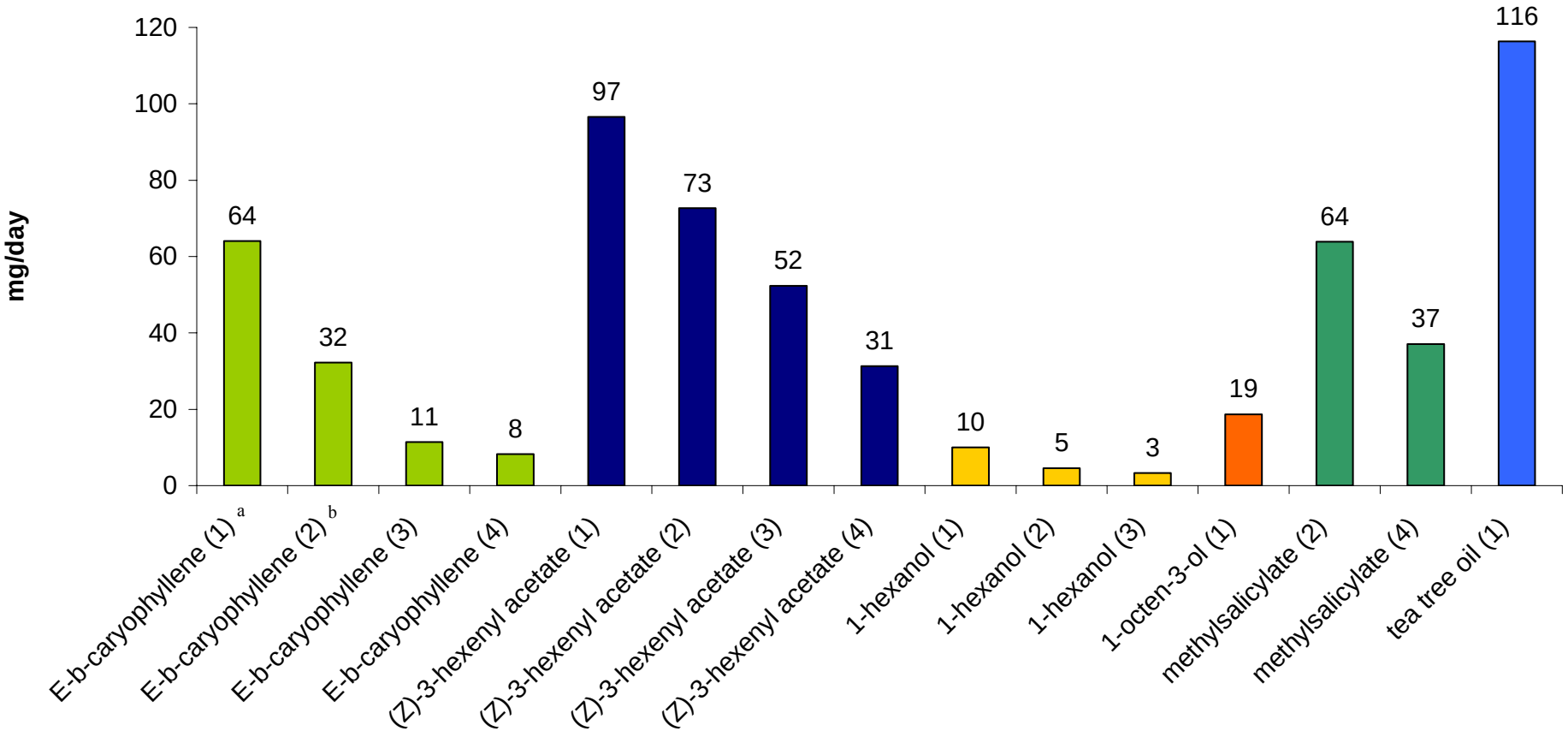
of 57ng present in the headspace sample. Plant volatiles were given off the rubber septa at least 11 times faster with an estimated amount of 605ng of (Z)-3-hexenol. Methyl salicylate had the highest release rate with an amount of 1206ng, which is 21 times more than the E7Z9-12:Ac. Release rates of plant volatiles from rubber septa seem to be particular for each different compound.

Tab. E. 2: Release ratios of the plant volatiles (Z)-3-hexenol, terpinen-4-ol and methylsalicylate relative to the main pheromone component E7Z9-12:Ac in a volatile blend emitted from a rubber septum.

	E7Z9-12:Ac	**(Z)-3-hexenol**	**terpinen-4-ol**	**methyl salicylate**
amount applied (µg/rs)[a]	10	10	10	10
amount collected (ng/rs)[b]	57	605	750	1206
release ratio	1	11	13	21

[a] (µg/rs)=µg per rubber septum
[b] (ng/rs)=ng per rubber septum

Annex F - Release rates of synthetic plant volatiles from polyethylene sachets with varying numbers of walls



[a] one-wall PE-sachet (wall thickness 0.1mm)

[b] double-wall PE-sachet (wall thickness of each layer 0.1mm)

Annex G - Influence of plant volatiles at an overdosed pheromone level

Wind tunnel experiments have been carried out where (E)-β-caryophyllene and 1-hexanol were admixed to an overdosed pheromone level of 100'000pg/min. (E)-β-caryophyllene was added to with the ternary pheromone blend (E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac at a ratio of 100:20:5) at the ratios of 10'000:1, 10'000:10 and 10'000:100 pheromone to plant compound. 1-hexanol was mixed with the ternary pheromone blend at ratios of 1000:1, 1000:10 and 1000:100 pheromone to plant compound.

Behavioural bioassays in a wind tunnel exposing grapevine moth males to different pheromone release levels showed that with increasing dose of the pheromone blend the proportion of males that successfully accomplished the whole flight decreased (see section 2.3). In the presence of high pheromone release levels most *L. botrana* males flew upwind to within a certain distance of the source but then showed in-flight arrestment or flew out of the odour plume. Only a few males flew to within 10cm of the source and made contact. To investigate if secondary plant products could serve to increase the number of grapevine moth males contacting the source at an overdosed pheromone level experiments have been carried out where different doses of (E)-β-caryophyllene and 1-hexanol were admixed to a pheromone level of 100'000pg/min. (E)-β-caryophyllene was added to with the ternary pheromone blend (E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac at a ratio of 100:20:5) at the ratios of 10'000:1, 10'000:10 and 10'000:100 pheromone to plant compound. 1-hexanol was mixed with the ternary pheromone blend at ratios of 1000:1, 1000:10 and 1000:100 pheromone to plant compound (Fig. G. 1, Fig. G. 2).

Grapevine moth males responded in a dose-dependent manner to the three release rates of (E)-β-caryophyllene with significantly more males being activated and taking off at a release rate of 100pg/min compared with 10pg/min or 1000pg/min. Releasing 100pg/min of (E)-β-caryophyllene rendered the overdosed pheromone more attractive with higher numbers of males engaging upwind flights and contacting the source, however the increase was not statistically significant compared to the pheromone alone.

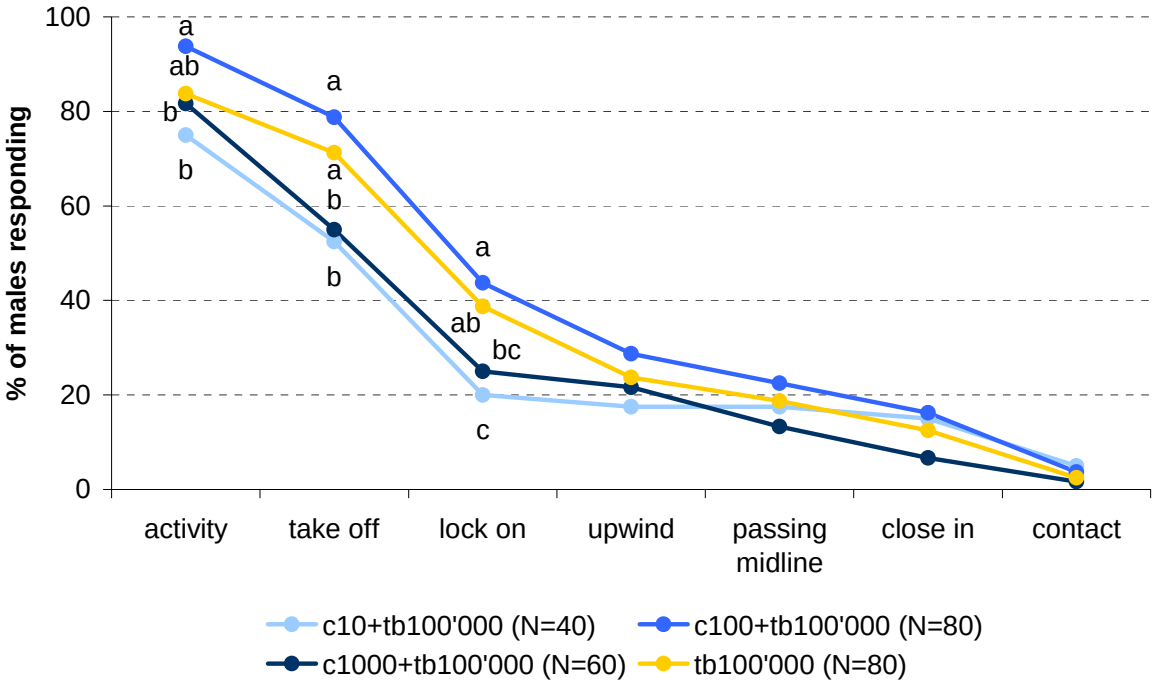


Fig. G. 1: Percentage of *L. botrana* males responding to an overdosed pheromone level (100'000pg/min; tb100'000) alone and in combination with three release rates of (E)-β-caryophyllene (10, 100, 1000pg/min; c10, c100, c1000). The activity of the ternary pheromone blend was moderately increased by adding (E)-β-caryophyllene at a ratio of 1000:1, but the difference was not significantly different. Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).

A dose-dependent response was also found by admixing the plant volatile 1-hexanol to the overdosed pheromone with the ratio of plant volatile to pheromone of 10:1000 being more attractive than ratios of 1:1000 or 100:1000. 1-hexanol released at 1000pg/min, that is a plant volatile to pheromone ratio of 1:100, rendered the pheromone more attractive with a higher number of males flying upwind and contacting the source, but the statistical analysis revealing only a moderate increase for upwind flight (p-value 0.08).

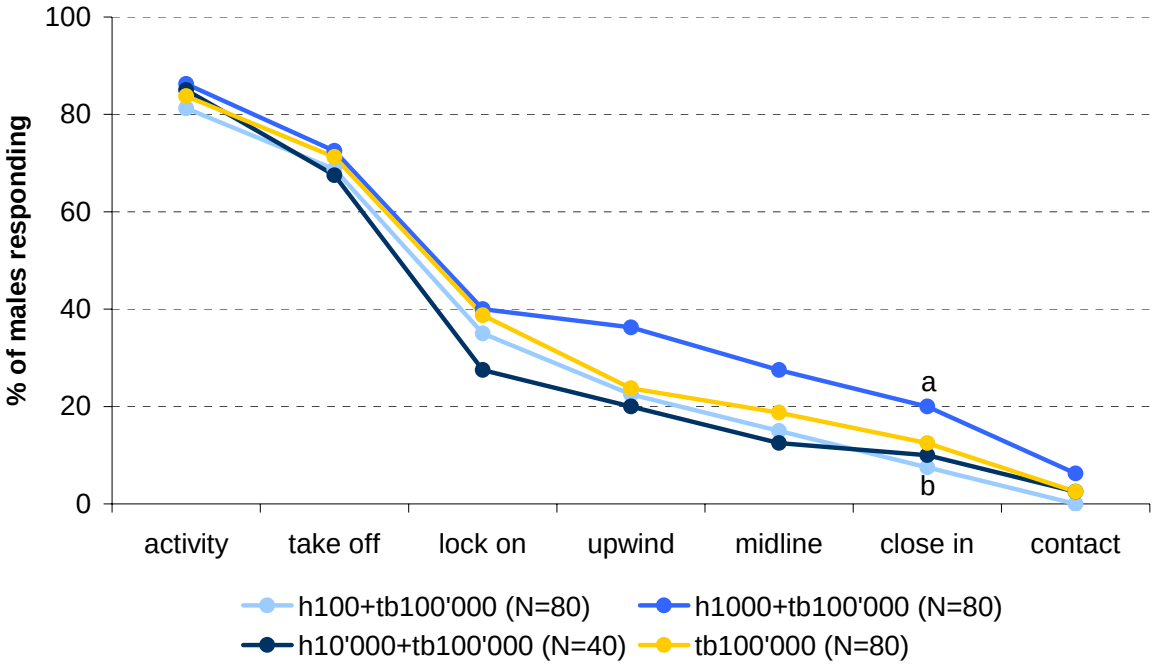
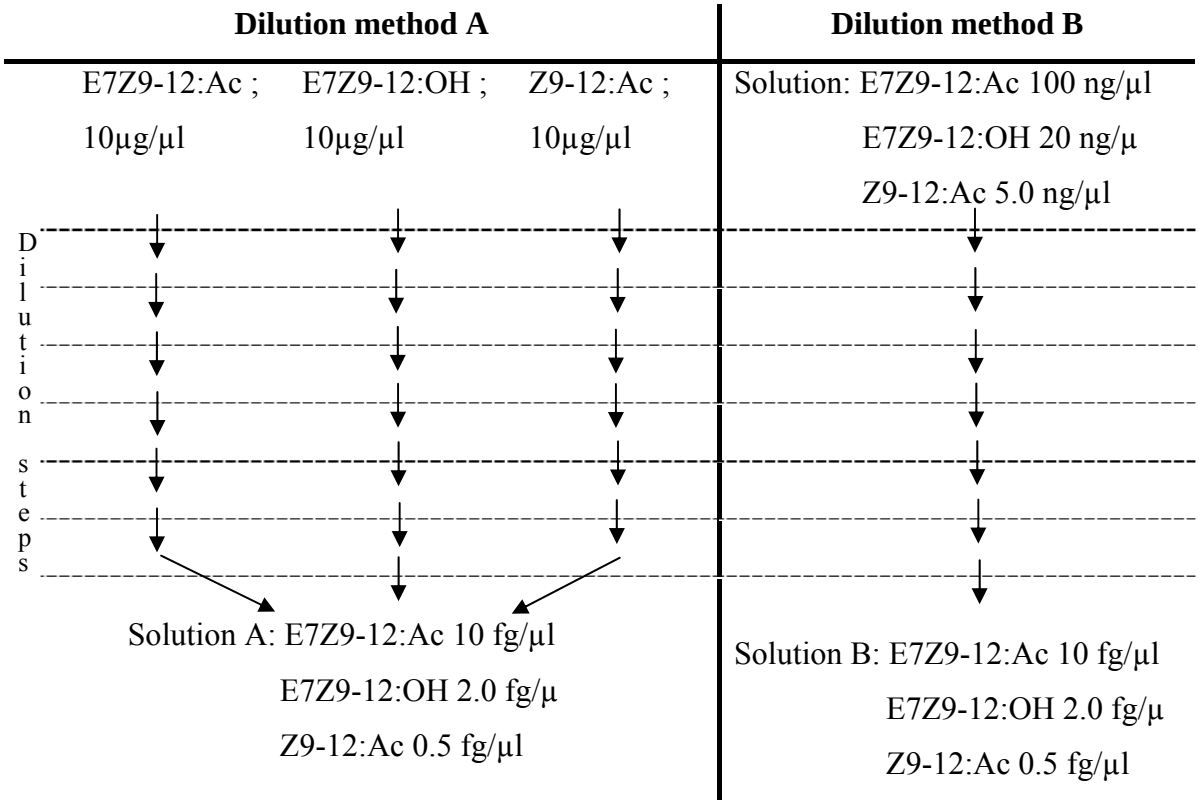


Fig. G. 2: Percentage of *L. botrana* males responding to an overdosed pheromone level (100'000pg/min; tb100'000) alone and in combination with three release rates of 1-hexanol (100, 1000, 10'000pg/min; h100, h1000, h10'000). The attractiveness of the ternary pheromone blend was slightly increased by adding 1-hexanol at a dose of 1000pg/min, but the differences were not significantly different (tendency for upwind with a p-value of 0.08). Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).

Annex H –The dilution of multicomponent blends can lead to distorted blend proportions

Grapevine moths are highly sensitive to minute amounts of their sex pheromone components. In the wind tunnel we observed oriented upwind flights of grapevine moth males down to levels of 1.0 femtograms per minute (amount refers to the main component E7Z9-12:Ac) of the ternary pheromone blend (E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac at 100:20:5). We used two different ways to make up solutions containing pheromone products at such low levels with ethanol as solvent (Tab. H. 1). With dilution method A the pheromone components were diluted singly and the ternary blend was formulated only at the end as the last step. With dilution method B, a solution was made consisting of the three main pheromone components. This stock solution was subsequently diluted in steps of 10, down to femtogram-levels.

Tab. H. 1: Dilution methods that were used to formulate pheromone blends for wind tunnel bioassays. In dilution method A each component was diluted singly and the formulation of multicomponent blend was the last step, whereas with dilution method B the multicomponent blend was formulated at the beginning followed by the dilution steps.



Wind tunnel assays revealed significant differences in the behavioural response of grapevine moth males between ternary pheromone blends formulated with the different dilution methods (Fig. H. 1). The ternary pheromone blend formulated according to dilution method B attracted significantly fewer males than the ternary pheromone blends formulated according dilution method A. The behavioural responses of grapevine moth males to the two ternary blends formulated with dilution method A were not significantly different and the response were repeatable.

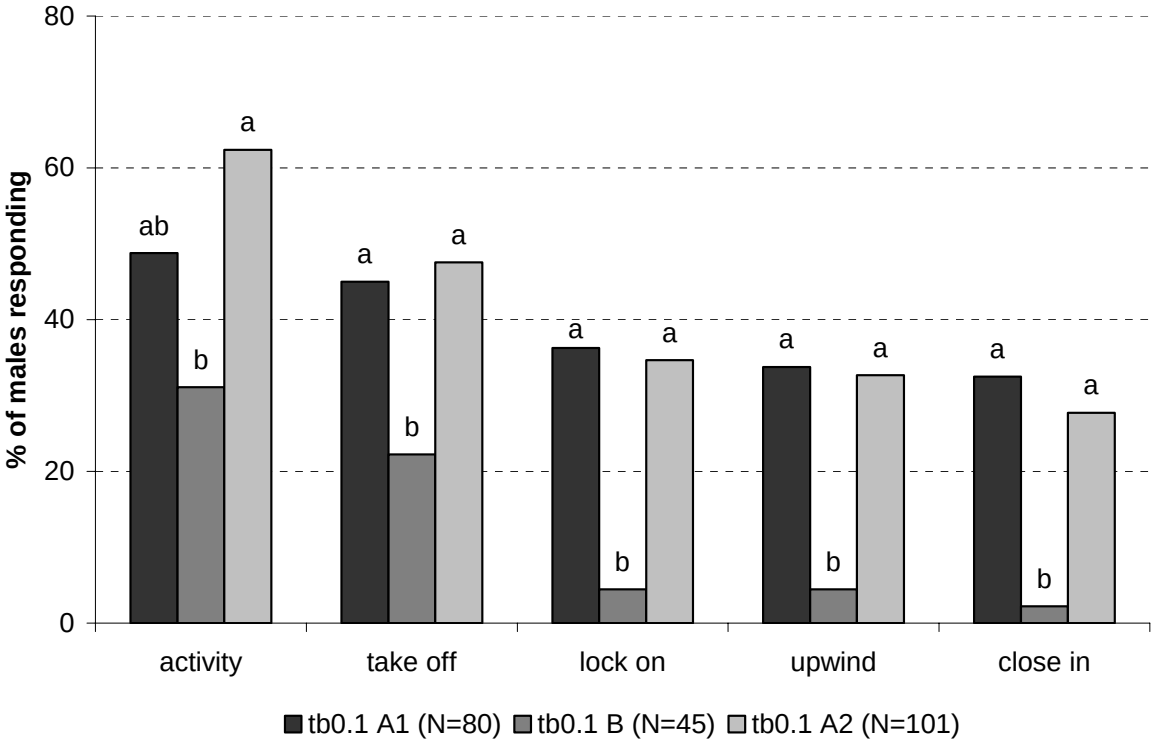


Fig. H. 1: Behavioural responses of grapevine moth males to three different ternary pheromone blends released at 0.1pg/min in a wind tunnel. Two ternary blends were formulated according to the dilution method A (tb0.1 A1 and tb0.1 A2; see Tab. H. 1) and one according to the dilution method B (tb0.1 B). Different letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$).

GC-MS analysis of three pheromone solutions formulated with dilution method B (stock solution and the two following dilutions) revealed blend distortions (Fig. H. 2). The relative amount of E7Z9-12:OH increased with each dilution step, whereas the ratio between the two acetates remained the same, suggesting that differences in solubility of the three pheromone components in ethanol influences the composition following each dilution step.

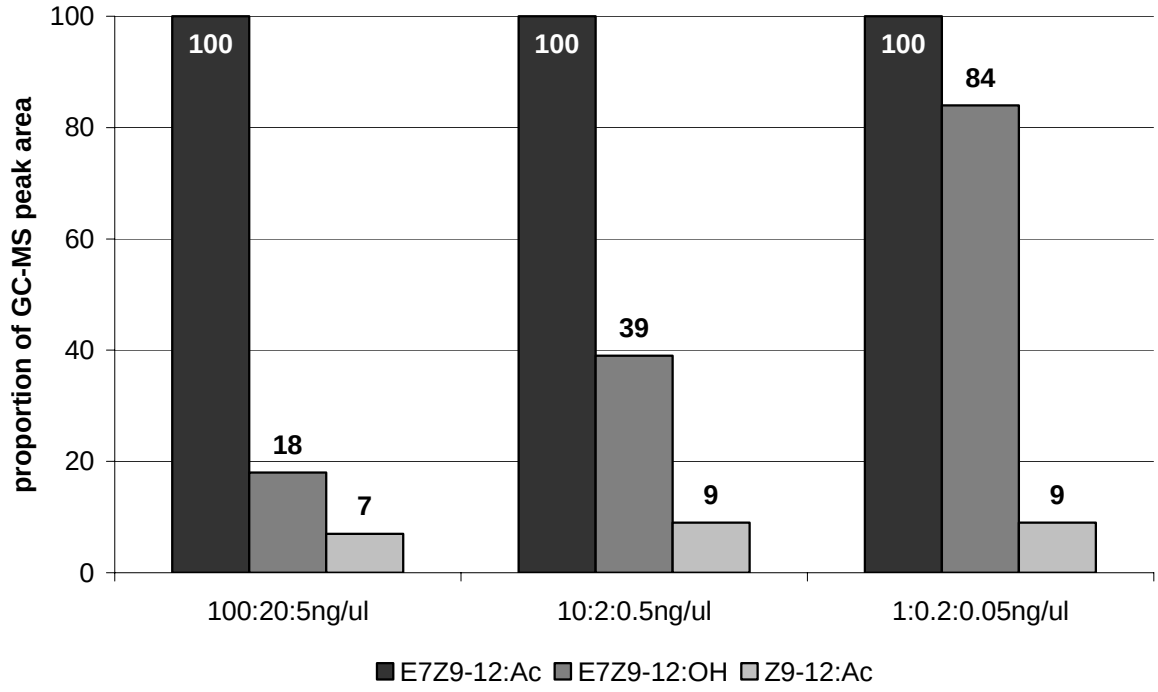


Fig. H. 2: Proportion of peak areas of the three main pheromone components (E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac) in the stock solution (100, 20 and 5 ng/µl) and in two following dilutions (10, 2 and 0.5 ng/µl and 1.0, 0.2 and 0.05 ng/µl) as determined by GC-MS analysis.

Care has to be excercised when formulating odour blends containing minute amounts of active compounds with different functional groups. We have indications that the properties of the solvent could considerably distort blend proportions but the today's analytical tools are not sensitive enough to detect and quantify low amounts of products down to concentrations of some femtograms per microliter. We suggest to formulate multicomponent blends using dilution method A and to avoid successively diluting multicomponent blends. Evidently, step wise dilution of each component separately assures the addition of the appropriate amount to the final mixture.