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*CLOSER TO THE FIELD: A LABORATORY BIOASSAY TO
ASSESS THE EFFECT OF A BACKGROUND ODOUR ON
THE ATTRACTION TO HOST-VOLATILES*

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"Education and training are the most powerful weapon to change the world. Education is the great engine of personal development. It is through education that the daughter of a peasant can become a doctor, the son of a miner's chief mine or a child born into a poor family, the president of a great nation. Not what we are given, but the ability to make the most of what we have is what distinguishes one person from another". (Nelson Mandela)

"L'istruzione e la formazione sono le armi più potenti per cambiare il mondo. L'educazione è il grande motore dello sviluppo personale. È grazie all'educazione che la figlia di un contadino può diventare medico, il figlio di un minatore il capo miniera o un bambino nato in una famiglia povera il presidente di una grande nazione. Non ciò che ci viene dato, ma la capacità di valorizzare al meglio ciò che abbiamo è ciò che distingue una persona dall'altra". (Nelson Mandela)

Abstract

In this research project I measured the laboratory response of an herbivorous insect, the apple fruit moth (AFM) *Argyresthia conjugella* (Zeller), to volatiles released by its primary and secondary host plants. AFM is a seed predator of rowan trees (*Sorbus aucuparia*). Since flowering and fruit setting of rowan are cyclic, populations of AFM build up in forests during good fruiting years and invade nearby apple (*Malus domestica*) orchards in the following year when rowan does not yield berries. Damage in orchards can reach up to the totality of the production. With the aim to develop a field attractant for the apple fruit moth to be used as a warning tool in apple, I measured the response of the moth to rowan and apple volatiles.

My research was entirely carried out in a laboratory wind tunnel. In single choice assay, I tested the response of the insect to both plant material and to sprayed volatile collections from this material. Then, I attempted to include the possible effect of the crop apple (background odour) on the attraction to rowan volatiles, through a choice assay.

The most attractive plant material was the rowan branch with green berries, used by the moth as a natural oviposition substrate. When using the sprayer, a similar response between flowering rowan, green berries and leaves was recorded.

The next step was to test the attraction of the two most attractive rowan odours against an apple background, mimicking the real setting of an orchard. Although both the apple background and the rowan headspace were attractive in single choice assays, females significantly preferred the volatiles from rowan (either as green berries or as a flowering cluster) to those from the apple branch in a dual choice experiment.

This shows that female apple fruit moth can discriminate across volatile signals from different host plants, with rowan being the most attractive plant over apple. The capacity of the rowan headspace to catch AFM females in an apple orchard could be tested with the aim to monitor or capture egg-laying females and thus to reduce the larval damage. We need however to consider that the rowan odour will have to compete with a much higher amount of apple volatiles than in the wind tunnel. An increase of the concentration of the odour (or of a related synthetic mimic) could provide a higher competition towards the apple background.

Finally, although aware of the limit of this project, because carried out only in the laboratory, I am convinced that the way forward now is to make the kairomone obtained and tested in the laboratory, more and more competitive, to be used in Scandinavian apple orchards as a way to optimize the use of insecticides.

Riassunto

Lo studio delle interazioni fra insetti e piante è un argomento che ha sempre impegnato molti scienziati, come testimoniano i recenti studi riguardanti i semiochimici, composti in grado di regolare l'interazione tra gli esseri viventi.

Nella mia indagine ho focalizzato l'attenzione sui cairomoni, sostanze volatili emesse dalle piante che portano beneficio solo al ricevente, che, in questo caso, è l'insetto che cerca la sua pianta ospite, per nutrirsi e deporre le uova, utilizzando un efficiente sistema nervoso.

Ogni pianta libera delle sostanze volatili che vengono captate anche da lunghe distanze dai sensilli posti nelle antenne dell'insetto, il quale, seguendo la scia di odori, raggiunge la propria pianta ospite dove avviene il riconoscimento dapprima con la vista ed infine l'accettazione dell'ospite attraverso stimoli tattili e gustativi. Considerata la straordinaria capacità di riconoscimento della propria pianta ospite da parte degli insetti, si sta ipotizzando di impiegare i cairomoni nella lotta integrata per ridurre gli insetticidi in agricoltura.

Per verificare questa possibilità, in Norvegia, ho compiuto osservazioni in laboratorio, impiegando le femmine di Apple fruit moth, *Argyresthia conjugella*, il più pericoloso carposfago del melo nell'intera Scandinavia, le cui larve danneggiano i frutti scavando numerose gallerie in tutta la mela fino ad arrivare ai semi dei quali si nutrono. L'ospite primario di questo insetto è il sorbo, *Sorbum aucuparia*; ma dato che la produzione di frutti di questa pianta varia di anno in anno, le femmine di *Argyresthia*, negli anni di scarsa produzione, non trovando un numero sufficiente di bacche per deporvi le uova, si trovano costrette ad emigrare nei frutteti vicini per ovideporre. Il melo è quindi l'ospite secondario di questo insetto, il quale tuttavia non ha mai attaccato altre piante appartenenti alla famiglia delle *Rosaceae*, quali pero, pesco e ciliegio.

In particolare ho svolto la mia ricerca interamente nel "tunnel del vento", nel quale, attraverso l'utilizzo di diversi campioni raccolti dalla pianta di sorbo, quali infiorescenze a diversi stadi, rami con sole foglie e rami con le bacche, ho potuto osservare le risposte positive degli insetti alle sostanze volatili emesse dai campioni di pianta impiegati nei test. Dopodiché, ho registrato che gli sprayer, utilizzati per rilasciare gli stessi odori del sorbo, hanno funzionato perché hanno mostrato la capacità di attrarre l'insetto, seppur in maniera minore rispetto ai corrispettivi campioni prelevati direttamente dalla pianta stessa.

Visti i risultati positivi dei test eseguiti con i campioni prelevati direttamente da alberi di sorbo e con gli sprayer, perché allora le femmine gravide, negli anni di scarsa produzione di bacche, emigrano nei meleti per ovideporre?

L'ipotesi è la seguente: l'ospite naturale dell'*Argyresthia* è l'albero di sorbo *Sorbum aucuparia* e la pianta di melo coltivata, *Malus domestica*, è solo un'alternativa obbligata che le femmine devono per forza scegliere negli anni di scarsa produzione da parte del sorbo.

Pertanto, ho eseguito dei test, nel "tunnel del vento", impiegando lo sprayer insieme ad un campione di pianta di melo, con l'obiettivo di verificare se l'apple background del melo possa in qualche modo influenzare l'insetto durante la ricerca dell'ospite e per individuare la sostanza volatile più attrattiva e di conseguenza preferita. La verifica di questa ipotesi è stato un passo fondamentale verso l'impiego dei cairomoni nella lotta integrata nei frutteti: lo sprayer, sintetizzato in laboratorio, avendo dimostrato di poter competere con le sostanze volatili emesse dal melo, potrà dunque essere impiegato direttamente in campo per monitorare la popolazione di Apple fruit moth. Inoltre le miscele di sostanze volatili sintetizzate in laboratorio potranno essere utilizzate nella costruzione di trappole provviste del cairomone del sorbo come erogatore, in modo da poter catturare gli individui di Apple fruit moth e prevenire così i danni ai meleti.

Infine, pur consapevole del limite di queste osservazioni eseguite esclusivamente in laboratorio, ma soddisfatto per gli ottimi risultati ottenuti, sono convinto che la strada da seguire ora sia quella di rendere il cairomone, ottenuto e testato in laboratorio, sempre più competitivo, per essere impiegato nei meleti scandinavi come alternativa agli insetticidi.

1. INTRODUCTION

1.1. Insect-plant interaction

Green plants constitute the most voluminous compartment of living matter, whilst insects are the leaders in number of species. Herbivorous insects and plants are interconnected in complex relationships. Insects, with their amazing variation in form and life history, are among the causes driving the evolution of green plants (Marquis R.J.,2004). A half of insect species feed on living plants: more than 400 000 herbivorous insect species live on 300 000 vascular plant species (Table 1). Probably no other interactions between two groups of organisms, comparable in type and extent, can be found elsewhere in the living world, thus rendering insect-plant interactions a unique and interesting area of biological research.

Table 1. Numbers of herbivorous species in different insect orders. (Data from various sources).

Insect order	Total n° of species	Herbivorous %
Coleoptera	349 000	35
Lepidoptera	119 000	100
Diptera	119 000	30
Hymenoptera	95 000	11
Hemiptera	59 000	91
Ortophtera	20 000	100
Thysanoptera	5 000	90
Phasmida	2 000	100

One of the most striking aspects of insect-plant relationships is the high degree of food specialization among herbivorous insects. Insects that in nature exploit only one or a few plant species are called *monophagous*. *Oligophagous* insects feed on a number of plant species that are not necessarily belonging to the same family. *Polyphagous* insects utilize many plants, often belonging to different plant families. Host-plant specialization seems to be the most frequent feeding regime, since less than 10% of herbivorous species feed on plants belonging to more than three different

families. Herbivorous insects living on herbaceous plants often show a higher degree of host-specialization in comparison with species adapted to shrubs and trees. This concept may be explained by the fact that herbaceous plants show a greater diversity, for example in life cycle and chemical composition, than woody plants. An additional explanation regards the relationships between the breadth of an insect's diet and its body size: smaller species are generally more specialized than larger species. In addition, insects show a degree of specialization according to the feeding site on their host: while a number of caterpillars, beetles and grasshoppers are leaf foragers, bugs often penetrate epidermal cells and ingest cell contents. Different species may excavate different layers of the leaf parenchyma (see leaf rollers as example), while others species often show a predilection for particular parts of a leaf (see leaf miners as example).

The phenomenon of host-plant specialization requires the recognition of the host among species-rich vegetation. Ovipositing females have a botanical instinct that helps them to recognize their host plants (*Städler, 2002*). The “botanical instinct” of some specialized feeders has in some cases helped botanists to classify some unknown plants. The gender *Thyridia* is a specialist feeding exclusively on the family *Solanaceae*. When this insect was observed to feed on an unknown plant (*Brunsfelsia*), the taxonomists guessed that this plant could also belong to the same family, according to the feeding habit of the herbivore.

Host-searching insects look for plants with a particular chemical profile that fits their search image. This profile may be narrow and restricted to plants belonging to a single species or somehow broader and more variable to embrace characteristics of a plant genus or a plant family. Insects may also prefer host plants that are nutritionally suboptimal but are not visited by some of their natural enemies. Such plants offer better possibilities to survive than more nutritious hosts where the herbivore would instead be more vulnerable to predation or parasitization. Some studies suggest that the strong influence of host plant on the risk of attack by parasitoids is a potentially important selective force driving the evolution of the herbivore diet (*Schoonhoven, 1991*).

1.2. *Host-location from a distance*

Insect herbivores use plant volatiles to recognize and to efficiently locate their host plants from a distance. Female and male insects perceive these signals through specialized olfactory receptor neurons housed in the antennae, and use them to discriminate food sources or larval food plants from the background chemical environment (*Bernays, 2001; Mustaparta, 2002*).

By deciphering the plant volatile signal, host-searching insects have access to food source, oviposition site and shelter (*Pichersky & Gershenzon, 2002; Bruce et al., 2005; Owen & Penuelas, 2005*). Plant volatile signals are sufficiently precise to allow insects distinguish between host and non-host plants and to choose plants in a suitable phenological or physiological state. Vegetative volatile cues carry information on the biotic condition of the releaser, which may reflect fitness opportunity from the receiver and its offspring (*e.g. Nordlund et al., 1981; Bell and Cardè, 1984; Cardè and Bell, 1995; Dicke, 1999*). Responding organisms are therefore expected to have evolved the ability to discriminate between signal and noise within a complex volatile background.

Plants emit a number of substances, termed plant allelochemicals, that have a sufficiently high vapour pressure to affect other organisms as volatile. Plants release volatile compounds through open stomata, leaf cuticles and gland walls. A higher release rate of plant volatiles occurs in case of plant damage.

In the past identification of plant volatiles began with extracts of chopped or macerated plant material. Recently, a more precise method termed *headspace collection* has been developed to obtain volatiles emitted by plants (Figure 1). This process reflects what is released from the plant into the surrounding air and, in combination with gas chromatography, gives a much more detailed information about the composition of naturally emitted volatiles than the method based on tissue maceration.

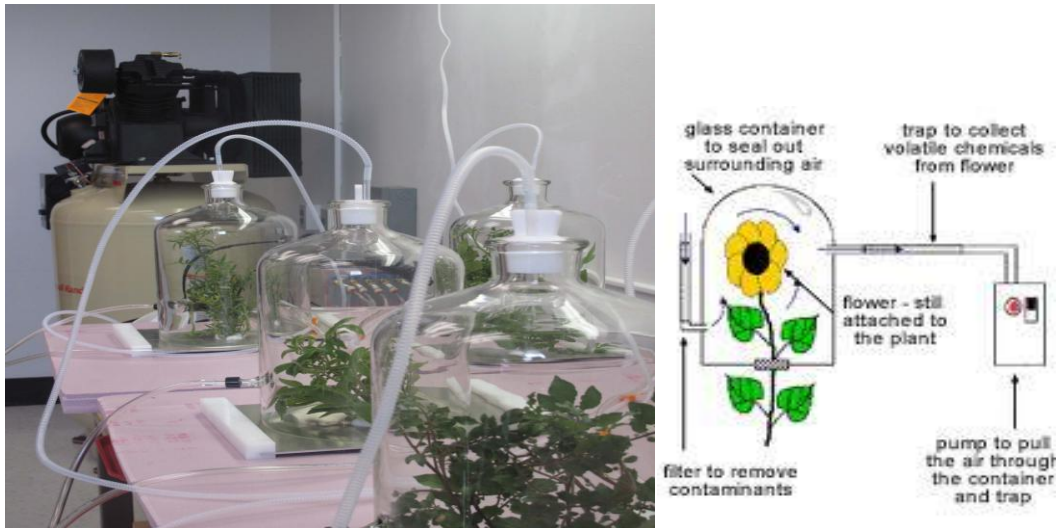


Figure 1. A system for the collection of a plant headspace (Photo by Bioforsk, explanation by schoolsience.uk).

The number of volatile substances emitted by a plant may reach several hundreds, although domination by one or a few major compounds is commonly reported. For example, the air around corn leaves contains at least 24 compounds, although a mayor fraction (75%) consists of only seven components (*Cantelo W. And Jacobson M., 1979*). The mayor headspace volatile is often a green leaf volatile (aldehyde or ester) or a terpenoid (*Bruce, T.J.A., Wadhams, L.J. & Woodcock, C.M., 2005*).

Herbivorous insect such as the *Colorado potato* beetles respond positively to the mixture of green leaf volatiles produced by the host-plant foliage. However, when the natural blend is distorted by changing the concentration of a single component, the response significantly decreases (*Jermy T., Szentesi A. and Horvath J., 1988*).

When damaged, the proportion of this principal component can either increase (soybean, eggplant) or decrease (pepper). Moreover, the odour emitted after mechanical damage differs from that induced by herbivore damage. In the latter, the amounts of compounds vary in accordance with the herbivore species. As an example, the headspace of apple leaves infested with the spider mite *Panonychus ulmi* contains 49% of 4,8-dimethyl-1,3(E),7nonatriene. However, when the leaves are infested by another spider mite, *Tetranychus urticae*, the proportion of the compound reaches only 9% (*Dicke M., 1994*).

Knowledge on insect host-location via volatiles will provide a basis for the development of innovative insect-control methods, through direct application of plant volatile compounds or through plant breeding for improved resistance (*Visser, 1988*).

1.3. Plant acceptance

Landing at the host represents the final step during host-location from a distance. The role of plant volatiles in eliciting landing has been suggested in many species, although the observed effects may often be attributed to attraction. For example, *Citrus* volatiles cause a higher frequency of host visit, increasing oviposition by *Papilio demoleus*. Similar results were reported for the black swallowtail butterfly, *Papilio polyxenes*, which laid a higher amount of eggs on artificial plants that were treated with carrot volatiles. Non-host volatiles, on the other hand, inhibited the landing of *P. polyxenes*. Stimuli that prevent or discourage landing on non-hosts or unsuitable hosts are suggested to play an important role in the selection of an oviposition. Once the seeking insect has landed on the host-plant, additional cues may be used to assess plant suitability. Tactile (mechanosensory) and contact chemosensory (taste or gustatory) stimuli may serve to this scope. Physical features of plant organs or tissues can influence the host-plant selection behaviour. Insects are equipped with a number of mechanosensory sensilla to acquire relevant information on plant surface structure and texture. The plant surface is often covered with trichomes, which may impede insect movement and feeding. In several cases glands associated with a trichome may liberate a secretion upon insect damage which may be repellent for the herbivore. Following this contact phase, the insect tends to restrict its movements to a small area. This step is called arrestment. Due to the limited mobility and energy reserves of the neonate larvae, the decision of a gravid female to accept a plant as oviposition resource is of crucial importance to the development of the future generation.

1.4. Oviposition

Plant chemistry is suggested as the most important source of information contributing to the final decision by a female to oviposit or not.

A number of chemicals from the plant surface are known to affect the oviposition behaviour of herbivorous insects. As an example, the spatially and temporally variable concentration of glucids and amino acids may affect the acceptance of the

plant by a searching-insect. Glucids are shown to promote oviposition in phytophagous insects. In moths, which do not injure plant tissues during egg-laying, the oviposition response is based on the perception of glucids and possibly additional stimuli at the leaf surface (*Renwick J., 1994*). Additional compounds, such as lipophilic constituents of leaf surfaces (alkanes, esters, fatty acids), may also promote oviposition in a number of insect species. In the cabbage white butterflies (*Pieris spp*), a single glucosinolate isolated from the surface of cabbage leaves stimulated oviposition when sprayed on artificial leaves of a non-host plants. However, glucosinolates differ in their stimulatory effect according to the tested herbivore. In two species of *Delia* flies, oligophagous on Brassicaceae, female showed a distinct order of preference towards different glucosinolates. The neural responses to glucosinolate-specific chemoreceptors located in sensory hairs on the tarsi, was correlated to the behavioural response of the fly (*Renwick J. et. al. 1992*).

1.5. The apple fruit moth

The apple fruit moth (AFM), *Argyresthia conjugella* (Figure 2), belongs to the order Lepidoptera, family *Argyresthiidae*. It is a seed predator of rowan trees (*Sorbus aucuparia*). Since flowering and fruit-setting of rowan are cyclic (*Sperens, 1997*), populations of AFM build up in forests during good fruiting years and invade nearby apple orchards in the following year when rowan do not yield berries. Damage in orchards can reach up to the totality of the production (*Ahlberg, 1927; Kopro, 1995*). Masting, the alternating production of berries, represents a strategy adopted by rowan trees to defend themselves against seed predation (*Kopro et al., 2003; Satake et al., 2004*).



Figure 2. Adult of *Argyroresthia conjugella* on a rowan tree (WildguideUK).

During May, adults (Figure 2) emerge from overwintering sites in the soil and, after mating, females oviposit from late June on rowan green berries or on green apple (*Kobro et.al. 2003*). Eggs hatch occurs approximately after two weeks from oviposition and newborn larvae immediately feed upon the fruit (Figure 3). After a feeding period of approximately 1-2 months, mature larvae reach the soil to overwinter (Figure 3). Depending on the climate, the apple fruit moth hibernates as a larva or as pupa, during a period of 6 to 8 months.

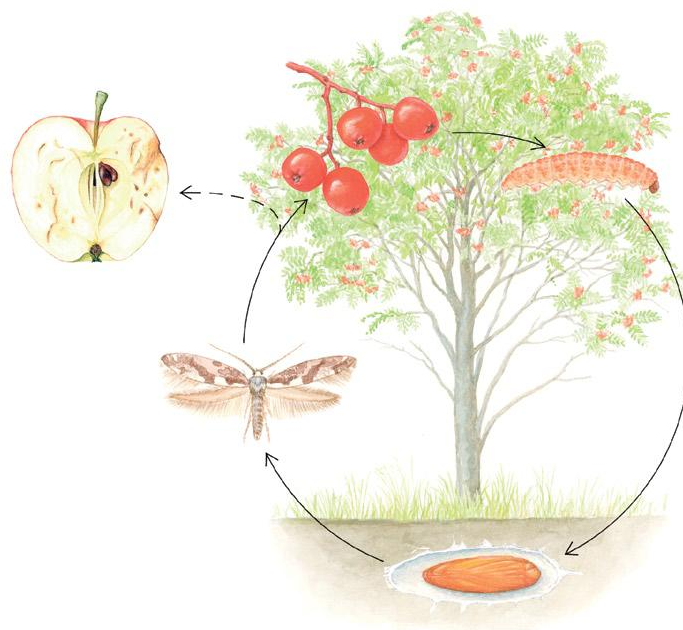


Figure 3. The biological cycle of Apple fruit moth (Drawing by Bioforsk).

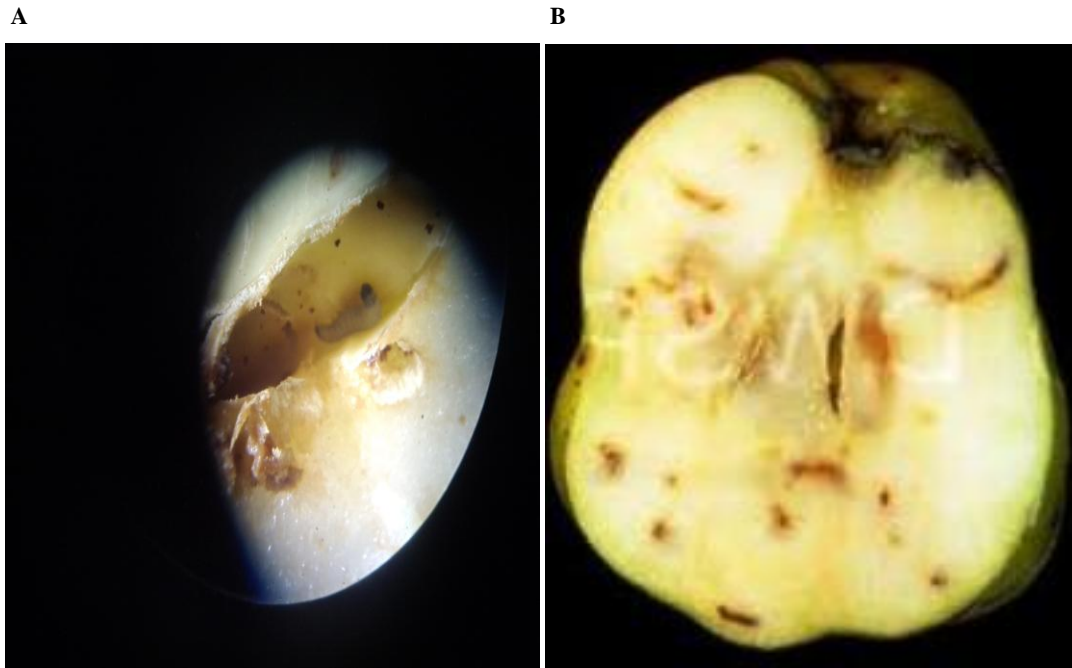


Figure 4. Larva (A) and damage (B) of AFM on apple. (Photo by Daniele Pasini (A) and Russell IPM (B)).

When migration to apple occurs, larvae damage the fruits by a rather random tunnelling (Figure 4). Damaged apples get a bitter taste and rot in advance. Although the larva can pupate in the seed cavity, generally the pupation occurs outside the fruit. The different nutritional value of rowan and apple is not exhaustively studied. Whilst in some cases it has been reported a higher fitness on rowan (Ahlberg, 1927; Edland 1979), in another it was proved otherwise (Kobro, 1995). In some cases, larvae developed in apples were larger than specimens developed in rowan berries. According to the literature, the apple fruit moth can reproduce also on apple without the need of rowan (Kobro, 1995). Although information on the ecology of this insect is available, knowledge on the sensory cues involved in the colonization of apple are not complete.

1.6. Literature on rowan volatiles

The apple fruit moth is particularly suitable for studying the odour that encodes recognition and attraction to different host plant. AFM females lay eggs on apple *Malus domestica* only during rowan intermasting years. Volatiles from both rowan and apple may accordingly account for attraction of *A. conjugella* females for oviposition.

Since single rowan trees interspersed within an apple orchard (Figure 5) could be colonized by AFM females moving from an adjacent forest, it is clear that host-seeking females were attracted to rowan by its odour signal over a distance. In addition, we can assume that they were able to discriminate a rowan tree with berries within the volatile background of an apple orchard with fruits (Figure 5).



Figure 5. Apple orchards in Lofthus, Norway (Photo by Bioforsk).

This observation supports the concept that rowan volatiles provided a preferred signal than apple volatiles during host-search (Kobro, 2003). Oviposition on apple may be regarded as a sensory confusion or mistake (Larsson & Ekbom, 1995; Menken & Roessingh, 1998), but may also be a step towards colonization of a new host. The same compounds may even attract females to food sources (Mc Neil & Delisle, 1989; Landolt & Phillips, 1997; Scheirs & De Bruyn, 2002).

Based on a comparative chemical and electroantennographic analysis of rowan and apple headspace, a blend of two compounds (2-phenyl ethanol and anethole) has been identified as an attractant for both sexes of the apple fruit moth in a rowan forest (Bengtsson *et al.*, 2006). In a further study, Knudsen *et al.* (2008) showed a discrepancies between the laboratory and the field response to this blend or its single components. This result emphasizes the relevance of background odour on the insect response to synthetic blends.

1.7 Hypothesis of my work

In this thesis work we examined the effect of a background odour (apple volatiles) on the attraction to rowan headspace in the laboratory. To this aim we studied the wind tunnel attraction of AFM females to rowan volatiles with or without an apple background. We hypothesized that the apple background may strongly affect the efficacy of a lure based on rowan volatiles. Results from such bioassay could provide knowledge to develop field attractants to efficiently monitor the apple fruit moth migration from the forest to the apple orchard.

2. MATERIALS AND METHODS

2.1. *Hosting Institution*

The experiments were carried at the Plant Health and Plant Protection Division by Bioforsk (Ås, Norway). Bioforsk is a Norwegian Research and Development Institute specialized in the fields of agriculture and food production, environmental protection and natural resource management. The division in which I worked is specialized in the field of plant diseases, weeds, pests, climate effects, genetics and biotechnology. Important areas include integrated plant protection, biological control and pest forecasting systems. The division is also involved in agrometeorology, ecotoxicology and risk analysis.

I elaborated the data and wrote my thesis at the Division of Integrated Plant Protection, Department of Crop Protection Biology by SLU (Alnarp, Sweden).

2.2. *Insects*

The insects used for the experiments were collected as overwintering larvae from infested rowan berries in August 2012. Berries came from different forests located in Southern Norway. Larvae overwintered inside corrugated cardboard rolls outdoors and became pupae during early spring. During April-May 2013, 5-10 rolls per week were transferred to Plexiglass cages for emergence under a 18:6 (Light:Dark) photoperiod, 20-24°C and 55-70% relative humidity. Emerged adults were allowed to freely mate inside the cage for 5 days. After this time were taken only the females for wind tunnel experiments and the males were thrown in the freezers. The adults had to be at least 5 days in the same cages because it was supposed that insects with less than of 5 days of age were not active. Insects were provided with water.

2.3. *Plants*

The plant material was collected daily from plants located outside the laboratory. Apple branches cv *Amorosa* with leaves and green fruits (Figure 7) and rowan branches with leaves or with a cluster at different phenological stages (flower buds, flowering (50% or 100%), green berries) (Figure 6 and 7) were cut off the plant and

immediately carried to the laboratory. These plant parts were used both as attractive sources in the wind tunnel and as samples for volatile collection.

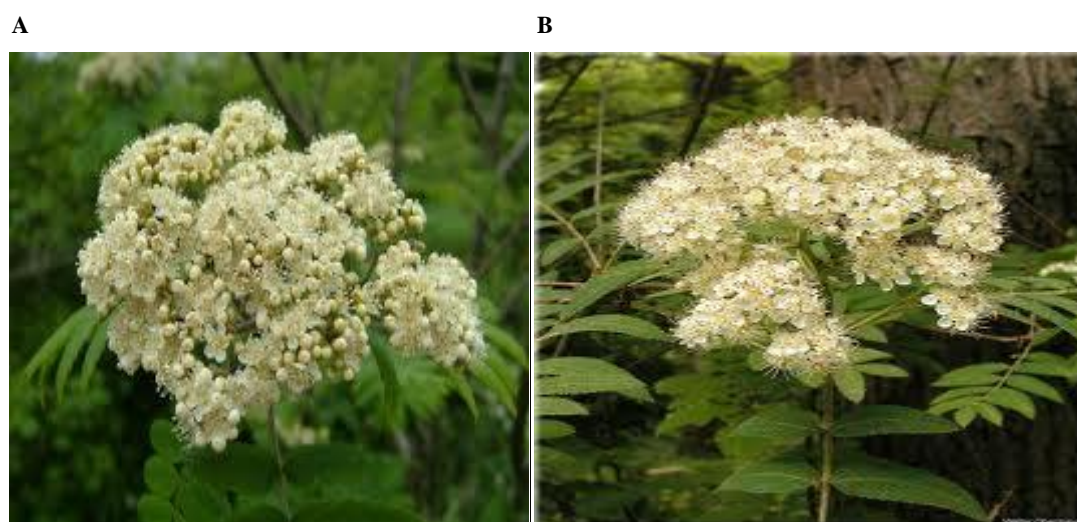


Figure 6. Rowan clusters flowering at 50 (A) and 100% (B).

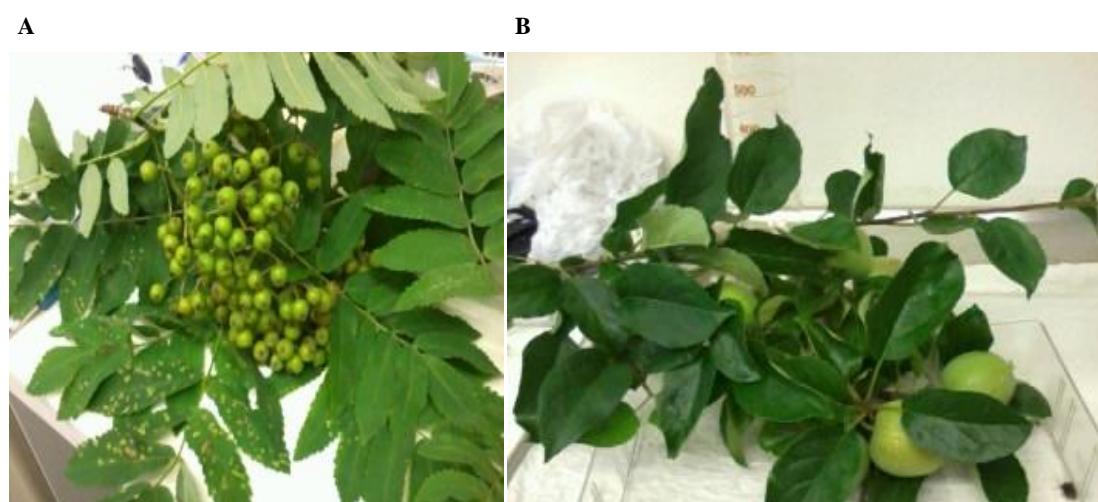


Figure 7. A rowan cluster with green berries (A) and an apple branch (cv Amorosa) with green fruits (B).

2.4. Headspace collection

Volatiles were collected from freshly cut plants (Figure 6 and 7). The sampling was done during AFM peak seasonal flight from middle of June to middle of July. The material was placed in a 3 liter glass jars closed at the top with a grounded glass fitting. A charcoal-filtered air stream (150 ml min) was pulled over the plant material from the bottom to the top of the jar. A volatile collecting trap (Super Q) was

connected with glass fittings to the outlet of the jar to entrap the headspace. All glassware were heated to 375°C during 8 h before use. Collections were done for 3 h and the trap was afterwards diluted with 0.3 ml of redistilled hexane. Then the filter was cleaned with 6 ml of hexane, 6 ml of ethanol and 6 additional ml of hexane before the next sampling.

Volatile collection were stored at -18°C until use.

2.5. Wind tunnel

The wind tunnel at Bioforsk (Ås, Norway) has a polycarbonate flight section of 67 x 88 x 200 cm. Air was blown into the tunnel by a fan (model D640/E35; Fischbach GmbH, Neunkirchen, Germany) through a dust filter (Camfill Farr, Trosa, Sweden) followed by active charcoal filters (Camfill Farr). The air exiting the tunnel was delivered through a similar filter system and released back into the room. Wind speed was calibrated to 30 cm/s.

Between the filter compartment and the flight arena, there was a 30 cm long in-between section with a perforated metal grid on each side to even the air flow. In experiments with choice (rowan headspace vs apple background), an apple branch with fruits was placed within this section to provide the background odour (see arrow, Figure 8).

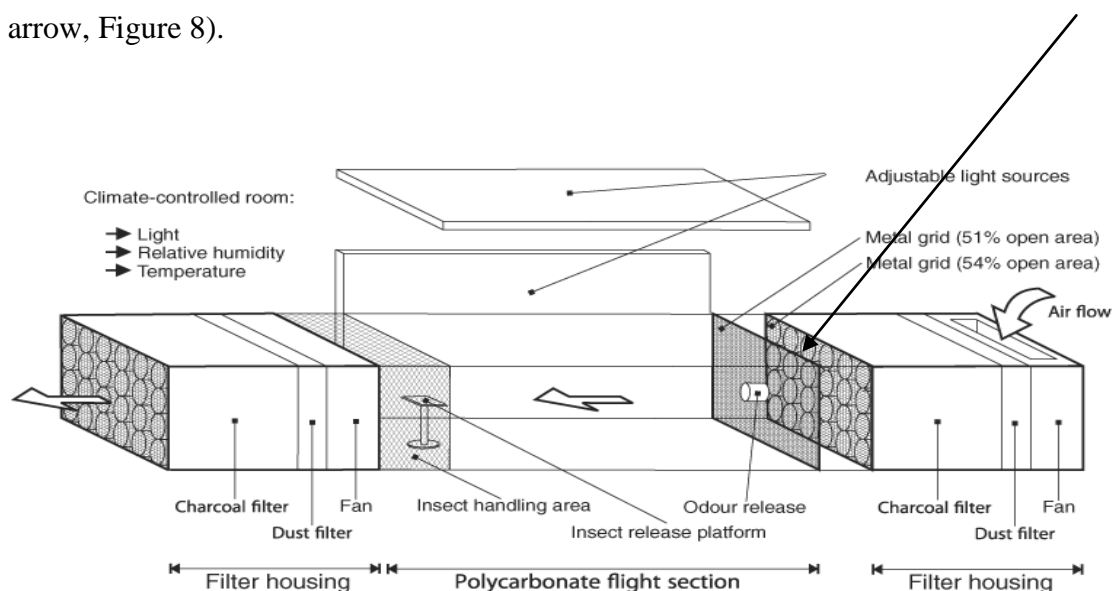


Figure 8. Schematic drawing of the wind tunnel used in this study (from Aak et al.)

Odours were released 30 cm from the ground, in the centre of the upwind end of wind tunnel (Figure 8). The plant material (or the sprayer) was covered by a glass cylinder, which was mounted on the perforated metal grid of the tunnel (see odour release, Figure 8). The opening of this cylinder facing downwind was covered with a metal mesh, which served as well as a landing platform. The temperature inside the wind tunnel during the experiments ranged from 20-22°C and the relative humidity between 55% and 60%. The light intensity was 6 lux.

2.6. Wind tunnel protocol

Wind tunnel tests were done every day during June and July. At 8 am 2 females were transferred into a glass tube. They rested for 60 minutes before the beginning of the wind tunnel session. Batches of 5 tubes (ie. 10 females) per treatment were prepared every day. At 9 am the light of the wind tunnel room was switched off in order to simulate the twilight. The olfactory stimulus (plant material or a sprayed volatile collection) was at this time set at the upwind end of the tunnel.

Test insects were exposed to the odour by positioning a glass tube with two females above a holder at the downwind end of the tunnel.

Females had 5 minutes to respond and then they were discarded. The following behavioural steps were recorded: no response (-); take off (exit from the tube with no oriented flight), oriented flight, oriented flight until half of tunnel, oriented flight and advance until two third of the tunnel and landing on the cylinder at the end of tunnel.

In no-choice experiments, a single odour source (as plant material or as sprayed headspace) was provided at the upwind end of the tunnel (see odour release point in Figure 8). Concerning the choice experiments with the apple background, I tested 3 different treatments: sprayed rowan headspace alone, an apple branch alone and the rowan headspace sprayed in front of the apple branch. In this case, the apple branch was placed behind the perforated screen as a background source of volatiles (see the arrow in Figure 8). Responding insects were initially orienting towards the odour released by the stimuli and then, when coming at the upwind side of the tunnel, chose to land either at the sprayed collection or on to the grid in front of the apple background.

2.7. Piezoelectric sprayer

Plant volatile collections were delivered in the wind tunnel through an ultrasonic sprayer (droplet size 18 μM , SonoTek 2002) (Figure 9). A syringe pump (CMA 102, CMA/Microdialysis AB, Solna, Sweden) (Figure 9) fed the sprayer with the test stimulus at 10 $\mu\text{L min}^{-1}$ and a broadband ultrasonic generator (NZL 120, SonoTek, New York, USA) (Figure 9) vibrated the nozzle at 120 KHz. Collections of volatiles were diluted with ethanol (99%) in order to reach a volume of 1800 μl to be delivered in the wind tunnel through the sprayer. This amount corresponded to a 3 hours collection time. Following a treatment, the sprayer was cleaned with 10 ml of pure ethanol.

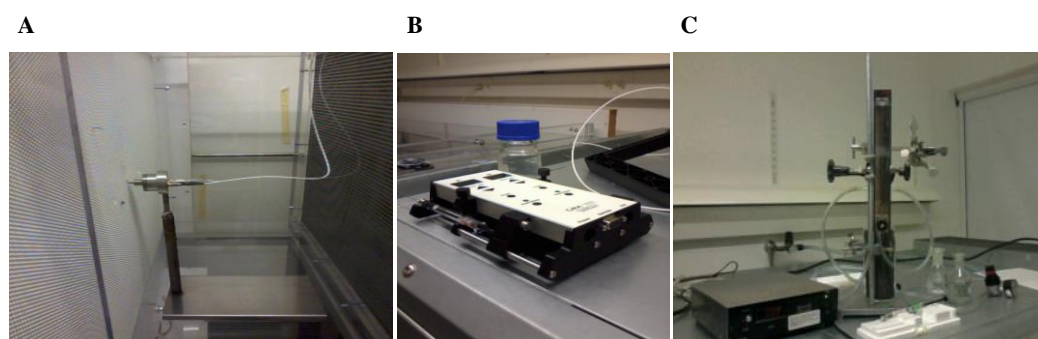


Figure 9. The in-between section of the wind tunnel with the piezoelectric sprayer. This chamber hosted also the apple branch as background stimulus during the two-choice assay. (A). The micro-dialysis pump feeds the volatiles to the sprayer (B). The broadband ultrasonic generator vibrates the nozzle (C).

2.8. Statistical analysis

Data concerning my wind tunnel experiments were daily registered on a binder and then transferred to excel sheets. Statistical analysis was done using the R Studio software (3.0.2, 2013). Data were submitted to a generalized linear model linked to a binomial distribution. I chose to use the binomial distribution because the insects tested in the wind tunnel had two possibilities: to fly or not to fly. The binomial function was also used in the case of the two-choice assay with the apple background. Two parameters (oriented flight in the first part of the tunnel and approaching the source, i.e. flying until the end of the tunnel and landing on the cylinder) were analysed. I chose these parameters because I observed that those insects that flew over the half of the tunnel could often continue until the end. On the other hand, those insects that were able to orient in the first part of the tunnel, often did not continue their oriented flight.

3. RESULTS AND DISCUSSION

3.1. No-choice test with plant material

I started my statistical analysis considering if the variable “day” had an effect on the response.

In the case of the experiments done with the samples of rowan tree, I obtained significant value for the day.

The samples was collected every day at the same time and not during the rainy days, because I knew that rain might affect the odour of the sample and consequently the response of the insect. Despite these precautions, in my opinion all experiments done with the plants are very different between them, because all samples were collected from different trees although grown in the same place; furthermore I think that each plant part is also different from the others. For example, if during one day I tested tree different branches with rowan leaves coming from three different trees, there could be some difference in the volatile release. This might give different intensity to the odours released by the rowan leaves and so you will have different responses by insects. In addition, a daily variation in insect behaviour may also be predicted.

After the statistical analysis using the binomial function, I examined the values without the voice day and this passage showed that values of “Rowan green berries” are different to the values of “Apple branch”, while the other values are similar (Figure 10).

PLANT MATERIAL

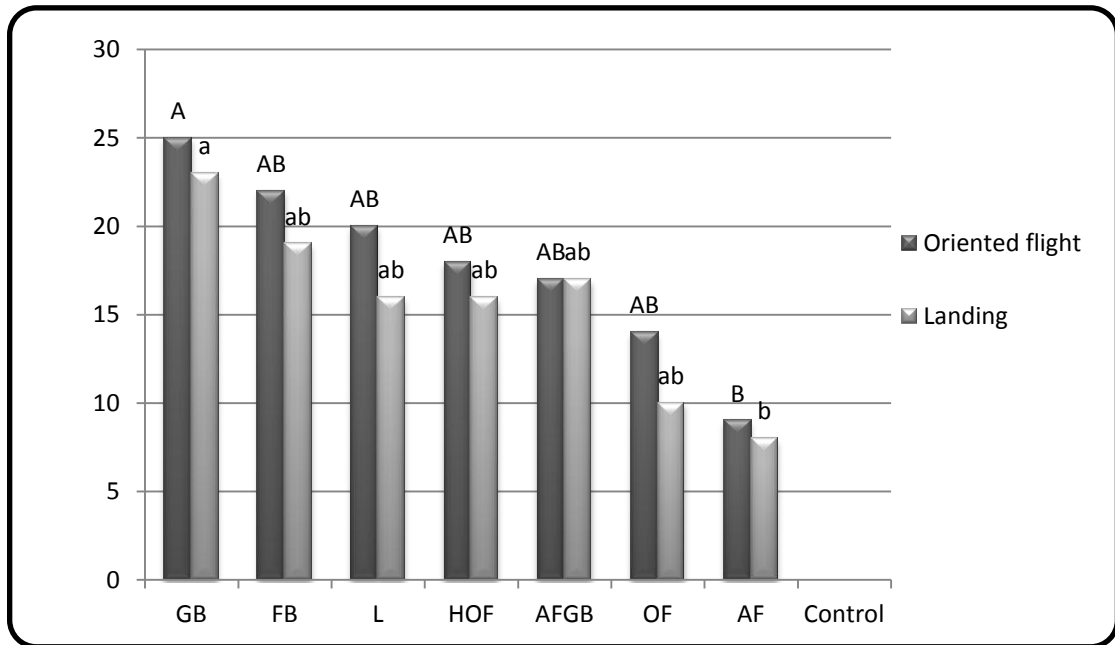


Figure 10. Wind tunnel attraction of female apple fruit moth (as % of oriented flight and landing insects) to rowan and apple (GB (60) = “Rowan green berries”, FB (27) = “Flower bud”, L (48) = “Rowan leaves”, HOF (44) = “50% flowering cluster”, AFGB (12) = “Apple cluster + Rowan green berries”, OF (29) = “Rowan full flowering” and AF (122) = “Apple branch”). Rowan green berries and apple branch significantly differ from the others (df=7, P=0.003 for oriented flight; df=7, P=0.008 for landing).

3.2. No-choice test with sprayed volatile collection

No significant effect of the factor day was found. Accordingly, I discarded this variable from the analysis. All the sprayed collections differed from each other in both parameters. The treatments green berries, open flowers and rowan leaves were different from the control (ethanol). The most attractive volatile collection was that from flowering rowan, and not the one from the green berries as I expected (Figure 11). In addition, the response to green berries was very close to that of leaves (Figure 11).

SPRAYED VOLATILE COLLECTION OF ROWAN

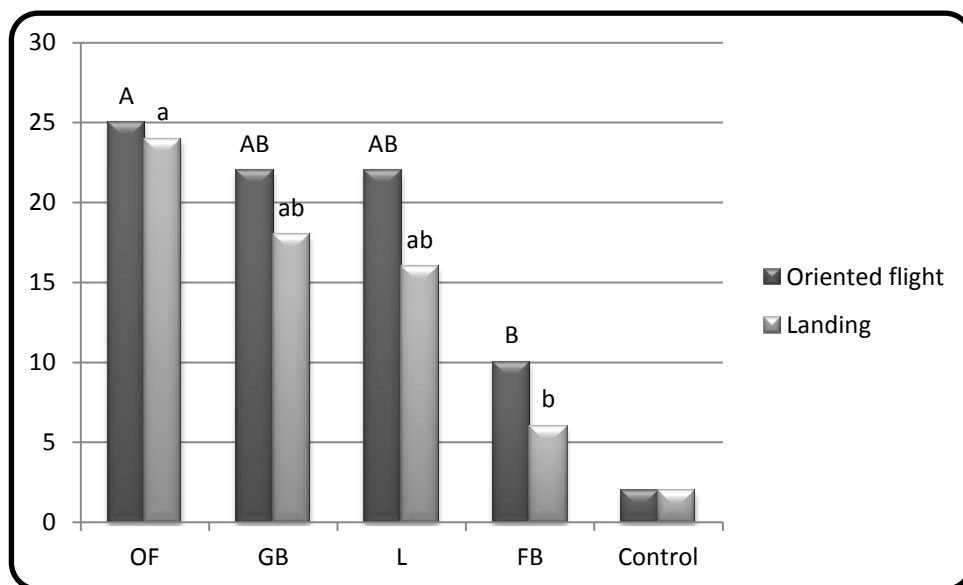


Figure 11. Wind tunnel attraction of female apple fruit moth (as % of oriented flight and landing insects) to plant headspace delivered through a piezoelectric sprayer (OF (50) = “Open flowers”, GB (50) = “Green berries”, L (50) = “Leaves” and FB (50) = “Flower bud”). Open flowers and flower bud significantly differ from the others (df=4, P=0.003 for oriented flight; df=4, P=0.0328 for landing).

3.3. Choice test

The two most attractive volatile collections (rowan green berries and rowan open flowers) were tested in a choice assay against an apple branch with fruits and leaves. The insects were allowed to choose between the odour of the apple branch and that of the rowan collection with the apple branch in the background. This test represents the true aim of my research project because it allowed me to measure the behavioural choice of the insect through a bioassay, which mimics an orchard.

Concerning the treatment relative to open flowers, 11 females chose to land at the rowan stimulus and only one at the apple background (see the response of females in Figure 12). In view of the no-choice experiments, I thought that the apple branch could be very attractive and I was unsure whether the sprayed rowan (open flowers) could or not be the most attractive source. The totality of the responding insects (7) chose to fly to the green berries headspace and no one flew to the apple branch (Figure 12).

Apple background

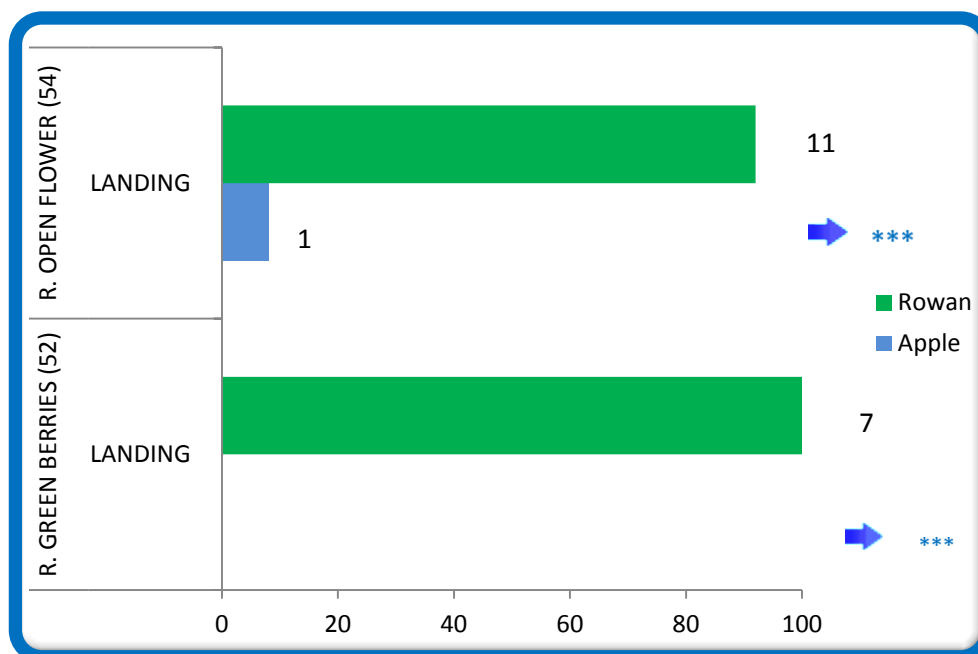


Figure 12. Response of female apple fruit moth (as % of landing insects) towards an apple branch or a rowan headspace on the top of the same branch in the wind tunnel choice assay. The numbers beside the bar represents the amount of females flying to that treatment.

4. CONCLUSION

In the wind tunnel, AFM females were significantly more attracted to a rowan cluster than to an apple cluster or to a volatile collection from a rowan cluster. Generally, during my experiments I measured a higher attraction to authentic plant material (leaves, flowers, berries) than to the related sprayed headspace.

This difference might be related to an alteration in the concentration of volatiles between the two stimuli or to a solvent (ethanol) disturbance during landing.

Rowan green berries were the most attractive part of the rowan tree to gravid females, since this represents the natural oviposition substrate. However, an attraction to leaves and flowers was also measured, although they seem to play a minor role in rowan location. In a study conducted in Norway, a single rowan tree situated inside an apple orchard was colonized by *A. conjugella* females arriving from an adjacent forest suggesting that flying females were attracted to the rowan tree by an odour signal over a distance. According to this study, females are capable of discriminating rowan within an apple orchard as long as rowanberries are available. In my wind tunnel test with choice, I measured the effect of an apple background on the attraction of a rowan volatile collection. Although both the apple background and the rowan headspace were attractive in single choice assays, females significantly preferred the volatiles from rowan to those from the apple branch in a dual choice experiment.

This shows that females apple fruit moth can discriminate across volatile signals from different host plants, with rowan being the most attractive plant over apple.

During intermasting years, when few rowan berries are available for egg laying, females are forced to find a substitute host to oviposit. Females fly therefore to apple because its secondary metabolites are similar to those of rowan, being both apple and rowan rosaceous plants. Other fruits, such as pear and plum, are not infested.

The capacity of the rowan headspace to catch AFM females in an apple orchard could be tested with the aim to capture egg-laying females and thus to reduce the larval damage. We need however to consider that the rowan odour will have to compete with a much higher amount of apple volatiles than in the wind tunnel. An increase of the concentration of the odour (or of a related synthetic mimic) could provide a higher competition towards the apple background.

A limitation of my project is that I have carried my work exclusively in the laboratory. Discrepancies in insect behaviour between the laboratory and the field

may result from visual and olfactory stimulus interaction (*Schoonhoven et al., 2005*). Moreover, a contrasting behavioural effect of single plant volatile in the laboratory and field re-emphasizes that it is crucial to study plant-insect communication in ecologically realistic settings (*Knudsen et al. 2008*). In addition, the use of point sources in the wind tunnel and trapping in the field may produce inconsistent results. Since females are attracted to rowan branches with fruit clusters both in the laboratory and in natural habitats, it will be necessary to repeat my wind tunnel test also in a natural setting, i.e. an apple orchard.

According to the results obtained in this project, I think that the use of traps loaded with a rowan odour (either as such or as a synthetic mimic) could lead to development of a monitoring and perhaps mass-trapping system for apple fruit moth in Scandinavia. In the future, if this technique will have success, it could be applied to additional pests and apple districts, contributing to an important reduction in the use of insecticides.

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