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Feeding damage of *Pandemis heparana* induces the release of specific volatile compounds from apple plants

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Introduction

Pandemis heparana Denis and Schiffermüller (Lepidoptera: Tortricidae) is among the major tortricid pests in European apple orchards and is considered a key pest on apple (Dickler, 1991). The species, also called apple brown tortrix, is widely distributed in Europe, as well as in many Asian countries and in North America. *P. heparana* is a polyphagous pest but the main hosts are apple and pear. The larvae feed primarily on the foliage by chewing the leaf surface and causing leaf curling, rolling or distortion. Larvae hide inside their shelters, formed by webbing the leaves or flower-clusters. Damage can be made also on the surface of fruitlets and fruits. In case of damage on younger fruitlets, the blemishes will be healed, resulting in rough corky spots, while unhealed dry abrasions will be present until harvest if the damage is on fruits. Without control, damage to apple fruits in an orchard with 55-100% *P. heparana* population, among other leafrollers could reach 10% or more (Płuciennik and Olszak, 2005). The damage made to the fruits can be of great concern, especially for apple-growing regions highly specialized in fresh apple production, such as Trentino-South Tyrol in Italy and Hawkes Bay in New Zealand. In Italy, *P. heparana* has two generations per year. The species overwinters mainly as second-instar larvae and begin to feed early in the spring, as soon as tree buds grow and blooming starts. Older larvae will also attack fruitlets and the second generation may make a webbing between fruits and/or adjacent leaves.

It is known that plants are able to release volatile compounds called herbivore-induced plant volatiles (HIPVs) in response to herbivore attack (for review, see Arimura *et al*., 2005). These volatile compounds can be perceived by natural enemies of the insect herbivores (e.g. predators and parasitoids), recruiting them as an indirect plant defence. Moreover, HIPVs can be perceived by different parts of the damaged plant and by neighbouring plants, contributing to the activation of direct plant defence (e.g. jasmonate signal pathway) (Dicke and Van Loon, 2000).

HIPV blends are complex mixtures that can contain several compounds, many of which may be present only in low amounts. They can vary greatly among plant species and may be influenced by abiotic factors and the species of the attacking herbivore.

Information about HIPVs released by apple plants is quite scarce. However, information available indicate that volatile compounds of apple plants under attack of the leaf miner *Phyllonorycter blancardella* Fabricius (Lepidoptera: Gracillariidae) are able to attract specific parasitoids (Dorn *et al.,* 1999; Lengwiler *et al.,* 1994). Therefore we aimed to characterise the specific HIPVs released by apple plants under attack of the pest insect *P. heparana,* comparing volatile compounds realised by undamaged plants, plant damaged mechanically and plant damaged by an artificial infestation of *P. heparana* larvae.

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Material and Methods

One-year-old apple seedlings originating from Golden Delicious cultivar seeds were used. They were grown in the following conditions: 26 ± 1°C, 50 ± 10% r.h., ca. 16L:8D photoperiod, for 1 month prior to the experiments as well as during the entire period of the experiments. The larvae of *P. heparana* were reared in a climate chamber (28 ± 0.4°C, 50 ± 10% r.h., 16L:8D photoperiod) and fed with a diet made of 70% water, 23.5% swollen beans, 3.5% brewing yeast, 1.4% agar, 0.4% ascorbic acid, 0.22% methyl 4-hydroxybenzoate, 0.11% sorbic acid and 0.87% formaldehyde (40%) until they reached the fourth larval stage.

In order to be able to discriminate between constitutive and herbivore-induced volatile compounds, the volatile profiles of apple seedlings were examined under three different conditions as follows: (1) undamaged; (2) mechanically-damaged leaves with a hole puncher on the first experimental day; (3) subjected to feeding by *P. heparana* larvae (twelve larvae per plant) during the entire experiment. Mechanical damage was inflicted on 12 leaves from each plant, starting from the first apical leaf with a surface area greater than 6 cm^2. Each leaf that had to be damaged was incised with a hole puncher every 3 cm^2 of the leaf surface (on average 4 holes per leaf were made).

The volatile organic compounds (VOCs) were collected 3 hours, 9 hours, 1 day and 2 days after the beginning of the experiment in three replicates. Each VOC collection lasted three hours, from 10 a.m. to 1 p.m. and/or from 4 to 7 p.m.

Volatile samples were collected using the closed-loop-stripping-analysis (CLSA) method (Kunert *et al*., 2009). Each selected plant was enclosed in a non-VOC-releasing polyethylene-terephthalate (PET) bag (Cuki® Cofresco S.p.A., Volpiano, Italy). A miniature vacuum graphite pump (Fürgut, Tannheim, Germany) fitted with a charcoal filter (CLSA filter, Daumazan sur Arize, France) was fixed at the top to circulate air within the PET bag such that volatiles were trapped on the CLSA filter. The negative control consisted of an empty PET bag. VOCs were eluted from the CLSA filters using a methanol-dichloromethane (Sigma-Aldrich, Milan, Italy) solution (1:2 v/v).

The volatile samples were analysed in a GC-MS system consisting of a gas chromatograph (7890A, Agilent Technologies, Santa Clara, USA) coupled with a mass spectrometer (5975C, Agilent Technologies) connected to a computer (Hewlett-Packard Company, CA, USA). The GC had a non-polar capillary column (HP-5MS, 30 m x 0.25 mm x 0.25 µm, Agilent Technologies). Sample injection (2 µl) was performed in splitless mode at an inlet valve temperature of 280 °C. The carrier gas was helium flowing at 1.2 ml/min. The temperature programme was the following: 50°C for 1.5 minutes, 7.50°C/min up to 250°C and 250°C for 10 minutes. ChemStation software (Agilent Technologies) was used for data acquisition and analysis.

The VOCs were tentatively identified by mass spectral comparison with the databases NIST11 (Gaithersburg, MD, USA) and Wiley7 (John Wiley, NY, USA). The identity of compounds was confirmed by comparison of retention indices with those of authentic standard compounds or with those present in literature listed on NIST web-book. Linear retention indices were calculated from the retention times of *n*-alkane standards from C_9 to C_{20}. The following analytical standards obtained from Sigma-Aldrich-Fluka were used to confirm the identification of the compounds (purity is indicated between parentheses): (*Z*)-3-hexenyl acetate (≥98%), (*Z*)-3-hexenyl benzoate (>97%), (*Z*)-3-hexenyl butyrate (>98%), 2-ethylhexanol (>99%), benzyl cyanide (98%), *β*-caryophyllene (>80%), indole (>99%), linalool (≥95%), methyl salicylate (≥99%), nonanal (95%) and *n*-alkanes from C_9 to C_{20} (>99%).

The peak area of each compound was analysed for each treatment and collection time. Arithmetic mean and standard error were calculated for each group of replicates. The peak areas were subjected to Kruskal-Wallis test (IBM SPSS, version 20; Armonk, NY, USA) and followed by Mann-Whitney U test (IBM SPSS) where significant differences were observed.

Results and Discussion

The VOC profiles of *P. heparana*-damaged plants were markedly different from those of mechanically-damaged ones. *P. heparana*-damaged plants showed the first qualitative changes in their volatile profile 9 hours after placing the larvae on the leaves, while a dramatic change occurred after 24 hours, when many induced compounds were detected (Figure 1 & 2). Unlike the VOC profiles of *P. heparana* damaged plants, the VOC profiles of mechanically-damaged plants showed a high increase in a single compound immediately after the damage, modest amounts of two new induced compounds 9 hours later and then gradually returned to its typical undamaged baseline level within 2 days (Figure 1).

Twelve VOCs were characterised in the headspace of undamaged apple plants. They included the green leaf volatile (GLV)-ester (*Z*)-3-hexenyl acetate, the branched alcohol 2-ethylhexanol, the aldehydes nonanal and decanal, five linear hydrocarbons, ranging from C_{14} to C_{18}, and three branched hydrocarbons, ranging from C_{18} to C_{20}. The dominant compounds were hexadecane and (*Z*)-3-hexenyl acetate (quantitative data not shown).

In the headspace of mechanically-damaged apple plants, 18 VOCs were identified. Twelve of them belonged to the typical undamaged-plant profile. Among them, (*Z*)-3-hexenyl acetate was the dominant compound during the VOC collection at 3 hours after the experiment started; its abundance increased strongly with respect to undamaged conditions, while the other constitutive compounds were detected in lower amounts. Three new compounds, namely the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene and the GLV-esters (*Z*)-3-hexenyl butyrate and (*Z*)-3-hexenyl benzoate were detected 3 hours after the damage while other two volatiles, *α*-farnesene and indole appeared only among the VOCs detected after 9 hours of damage. The sesquiterpene *α*-farnesene is a well-known damage-related volatile in many plant species, including poplar and maize. To the best of our knowledge, indole has never been detected in the headspace of mechanically-damaged leaves of apple plants.

Twenty-six compounds were identified in the volatile blend of *P. heparana*-damaged apple plants. Twelve belonged to the typical undamaged-plant profile. Five had earlier been found in the mechanical-damage profile, so they were classified as "damage-related" (Figure 1). The remaining eight compounds were detected for the first time in this study, so they were considered as "*P. heparana*-induced". They are the terpenes (*E*)-*β*-ocimene, linalool, *β*-caryophyllene, germacrene D, calamenene and cadalene, and the benzenoids, benzyl cyanide and methyl salicylate (Figure 2).

Terpenes are known to play an important role in plant direct defence, since they are toxic or deterrent to a wide range of organisms (Gershenzon and Dudareva, 2007). For instance, germacrene D was reported to have insecticidal activity against mosquitoes (Kiran and Devi, 2007), as well as repelling aphids (Bruce *et al*., 2005) and ticks (Birkett *et al*., 2008), while linalool is very toxic for many beetle species (e.g. Rozman *et al*., 2007).

Among the benzenoids, methyl salicylate is a proven attractant for many beneficial insects including parasitic wasps (James and Price, 2004), while benzyl cyanide is toxic to most larvae belonging to the family Lepidoptera (Hopkins *et al*., 2009). Indeed, toxic effects were observed in our experiment since many *P. heparana* larvae stopped their feeding activity in the second experimental day and some of them died in the following days.

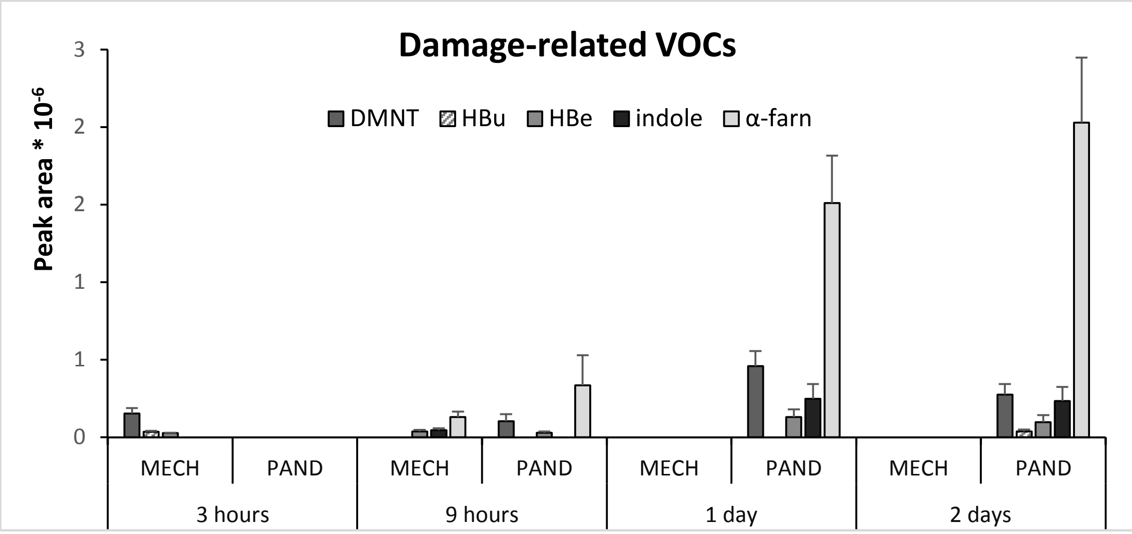

Figure 1. Damage-related VOCs identified in the headspace of mechanically-damaged (MECH) and *P. heparana*-damaged (PAND) apple seedlings (*Malus domestica*, one-year-old) in four collection times, namely 3 hours, 9 hours, 1 day and 2 days after the beginning of the experiment. DMNT, (*E*)-4,8-dimethyl-1,3,7-nonatriene; HBu, (*Z*)-3-hexenyl butyrate; HBe, (*Z*)-3-hexenyl benzoate; *α*-farn, *α*-farnesene. Each bar is the mean of three replicates ± SE.

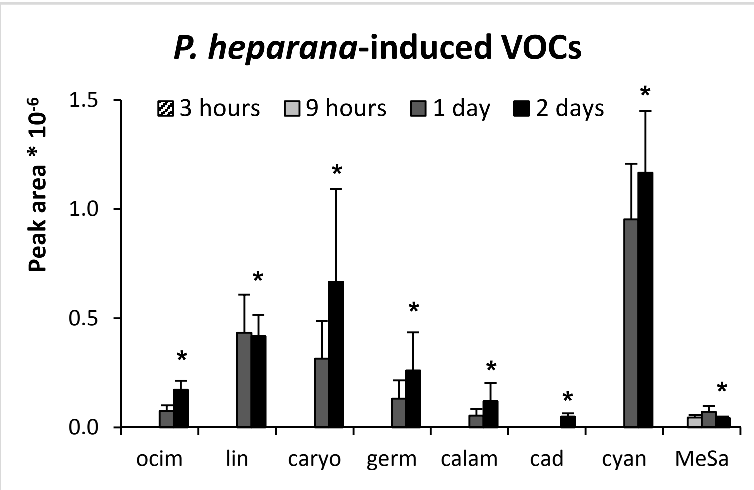

Figure 2. Herbivore-induced VOCs in the headspace of *P. heparana*-damaged apple seedlings (*Malus domestica*, one-year-old) in four collection times, namely 3 hours, 9 hours, 1 day and 2 days after the beginning of the experiment. Ocim, (*E*)-*β*-ocimene; lin, linalool; caryo, *β*-caryophyllene; germ, germacrene D; calam, calamenene; cad, cadalene; cyan, benzyl cyanide; MeSa, methyl salicylate. Each bar is the mean of 3 replicates ± SE. Asterisks above each group of bars indicate significant differences between the first and last collection times. Statistical significance was determined at $p < 0.05$ based on Mann-Whitney U test.

Conclusion

To the best of our knowledge, our experiments have shown for the first time that apple plants release specific VOCs in response to the feeding damage of *P. heparana*. In fact, the volatile profile of *P. heparana*-damaged apple plants included several compounds that were not detected in either undamaged or mechanically-damaged plants. These HIPVs could be important for the development of new eco-friendly techniques of insect pest control in apple orchards.

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