Root damage to apple plants by cockchafer larvae induces a change in volatile signals below- and above-ground

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Abstract
Volatile organic compounds (VOCs) mediate communication between plants and insects. Plants under insect herbivore attack release VOCs either at the site of attack or systemically, indicating within-plant communication. Some of these VOCs, which may be induced only upon herbivore attack, recruit parasitoids and predatory insects to feed on the attacking insects. Moreover, some plants are able to ‘eavesdrop’ on herbivore-induced plant volatiles (HIPVs) to prime themselves against impending attack; such eavesdropping exemplifies plant–plant communication. In apple orchards, the beetle Melolontha melolontha L. (Coleoptera: Scarabaeidae) is an important insect pest whose larvae live and feed on roots for about 4 years. In this study, we investigated whether the feeding activity of M. melolontha larvae (1) alters the volatile profile of apple roots, (2) induces the release of HIPVs systemically in the leaves, and (3) whether infested plants communicate to neighbouring non-infested conspecifics through HIPVs. To answer these questions, we collected constitutive VOCs from intact M9 roots as well as M. melolontha larvae-damaged roots using a newly designed ‘rhizobox’, to collect root-released volatiles in situ, without damaging the plant root system. We also collected VOCs from the leaf-bearing shoots of M9 whose roots were under attack by M. melolontha larvae and from shoots of neighbouring non-infested conspecifics. Gas chromatography-mass spectrometry analysis showed that feeding activity of M. melolontha larvae (1) alters the volatile profile of apple roots, (2) induces the release of HIPVs systemically in the leaves, and (3) whether infested plants communicate to neighbouring non-infested conspecifics through HIPVs. To answer these questions, we collected constitutive VOCs from intact M9 roots as well as M. melolontha larvae-damaged roots using a newly designed ‘rhizobox’, to collect root-released volatiles in situ, without damaging the plant root system. We also collected VOCs from the leaf-bearing shoots of M9 whose roots were under attack by M. melolontha larvae and from shoots of neighbouring non-infested conspecifics. Gas chromatography-mass spectrometry analysis showed that feeding activity of M. melolontha larvae induces the release of specific HIPVs; for instance, camphor was found in the roots only after larvae caused root damage. Melolontha melolontha also induced the systemic release of methyl salicylate and (E,E)-α-farnesene from the leaf-bearing shoots. Methyl salicylate and (E,E)-α-farnesene were also released by the shoots of non-infested neighbouring conspecifics. These phenomena indicate the induction of specific VOCs below- and above-ground upon M. melolontha larvae feeding on apple roots as well as plant–plant communication in apple plants.

Introduction
Plants under insect herbivore attack are able to defend themselves by releasing a range of chemicals that directly protect them, including alkaloids, terpenes, and phenolic compounds (Baldwin et al., 2006). They may also release chemicals that could defend them indirectly by attracting ‘enemies of their enemies’ (i.e., predators and/or parasitoids), a phenomenon that is described as ‘crying for help’ (Whitfield, 2001; Gershenzon, 2007; Bruinsma & Dicke, 2008; Dicke, 2009; Dicke & Baldwin, 2010; Kaplan, 2012). It has also been established that plants under attack from herbivorous insects are able to ‘warn’ their neighbours of impending danger; additionally, neighbours of plants under attack ‘eavesdrop’ on chemicals released by these plants and by so doing, prepare to defend themselves before they are attacked (Baldwin & Schultz, 1983; Karban et al., 2000; Baldwin et al., 2006; Heil & Silva Bueno, 2007). This occurrence is now popularly known as the ‘talking trees’ phenomenon.

It is clear from these phenomena that plants are able to communicate and they do so through the ‘language’ of volatile organic compounds (VOCs). Furthermore,
predatory arthropods are known to follow odour plumes of herbivore-induced plant volatiles (HIPVs) to navigate their way towards target prey which may be feeding or ovipositing on host plants (Fatouros et al., 2012). For example, recent olfactometer and wind tunnel studies showed that the parasitic wasps Trichogramma brassicae Westwood and Cotesia glomerata L. were attracted to volatile compounds of a wild crucifer (Brassica nigra L.) induced through contact of their leaves with eggs of Pieris brassicae L. (Fatouros et al., 2012). In addition, neighbours of plants under herbivore attack are primed for impending attack through sensing HIPVs (Karban et al., 2000; Engelberth et al., 2004; Baldwin et al., 2006; Kessler et al., 2006). For example, (Z)-3-hexen-1-yl acetate was produced in undamaged maize plants when exposed to wound-induced green leaf volatiles (GLVs), indicating plant–plant communication (Yan & Wang, 2006). This further triggered the release of the terpenoid (E)-4,8-dimethyl-1,3,7-nonatriene, a typical defence compound likely to prime them against attack (Yan & Wang, 2006). Moreover, herbivory on Lima bean plants (Phaseolus lunatus L.) decreased when they were exposed to insect-induced volatiles released by conspecifics, another example of priming among plants (Heil & Silva Bueno, 2007).

Besides plant–plant communication in which receiving plants are primed, within-plant communication is also possible. It has been found that herbivory by the tobacco hornworm, Manduca sexta L., on lower leaves of tomato plants (Solanum lycopersicum L.) elicited the release of the monoterpenes α-pinene, β-pinene, 2-carene, and β-phellandrene, and the sesquiterpenes β-caryophyllene, α-humulene, and δ-elemene, both at the site of damage and systemically from the upper undamaged leaves (Farag & Paré, 2002). Thus, HIPVs may be released either at the site of herbivore attack or systemically at other parts of the plant not under attack (Heil & Ton, 2008) probably for priming. Indeed, priming of plants against impending herbivore attack helps them avoid possible heavy damage by herbivores and increases resistance to the attack (Engelberth et al., 2004; Baldwin et al., 2006; Turlings & Ton, 2006; Rodriguez-Saona & Frost, 2010; Pastor et al., 2013). Moreover, when undamaged leaves of P. lunatus were exposed to volatiles released from damaged leaves of the same plant, extra-floral nectar (EFN) secretions by the undamaged leaves increased (Heil & Silva Bueno, 2007). On the other hand, when damaged leaves were covered so that only internal signalling pathways were possible, the release of EFN on undamaged leaves was significantly lower, indicating that the VOCs were necessary to induce a full systemic response within the plant (Heil & Silva Bueno, 2007). Thus, apparently the same mechanism that primes neighbouring plants is also responsible for intra-plant communication.

In apple [Malus × domestica Borkh. (Rosaceae)] orchards, Melolontha melolontha L. (Coleoptera: Scarabaeidae) is one of the major insect pests (Zelger, 1996; Strasser, 2004). This pest spends about 98% of its 3- to 4-year life cycle in the soil as egg, larva, and pupa (Fidler, 1936; Huiting et al., 2006; Ester et al., 2007). Melolontha melolontha have three larval instars during their development, feeding voraciously on the roots of many plant species including annual and perennial plants (Huiting et al., 2006). Although much is known about the ecology of M. melolontha adults above-ground (e.g., Reinecke et al., 2002a,b, 2005, 2006), very little information on the chemical ecology of the larvae is documented (e.g., Eilers et al., 2012; Weissteiner et al., 2012). In below-ground interactions, there is evidence that VOCs play a role in the search for food resources (Wenke et al., 2010). Similarly, host plants also utilise VOCs released below-ground to protect themselves from herbivory (Rasmann et al., 2012). For instance, it was documented that feeding damage by larvae of the maize pest, Diabrotica virgifera virgifera LeConte, induces the release of (E)-ß-caryophyllene, a sesquiterpene that is attractive to entomopathogenic nematodes (Rasmann et al., 2005). A similar study was conducted on citrus where it was found that feeding damage by the root weevil, Diaprepes abbreviatus L., induces the release of terpenes which are attractive to the entomopathogenic nematode Steinernema diaprepes sp. nov. (Ali et al., 2010). These two examples indicate that HIPVs released below-ground have similar ecological functions as those released above-ground in response to insect herbivore attack. Unfortunately, the revelation that D. abbreviatus-induced terpenes from the roots of citrus are attractive to entomopathogenic nematodes is hampered by a new revelation that the same terpenes also attract phytopathogenic nematodes (Ali et al., 2011). This means those terpenes cannot be used in controlling the root weevils.

As the larvae of our study insect, M. melolontha, live in soil and feed for a long time, we investigated whether the feeding activity of M. melolontha larvae (1) alters the volatile profile of the roots of apple plants; (2) induces a systemic release of HIPVs above-ground in response to root damage; and (3) induces chemical communication between infested plants and their neighbouring non-infested conspecifics.

**Materials and methods**

**Novel rhizobox for root volatile collection**

To investigate the apple root volatiles without the need to remove the root from the soil and the risk of causing...
mechanical damage, a novel ‘rhizobox’ was designed to allow direct collection of VOCs released by the root into a second empty compartment through a porous membrane (Figure 1). The rhizobox was constructed with a white foamed PVC Forex sheet (Tecnomag, Bolzano, Italy). It consisted of two compartments (A: 520 ml, B: 430 ml) separated by a diagonal window with a nylon monofilament gauze (30 μm mesh; Georg Becker Laboreinrichtungen, Vienna, Austria). We placed our plants, soil, and, if applicable, insect larvae in compartment A. The bottom of this compartment had four drainage holes, allowing extra water to drain. Plant roots developed and branched in A near the diagonal gauze. Compartment B was used to collect the volatile compounds released through the gauze from the root-containing soil. The upper part of this compartment had two customised holes, to insert two Teflon tubes for the circulation of air inside the compartment.

Plants

*Malus × domestica* rootstock M9 is one of the most popular rootstocks used in commercial apple production orchards. It is a dwarving rootstock with a better water-use efficiency than other rootstocks, making it popular among apple growers (Soumelidou et al., 1994; Li et al., 2002). Clones of overwintering M9 rootstocks without leaves were obtained from a plant nursery (Fairplant, Luttelgeest, The Netherlands). To enable the collection of constitutive volatiles from the M9 roots, 10 rootstocks were cut to 12 nodes long and planted singly in rhizoboxes. Another 28 M9 rootstocks (also 12 nodes long) were planted in 800-ml plastic pots. The planting medium for both rootstocks in rhizoboxes and plastic pots was TerraBrill/peat moss (Agrochimica, Bolzano, Italy). The planted M9 rootstocks were placed in a growth chamber (Snijders Scientific, Tilburg, The Netherlands) with ca. 70−10% r.h., L16(26 °C):D8(20 °C) photoperiod, and ca. 10 000 lux illuminance. The rootstocks were watered 3× per week and they developed leaves after 3 weeks.

Insects

Third instars of *M. melolontha* with head capsule width measuring 5.5−6.5 mm (Malysh & Frolov, 2009) were collected from an infested apple orchard in Trentino, Italy. They were reared in pairs in plastic boxes filled with peat moss in the laboratory at ca. L16(26 °C):D8(20 °C) photoperiod, ca. 2 500 lux illuminance, and ca. 70 ± 10% r.h. They were fed pieces of organically grown carrots [*Daucus carota* L. (Apiaceae)] 2× per week. They were fed only once during the week prior to the experiments.

Volatile samples collection

VOCs were collected from *M. × domestica* M9 by closed-loop-stripping-analysis (CLSA) (Boland et al., 1984; Kunert et al., 2009). An absorbent trap loaded with 1.5 mg of activated charcoal (CLSA-filter; Grünicher Daniel, Dau-mazan sur Arize, France) was attached to a Teflon tube fixed to the inlet valve of a small 12-V graphite vacuum pump (Fürgut, Tannheim, Germany); a Teflon tube was also attached to the outlet valve. These were passed through the two holes at the top of the rhizobox (Figure 1), such that the pump circulated air at a rate of 1 l per min within the rhizobox and trapped VOCs from the root-containing soil onto the activated charcoal. In the case of VOC sampling from leaf-bearing shoots of M9, the shoots bearing ca. 25−30 leaves were enclosed within a Cuki® oven bag (Cofresco, Volpiano, Italy) and the graphite vacuum pump with the CLSA filter attached was fixed at the top of the enclosure. The pump was powered by 7.5 V of energy from a laboratory DC power supply (GW Instek, Shanghai, China). VOCs were collected in three
experiments described below; each collection process lasted 3 h. All trapped VOC samples were eluted from the CLSA filters with 100 µl of GC grade dichloromethane (Sigma-Aldrich, Milan, Italy). The elutes were stored in a freezer at −80 °C pending gas chromatography-mass spectrometry (GC-MS) analysis.

**Experiment 1**
To investigate whether feeding damage on roots by *M. melolontha* larvae alters the volatile profile of the roots, we collected constitutive and *M. melolontha* damage-induced VOCs from the roots of M9 rootstocks planted in rhizoboxes. Constitutive VOCs were collected from undamaged roots in 10 replicates, as described above. Immediately after the collection of constitutive VOCs from the roots, three third instars of *M. melolontha* were introduced into the peat moss and VOCs were collected after 3, 7, and 14 consecutive days (10 replicates per sampling time). As control, the volatiles released by the rhizobox filled only with peat moss and three *M. melolontha* larvae were collected using the same protocol (four replicates).

**Experiment 2**
To investigate whether systemic release of HIPVs above-ground occurs in response to *M. melolontha* larval damage on the roots, we collected both constitutive and induced VOCs from the leaf-bearing shoots of M9 rootstocks planted in pots with intact and *M. melolontha*-damaged roots, respectively (n = 10 each). After the collection of constitutive VOCs from root-undamaged M9 shoots, three third-instar *M. melolontha* were immediately introduced into the peat moss in which the M9 were planted and the VOCs collection process was repeated 3, 7, and 14 consecutive days after the introduction of the larvae with the same protocol. To detect potential contaminants released by the oven bags, the VOCs within six empty oven bags were collected.

**Experiment 3**
To investigate whether chemical communication occurs between infested M9 plants and their neighbouring non-infested conspecifics, we collected VOCs from the leaf-bearing shoots of M9 rootstocks, which were under attack by *M. melolontha* larvae and those of neighbouring conspecifics which were not under attack. For experimental purposes, we placed a non-infested M9 rootstock 20 cm away from each of their conspecifics which were under *M. melolontha* attack. VOCs were collected from the leaf-bearing shoots of both *M. melolontha*-infested plants and non-infested ones simultaneously after 3, 7, and 14 consecutive days after introduction of the larvae (n = 6 per sampling time). As control, another set of non-infested M9 rootstocks was placed in another growth chamber with the same conditions and their VOCs were collected in the same sequence as above (n = 6).

**Standard compounds**
Reference standard compounds were purchased from Sigma-Aldrich to aid identification and quantification of volatile compounds collected from the M9 rootstock: (Z)-3-hexen-1-yl acetate, ≥98%; 2-ethyl hexan-1-ol, ≥99.6%; 3-methylbutyl butanoate, ≥98%; linalool, ≥95%; nonanal, 95%; camphor, ≥95.5%; (Z)-3-hexen-1-yl butanoate, ≥98%; methyl salicylate, ≥99%; decanal, ≥98%; β-caryophyllene, ≥80%; farnesene, mixture of isomers; and 1-hexadecanol, ≥90%.

**GC-MS analysis**
The VOCs were analysed in a networked GC system (7890A) coupled with an MS (5975C Network) (Agilent Technologies, Santa Clara, CA, USA). The GC had a non-polar HP-5MS column (30 m × 0.25 mm ID, 0.25 μm film thickness; Agilent Technologies). Aliquots of 2 µl of volatile sample were injected into the GC in the splitless mode when the inlet valve was 280 °C. Helium was used as carrier gas at a flow rate of 1.2 ml per min and a velocity of 39.92 cm s⁻¹. Starting temperature was 50 °C held for 1.5 min, followed by an increase of 7.5 °C per min until a temperature of 250 °C was reached and held for 10 min. The mass range of the mass spectra was 20–400 amu. Data acquisition was carried out using ChemStation software (Agilent Technologies). All volatile compounds were initially identified by comparing their mass spectra with those in the databases of NIST 11 (Gaithersburg, MD, USA) and Wiley 7N (Wiley, Hoboken, NJ, USA). The identities of 12 of 14 compounds were confirmed by comparing their mass spectra and retention times with those of the reference standard compounds. The linear retention indices of the compounds were calculated using the retention times of n-alkane series from C9 to C20 as reference compounds (Van den Dool & Kratz, 1963) and were compared with those already published.

**Statistical analysis**
To estimate the amount of volatiles emitted by the M9 rootstock, we purchased standards of the 12 available compounds out of the total 14 identified. Five-point calibration curves were prepared for each corresponding standard compound at 0, 0.01, 1, 100, and 10 000 µg g⁻¹ in dichloromethane considering the total
ion current (TIC). We compared the amount of volatile compounds emitted among experimental treatments using the Kruskal–Wallis test (SPSS version 20; IBM SPSS, Armonk, NY, USA), followed by Mann–Whitney U-test (IBM SPSS), where significant treatment effects were observed.

Results

VOCs from roots of M9 rootstocks with undamaged and Melolontha melolontha-damaged roots

Analysis of volatile samples from intact and undamaged M9 roots revealed five constitutive volatile compounds (2-ethyl hexan-1-ol, nonanal, methyl salicylate, decanal, and 1-hexadecanol), which were not present in control rhizoboxes filled with only peat moss and M. melolontha larvae (data not shown). Upon feeding by larvae of M. melolontha, camphor (root bark oil) was released (Table 1). Methyl salicylate, that had been released constitutively, was not detected in the volatile profile of roots fed on by larvae of M. melolontha (Table 1; Figure 2B).

The amount of 2-ethyl hexan-1-ol released by undamaged roots of M9 decreased significantly after feeding by larvae of M. melolontha for 7 and 14 days ($\chi^2 = 14.82$, d.f. = 3, $P = 0.002$; Figure 2A). The amount of decanal released by undamaged roots was also significantly higher than the amount released after 7 days of feeding by M. melolontha larvae ($U = 20.0$, $P = 0.023$). Furthermore, the amount of 1-hexadecanol released by undamaged roots was significantly higher than the amount released after 3 days ($U = 23.5$, $P = 0.045$) and 14 days ($U = 17.5$, $P = 0.014$) of M. melolontha larvae feeding damage. There was no difference in the amount of non-anal released by undamaged and M. melolontha-damaged roots ($\chi^2 = 5.83$, d.f. = 3, $P = 0.12$; Figure 2B). Camphor was released only when the roots were fed on by larvae of M. melolontha and the amount released after 3 days of damage was significantly higher than that released after 7 days ($U = 21.0$, $P = 0.024$) and 14 days ($U = 19.0$, $P = 0.012$) (Figure 2B).

Table 1 Mean (± SEM) volatile organic compound emission rate (ng per 3 h) from the roots of non-infested (n = 10) and Melolontha melolontha-infested (n = 30) M9 apple rootstocks

<table>
<thead>
<tr>
<th>No.</th>
<th>Reference LRI</th>
<th>LRI</th>
<th>Compound</th>
<th>Non-infested root</th>
<th>Infested root</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1030</td>
<td>1031</td>
<td>2-Ethyl hexan-1-ol</td>
<td>763 ± 85</td>
<td>499 ± 56</td>
<td>0.002</td>
</tr>
<tr>
<td>2</td>
<td>1107</td>
<td>1105</td>
<td>Nonanal</td>
<td>63 ± 8</td>
<td>52 ± 7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>3</td>
<td>1149</td>
<td>1151</td>
<td>Camphor (root bark oil)</td>
<td>0</td>
<td>24 ± 12</td>
<td>0.01</td>
</tr>
<tr>
<td>4</td>
<td>1198</td>
<td>1197</td>
<td>Methyl salicylate</td>
<td>32 ± 14</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5</td>
<td>1208</td>
<td>1208</td>
<td>Decanal</td>
<td>102 ± 18</td>
<td>70 ± 11</td>
<td>0.04</td>
</tr>
<tr>
<td>6</td>
<td>1879</td>
<td>1879</td>
<td>1-Hexadecanol</td>
<td>43 ± 8</td>
<td>27 ± 5</td>
<td>0.028</td>
</tr>
</tbody>
</table>

LRI, linear retention index; reference LRI, retention indices already published in peer-reviewed literature and listed on NIST WebBook. P values are based on Mann–Whitney U-tests.

VOCs from leaves of M9 rootstocks with undamaged and Melolontha melolontha-damaged roots

Ten constitutive VOCs were characterised from the leaf-bearing shoots of non-infested M9 rootstocks (Table 2). When the roots of the M9 rootstocks were fed on by larvae of M. melolontha, one of the constitutive VOCs (3-methylbutyl butanoate) was no longer detected in the volatile profile; however, two HIPVs, [methyl salicylate and (E,E)-α-farnesene] were released in addition to nine of the constitutive VOCs (Table 2).

(Z)-3-hexen-1-yl acetate and 2-ethyl hexan-1-ol were released in higher amounts than virtually any other compound released by the leaf-bearing shoots of M9 rootstocks (Figure 3A). The amount of (Z)-3-hexen-1-yl acetate released constitutively was significantly higher than that released by leaf-bearing shoots of M9 whose roots had been damaged by M. melolontha larvae for 3, 7, and 14 consecutive days ($\chi^2 = 19.52$, d.f. = 3, $P < 0.001$). There was no difference in the amount of 2-ethyl hexan-1-ol released constitutively and that released after M. melolontha larvae damage ($\chi^2 = 3.63$, d.f. = 3, $P = 0.31$) (Figure 3A).

There were differences in the amounts released of linalool, (Z)-3-hexen-1-yl butanoate, and β-caryophyllene ($\chi^2 = 24.93$, 20.34, and 19.15, respectively; all d.f. = 3, $P < 0.001$); the amounts released constitutively were always higher than those released after M. melolontha damage to the roots (Figure 3B). 3-Methylbutyl butanoate was released only constitutively. There were no statistical differences in the amounts of nonanal, methyl salicylate, decanal, and (E,E)-α-farnesene (Figure 3B). The amounts of 2-methyl pentadecane and 3-methyl pentadecane released could not be quantified because the respective analytical standards are currently not available on the market.
VOCs from leaves of Melolontha melolontha-damaged M9 and neighbouring non-infested conspecifics

The leaf-bearing shoots of M9 rootstocks whose roots were infested with *M. melolontha* larvae released methyl salicylate and \((E,E)\)-\(\alpha\)-farnesene in addition to nine constitutive volatiles (Table 2). The non-infested neighbouring M9 plants located 20 cm away also released methyl salicylate and \((E,E)\)-\(\alpha\)-farnesene from their leaf-bearing shoots. Moreover, methyl salicylate and \((E,E)\)-\(\alpha\)-farnesene were not detected in the volatile profiles of a set of non-infested M9 rootstocks used as control (Table 2). All volatile compounds that were released by the empty oven bags were removed from the volatile profile of leaf-bearing M9 shoots (data not shown).

The amount of methyl salicylate released by the leaf-bearing shoots of M9 rootstocks whose roots had been infested with *M. melolontha* larvae for 7 days was significantly higher than the amount released by the leaves of their neighbouring non-infested conspecifics (\(U = 3.5, P = 0.003\)). However, the amounts of methyl salicylate released from the leaf-bearing shoots of both infested M9 and neighbouring non-infested rootstocks were not significantly different after 3 days (\(U = 19.0, P = 0.21\)) and 14 days (\(U = 28.0, P = 0.82\)) (Figure 4A).

Similarly, the amount of \((E,E)\)-\(\alpha\)-farnesene released by the leaf-bearing shoots of M9 rootstocks whose roots had been infested with *M. melolontha* larvae for 7 days was significantly higher than the amount released by the leaf-bearing shoots of their non-infested neighbours (\(U = 2.0, P = 0.002\)), whereas the amounts were not significantly different 3 days (\(U = 18.0, P = 0.19\)) and 14 days (\(U = 25.0, P = 0.59\)) after the roots of the *M. melolontha*-infested M9 were damaged (Figure 4B).

**Discussion**

The results we have obtained using our novel rhizobox have demonstrated the validity of this technique for collecting volatile samples from roots in situ without touching the roots after earlier trials (e.g., Ali et al., 2010, 2011, 2012). This new technique adds to the very few available for intact root volatile collection (Campos-Herrera et al.,
This technique enabled us to detect differences in the volatile profiles of undamaged and *M. melolontha*-damaged roots of M9. The roots released typical plant volatiles comprising five constitutive volatile compounds, consisting of two alcohols (2-ethyl hexan-1-ol and 1-hexadecanol), two aldehydes (nonanal and decanal), and one sesquiterpene (methyl salicylate). Upon feeding by *M. melolontha* larvae, camphor was released by the M9 roots; proving that feeding activity of *M. melolontha* larvae on roots alters the volatile profile of *M. domestica*. Interestingly, camphor was also detected in a recent investigation as an induced compound from the roots of *Quercus petraea* (Mattuschka) Lieblein and *Quercus robur* L. damaged by *Melolontha hippocastani* Fabricius, a closely related species of *M. melolontha* (Weissteiner et al., 2012). Although in that study camphor was detected in the volatile blends of undamaged, mechanically damaged, and *M. hippocastani*-damaged *Quercus* spp., the concentration detected in *M. hippocastani*-damaged *Quercus* spp. was 6× higher than that detected in mechanically damaged, and 20× higher than in undamaged *Quercus* spp. (Weissteiner et al., 2012). This together with our finding suggests that the release of camphor from roots may be induced generally by the feeding activity of *Melolontha* spp. Moreover, it is worth noting that the ‘undamaged’ roots of *Quercus* spp. referred to in Weissteiner et al. (2012) were actually washed with water after the soil on them have been removed. These processes may have caused some degree of mechanical damage to the roots, hence the emission of camphor.

In our study, methyl salicylate and (*E,E*)-α-farnesene were released systemically in the leaf-bearing shoots, when there was *M. melolontha* attack on the roots. This indicates within-plant chemical communication and proves that there is an induced systemic release of HIPVs above-ground in response to *M. melolontha* damage on the roots of the host plant. Methyl salicylate and (*E,E*)-α-farnesene have already been characterised as induced compounds released by plants under attack to defend themselves (Engelberth et al., 2004; Kessler et al., 2006). As HIPVs, they could either be released at the site of attack, or systemically at other parts of the plant not under attack (Heil &

### Table 2

<table>
<thead>
<tr>
<th>No.</th>
<th>LRI</th>
<th>Reference LRI</th>
<th>Compound</th>
<th>Leaves of M9 with</th>
<th>Leaves of M9 with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Non-infested root</td>
<td>Infested root</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>root at a different location</td>
</tr>
<tr>
<td>1</td>
<td>1008</td>
<td>1008</td>
<td>(Z)-3-hexen-1-yl acetate</td>
<td>9160 ± 2839</td>
<td>1289 ± 318</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5868 ± 1752</td>
</tr>
<tr>
<td>2</td>
<td>1030</td>
<td>1031</td>
<td>2-Ethyl hexan-1-ol</td>
<td>2444 ± 631</td>
<td>1451 ± 325</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>452 ± 73</td>
</tr>
<tr>
<td>3</td>
<td>1059</td>
<td>1058</td>
<td>3-Methylbutyl butanoate</td>
<td>110 ± 56</td>
<td>–</td>
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<td></td>
<td></td>
<td></td>
<td>97 ± 17</td>
</tr>
<tr>
<td>4</td>
<td>1102</td>
<td>1103</td>
<td>Linalool</td>
<td>173 ± 26</td>
<td>28 ± 9</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>83 ± 9</td>
</tr>
<tr>
<td>5</td>
<td>1107</td>
<td>1105</td>
<td>Nonanal</td>
<td>120 ± 10</td>
<td>113 ± 16</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>63 ± 7</td>
</tr>
<tr>
<td>6</td>
<td>1188</td>
<td>1187</td>
<td>(Z)-3-hexen-1-yl butanoate</td>
<td>81 ± 11</td>
<td>27 ± 5</td>
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<td></td>
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<td></td>
<td>45 ± 8</td>
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<tr>
<td>7</td>
<td>1198</td>
<td>1197</td>
<td>Methyl salicylate</td>
<td>0</td>
<td>94 ± 41</td>
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<td>0</td>
</tr>
<tr>
<td>8</td>
<td>1208</td>
<td>1208</td>
<td>Decanal</td>
<td>79 ± 6</td>
<td>80 ± 13</td>
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<td>34 ± 4</td>
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<tr>
<td>9</td>
<td>1428</td>
<td>1428</td>
<td>β-Caryophyllene</td>
<td>378 ± 70</td>
<td>106 ± 28</td>
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<td></td>
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<td>114 ± 38</td>
</tr>
<tr>
<td>10</td>
<td>1510</td>
<td>1510</td>
<td>(<em>E,E</em>)-α-farnesene</td>
<td>0</td>
<td>82 ± 35</td>
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<td>0</td>
</tr>
<tr>
<td>11</td>
<td>1563</td>
<td>1564</td>
<td>2-Methyl pentadecane</td>
<td>+</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>1570</td>
<td>1570</td>
<td>3-Methyl pentadecane</td>
<td>+</td>
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</tr>
</tbody>
</table>

+/– indicates presence/absence of VOCs that could not be quantified; LRI, linear retention index; reference LRI, retention indices already published in peer-reviewed literature and listed on NIST WebBook.

1Identity confirmed by authentic standard compound.

2Identity confirmed by published LRI.
To the best of our knowledge, our finding is the first record of systemic release of HIPVs in apple plants. Previous studies have shown that other plants are able to release chemicals systemically to protect themselves from insect herbivory. This has been demonstrated in tomato plants (S. lycopersicum) (Farag & Paré, 2002), Lima bean plants (P. lunatus) (Heil, 2004; Heil & Silva Bueno, 2007; Heil & Adame-Alvarez, 2010), and blueberry (Vaccinium corymbosum L.) (Rodriguez-Saona et al., 2009). Recently, Rodriguez-Saona & Frost (2010) further suggested that the release of HIPVs within a plant is primarily for the protection of undamaged parts of that plant against impending attack.

Earlier studies also demonstrated that plants under attack are capable of communicating to their conspecific neighbours that are not under attack through the release of HIPVs. This has been demonstrated in tomato plants (S. lycopersicum) (Farag & Paré, 2002), Lima bean plants (P. lunatus) (Heil, 2004; Heil & Silva Bueno, 2007; Heil & Adame-Alvarez, 2010; Heil & Karban, 2010). In fact, communication between plants has been shown to be more efficient between plants that are genetically identical (clones) (Karban & Shiojiri, 2009). M9 rootstocks are clones, and plant–plant communication has been demonstrated by our experiments, indicating that the volatile profile of non-infested M9 plants was altered after they were placed within 20 cm radius of M. melolontha-infested plants. In particular, the non-infested M9 released relatively small amounts of methyl salicylate and (E,E)-α-farnesene when their neighbours were under M. melolontha attack in preparation of any eventual insect herbivore attack. Through this plant–plant communication, the M9 rootstocks that were under attack were likely warning their neighbouring clones of impending attack.

The release of camphor at the site of M. melolontha attack and induction of methyl salicylate and (E,E)-α-farnesene systemically in the leaf-bearing shoots of both M9 with M. melolontha-damaged roots and neighbouring non-infested conspecifics is likely a defence strategy of the M9 rootstock, as these VOCs are among typical HIPVs (Arimura et al., 2005; Dicke & Baldwin, 2010). Camphor is known to have insecticidal and repellent properties (Kumar & Ando, 2003; Chen et al., 2013), achieving a repellence of 80–100% in the beetles Sito-philus granarius (L.), Sitophilus zeamais Motschulsky,
Tribolium castaneum (Herbst), and Prostephanus truncatus Horn (Obeng-Ofori et al., 1998). Recently, camphor has also been confirmed to have toxic effect on T. castaneum although at relatively high doses (10.0 l per adult) achieving 78.5% mortality (Liska et al., 2010). Likely, M9 rootstock release camphor at the site of attack in an attempt to defend itself against the feeding M. melolontha. However, behaviour studies with pure (1R)-camphor and (1S)-camphor on M. hippocastani did not show a clear repellent effect, although some M. hippocastani were repelled (Weissteiner et al., 2012). The induction of methyl salicylate and (E,E)-α-farnesene in the leaf-bearing shoots of the M. melolontha-infested M9 apple rootstocks and their neighbouring conspecific after the infested M9 had been fed on for 3, 7, and 14 consecutive days. Means within a day capped with different letters are significantly different (Mann–Whitney U-test: P<0.05).

Cockchafer larvae induces HIPVs in Malus domestica

Figure 4 Mean (+ SEM) emission rate (ng per 3 h) of (A) methyl salicylate and (B) (E,E)-α-farnesene from the leaf-bearing shoots of Melolontha melolontha-infested M9 apple rootstocks and their neighbouring conspecific after the infested M9 had been fed on for 3, 7, and 14 consecutive days. Means within a day capped with different letters are significantly different (Mann–Whitney U-test: P<0.05).

In conclusion, with this study we have demonstrated that feeding damage by larvae of M. melolontha on M9 rootstocks causes a release of HIPVs at the site of attack and systemically from the leaf-bearing shoots that are not under attack. Moreover, neighbouring M9 that are not under attack are able to eavesdrop on the volatiles released by the shoots of those whose roots are under attack. This shows that there is chemical communication within and between M9 rootstocks through the induction of HIPVs. Our study adds to knowledge on tritrophic interactions in apple orchards and may lead to the use of natural plant defence strategies in apple-growing regions. Our results

or parasitoids, plants are able to prepare to defend themselves even before an attack occurs; however, the downside is that pollinators may be adversely affected (Kaplan, 2012; Farré-Armengol et al., 2013). This also amounts to deceit of predators as in the case of the flowers of Epipactis helleborine (L.) Crantz and Epipactis purpurata Sm., which release damage-induced GLVs that attract the wasps Vespula germanica (Fabricius) and Vespula vulgaris L. (Brodmann et al., 2008).
could contribute to finding eco-friendly ways of controlling the Melolontha spp. damage in apple orchards and forest ecosystems.

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References


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