Caterpillars of *Euphydryas aurinia* (Lepidoptera: Nymphalidae) feeding on *Succisa pratensis* leaves induce large foliar emissions of methanol

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**Summary**

- A major new discovery made in the last decade is that plants commonly emit large amounts and varieties of volatiles after damage inflicted by herbivores, and not merely from the site of injury. However, analytical methods for measuring herbivore-induced volatiles do not usually monitor the whole range of these compounds and are complicated by the transient nature of their formation and by their chemical instability.

- Here we present the results of using a fast and highly sensitive proton transfer reaction–mass spectrometry (PTR-MS) technique that allows simultaneous on-line monitoring of leaf volatiles in the pptv (pmol mol⁻¹) range.

- The resulting on-line mass scans revealed that *Euphydryas aurinia* caterpillars feeding on *Succisa pratensis* leaves induced emissions of huge amounts of methanol – a biogeochemically active compound and a significant component of the volatile organic carbon found in the atmosphere – and other immediate, late and systemic volatile blends (including monoterpenes, sesquiterpenes and lipoxygenase-derived volatile compounds).

- In addition to influencing neighboring plants, as well as herbivores and their predators and parasitoids, these large emissions might affect atmospheric chemistry and physics if they are found to be generalized in other plant species.

**Key words:** biogenic volatile organic compounds (VOCs), *Euphydryas aurinia*, herbivory and leaf wounding, lipoxygenase-derived (LOX) volatiles, methanol, monoterpenes, sesquiterpenes, *Succisa pratensis*.

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**Introduction**

Numerous studies carried out over the last decade have shown that plants release volatiles in response to herbivore attack (Dicke *et al*., 1990; Turlings *et al*., 1990; Paré & Tumlinson, 1999; Llusià & Peñuelas, 2001), both from the exact area of feeding damage and from undamaged plant parts (Karban & Baldwin, 1997; Mumm *et al*., 2003). This induction of volatiles results in a change in plant odor that may be quantitative (i.e. the same volatiles are released by the undamaged and damaged plants, although in differing amounts) or qualitative (i.e. the damaged plant produces components that are not emitted by the undamaged plant) (Boland *et al*., 1999; Dicke, 1999; Paré & Tumlinson, 1999). Interest in these herbivore-induced volatile emissions has greatly increased in recent years given that these emissions influence neighboring plants, as well as herbivores and their predators and parasitoids (Turlings *et al*., 1990; Peñuelas & Llusià, 2001; van Poecke & Dicke, 2004). However, there is another reason why these emissions are worth considering closely: they may also considerably affect atmospheric chemistry and physics (Peñuelas & Llusià, 2003).

The analytical methods for measuring these herbivore-induced volatile organic compounds (VOCs) do not usually
monitor their whole range and are complicated by their transient nature after leaf herbivore grazing and by their chemical instability. Sampling and analysis of plant VOCs by gas chromatography can be very time-consuming and only recently has it become generally feasible to follow the dynamics of VOC emissions caused by most kinds of biotic stresses (Steeghs et al., 2004). Here we used a relatively recent methodology using proton transfer reaction–mass spectrometry (PTR-MS) that allows for the almost simultaneous on-line monitoring of these leaf volatile compounds in the pptv (pmol mol\(^{-1}\)) range. This technique has emerged as a useful tool that allows us to study accurately large numbers of different VOCs with a rapid time response (< 1 s) and with a low detection threshold (10–100 pptv) (Lindinger et al., 1998; Warneke et al., 2003). Moreover, the lack of sample treatment in PTR-MS facilitates the detection of species such as organic acids, peroxides, and doubly oxygenated species that are otherwise difficult to measure. This technique is thus a more general detection method than, for example, gas chromatography–mass spectrometry (GC-MS), in which different columns may be required to target different classes of compounds. However, the identity of the compound still needs to be confirmed by other methods such as GC-MS (Steeghs et al., 2004).

We used both PTR-MS and GC-MS to asses the dynamics and the quantitative significance of herbivore-induced emissions. We studied the emissions induced by *Euphydryas aurinia* larvae (Lepidoptera: Nymphalidae) feeding on *Succisa pratensis* (Dipsacaceae), one of the two host plants commonly used by the butterfly in north-east Spain and the only host plant used in damp meadows throughout much of its European range (Wahlberg et al., 2002). We analysed the compounds emitted by *E. aurinia* caterpillars immediately after leaf attack (first hour) to check for the presence of a rapid-induced response and after 1 d of continuous herbivorous attack to check for the presence of a more delayed induced response, as well as those emitted by leaves not attacked on attacked plants (systemic response). We also monitored the compounds emitted by the caterpillars.

**Materials and Methods**

**System studied: caterpillars, plants and experimental design**

In spring females of *E. aurinia* Rottemburg lay large batches of about 200–300 eggs on the underside of the leaves of *S. pratensis* Moench. Larvae hatch in 3 wk and spin a large silken web around the leaves, within which they feed for c. 3 wk. Immediately after their third molt, larvae enter diapause in a winter web at the base of the plant and do not resume feeding until early next spring.

The experiments were carried out with 60 larvae from a single egg-cluster and six *S. pratensis* plants from a single population. The plants were chosen randomly and were transplanted with their roots intact in Mediterranean-like environmental conditions in a glasshouse until the experiment started 3 d later. As soon as they hatched, the larvae were fed with fresh cut leaves of a wild *S. pratensis* plant and, after molting into their second instar, they were divided into three groups of 20 and each group was placed on a leaf of a different potted *S. pratensis* plant. The other three potted *S. pratensis* plants (i.e. those with no larvae) were used as controls. We measured the VOC emission rates and the net photosynthetic rates of leaves from unattacked *S. pratensis* plants, of attacked leaves (20 larvae per leaf, Fig. 1) and of unattacked leaves from plants with an attacked leaf (systemic response). All these gas exchange measurements were conducted before the attack, immediately after, and 1 d later. After these foliar gas exchange rate measurements had been conducted, the caterpillars were removed from the leaves and their own VOC emissions were also measured.

In all the measurements leaves were clamped in a 90 cm\(^3\) PLC-2 ADC cuvette connected to an IRGA porometer (LCA-4, ADC; Hoddeson, Hertfordshire, UK). The VOC-free zero air was fluxed into the cuvette and the air coming out of the cuvette flowed through a T-system to either a PTR-MS system or to an absorption glass tube and was analysed by GC-MS. Measurements of CO\(_2\) and H\(_2\)O exchange and VOC sampling
and analysis were thus conducted simultaneously. Because the flow through the cuvette was higher than the flow needed in the PTR-MS, part of the flow was channeled out of the laboratory through an overflow outlet. The cuvette was lined with Teflon and only Teflon tubing, connectors, and valves were used in order to reduce the memory effects caused by surface interactions in the system. The gas exchange measurements were also conducted with the empty cuvette as an additional control. A lamp suspended 20 cm above the cuvette provided photosynthetic photon fluence rate (PPFR) of 700 μmol m⁻² s⁻¹. Leaf temperature was kept at 25°C by air conditioning. All these leaves were afterwards harvested to measure their surface area in a Li-Cor 3100 Area Meter (Li-Cor Inc., Lincoln, NE, USA), and to calculate their dry mass (by drying at 60°C until constant weight was reached).

Analysis of VOCs by PTR-MS and GC–PTR-MS

Part of the air exiting the leaf cuvette flowed through a T-system to the PTR-MS inlet. The PTR-MS apparatus was a highly sensitive device (PTR-MS-FTD hs; Ionicon Analytik, Innsbruck, Austria) consisting of three parts: the ion source, where ions are produced by a hollow cathode discharge using water vapor as the molecular source of ions; the drift tube, where proton transfer reactions to the trace constituents in the air occur (VOCs with a higher proton affinity than that of water (166.5 kcal mol⁻¹), including most unsaturated and almost all oxygenated hydrocarbons, undergo a proton-transfer reaction with H₂O⁺); and the ion detector, which provides sensitive detection of the mass selected ions that are characteristic of the molecules of interest. Both PTR-MS and its use in VOC analysis has been described in detail elsewhere (Lindinger et al., 1998; Fall et al., 1999). Here, the PTR-MS drift tube was operated at 2.1 mbar and 40°C, with a drift field of 600 V cm⁻¹. The parent ion signal was maintained at c. 2 x 10⁶ counts s⁻¹ during the measurements. We conducted scans of all masses between 21 and 205 to determine which masses changed emissions during the different periods after different herbivore attack conditions.

For each of the measurement periods in unattacked and attacked leaves of attacked plants, as well as in the control plants and caterpillar emissions, we also sampled VOCs in carbon trap adsorption tubes and conducted GC-MS analyses in order to confirm VOC identities by an alternative method. Part of the air exiting the chamber flowed through a T-system into a glass tube (11.5 cm long and 0.4 cm internal diameter) manually filled with terpene adsorbents Carbotrap C (300 mg), Carbotrap B (200 mg), and Carboseive S-III (125 mg) (Supelco, Bellefonte, PA, USA) separated by plugs of quartz wool and treated as described by Peñuelas et al. (2005). After VOC sampling, the adsorbent tubes were stored at −30°C until analysis (within 24−48 h). The VOC analyses were conducted in a GC-MS (HP59822B; Hewlett Packard, Palo Alto, CA, USA), as described by Peñuelas et al. (2005).

Statistical analyses

We used t-tests and one-way ANOVA and post hoc tests (Statistica; StatSoft Inc., Tulsa, OK, USA) to compare the emissions from leaves before the attack with (1) emissions from the same leaves immediately after the attack, (2) emissions from the same leaves 24 h after the attack, (3) emissions from unattacked leaves on attacked plants, and (4) emissions from unattacked leaves on control plants. There were three plants per group. A t-test was also used to compare VOC emissions from the caterpillars alone with the empty chamber.

Results and Discussion

Volatile emitted de novo or in greater amounts

Time-course The PTR-MS technique and the verification with GC revealed that a blend of 13 compounds was consistently emitted de novo or in greater amounts by leaves immediately after attack by E. aurinia caterpillars: acetaldehyde, ethanol, hexanals, hexenals, hexenols, mass 88, hexadienal, mass 116, monoterpenes, hexenyl acetates, mass 163, mass 172 and the sesquiterpene α-caryophyllene (Table 1). The time-course dynamics showed that the emission of these volatiles started to increase after the caterpillar attack (see examples in Fig. 1).

Another blend of 15 compounds, was observed 1 d after the attack started: methanol, mass 58, butanone, hexenols, hexanals, hexanols, hexyl acetates, mass 102, heptanals, mass 124, mass 127, monoterpenes, mass 139, mass 175, and the sesquiterpene α-caryophyllene. Finally, a blend of four of these compounds (mass 58, butanone, mass 127, monoterpenes, and hexenyl acetates), was also found to be emitted systemically (i.e. emitted by unattacked leaves of attacked plants; Table 1). Increased emissions were not found either when measuring caterpillars alone or with no leaves in the gas exchange chamber, or when measuring the unattacked control plants (data not shown).

Emission rates When studied 24 h after the start of the caterpillar feeding (Fig. 2), attacked leaves emitted up to 20 nmol m⁻² s⁻¹ of methanol more than unattacked leaves. Emissions from unattacked leaves (3.6 nmol m⁻² s⁻¹) were within the range of methanol foliar emissions reported in the available literature in data sets for other species (Gallbally & Kirstine, 2002); emissions of attacked leaves were within the maximum upper range of fluxes described after grass-cutting for hay (Karl et al., 2001).

This large increase in methanol emissions accounted for almost 0.5% of the net photosynthetic rates in S. pratensis leaves, which were, on average, 4.8 μmol m⁻² s⁻¹ before the caterpillar attack (Table 2). This 0.5% is higher than the range found to date for the ratio of methanol emission to net photosynthetic rates for various types of higher plants (between 0.01%)
The CO₂ and H₂O exchange induced by leaves in the measuring chamber (Table 2) and the CO₂ and H₂O exchange explained by caterpillars alone (Table 1) have been taken into account in the calculations for attacked leaves. Different letters after values indicate significant differences (P < 0.05). Only those masses with emission rates higher than 1 pmol m⁻² s⁻¹ are given.

**Table 1** Blend of volatile compounds emitted by *Succisa pratensis* leaves at higher rates after feeding by *Euphydryas aurinia* larvae

<table>
<thead>
<tr>
<th>Compound</th>
<th>Leaf before any feeding (pmol m⁻² s⁻¹)</th>
<th>Attacked leaf (at 1st hour) (pmol m⁻² s⁻¹)</th>
<th>Attacked leaf (at 24 h) (pmol m⁻² s⁻¹)</th>
<th>Unattacked leaf (at 24 h) (pmol m⁻² s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m33 (methanol)</td>
<td>3636.49 (1507.75)</td>
<td>7405.15 (2507.80)</td>
<td>24097.93 (1738.69)</td>
<td>3765.14 (2795.81)</td>
</tr>
<tr>
<td>m45 (acetaldehyde)</td>
<td>34.45 (2.79)</td>
<td>62.98 (2.95)</td>
<td>51.31 (16.96)</td>
<td>50.71 (34.09)</td>
</tr>
<tr>
<td>m51 (methanol-water cluster)</td>
<td>30.08 (12.50)</td>
<td>76.23 (20.37)</td>
<td>208.32 (40.99)</td>
<td>32.86 (35.95)</td>
</tr>
<tr>
<td>m58 (isotope of hexenal)</td>
<td>−8.38 (4.94)</td>
<td>5.13 (2.03)</td>
<td>18.65 (5.04)</td>
<td>13.77 (4.66)</td>
</tr>
<tr>
<td>m65 (ethanol-water cluster)</td>
<td>−1.42 (1.65)</td>
<td>3.53 (0.36)</td>
<td>1.64 (2.77)</td>
<td>−0.12 (0.34)</td>
</tr>
<tr>
<td>m73 (butanone)</td>
<td>−27.98 (16.18)</td>
<td>−13.82 (47.98)</td>
<td>34.91 (7.92)</td>
<td>27.64 (12.80)</td>
</tr>
<tr>
<td>m83 (hexenol + hexanal)</td>
<td>6.68 (5.62)</td>
<td>72.31 (21.08)</td>
<td>98.30 (32.61)</td>
<td>24.42 (27.34)</td>
</tr>
<tr>
<td>m85 (hexanol, hexyl acetate)</td>
<td>1.15 (1.75)</td>
<td>9.82 (13.44)</td>
<td>12.53 (1.71)</td>
<td>12.83 (5.72)</td>
</tr>
<tr>
<td>m88</td>
<td>−2.35 (2.19)</td>
<td>4.23 (1.35)</td>
<td>3.47 (5.01)</td>
<td>0.68 (2.88)</td>
</tr>
<tr>
<td>m97 (2E, 4E hexadienal)</td>
<td>−8.48 (6.68)</td>
<td>8.84 (2.43)</td>
<td>−8.80 (6.75)</td>
<td>−1.29 (4.84)</td>
</tr>
<tr>
<td>m101 (hexenal)</td>
<td>−4.90 (6.80)</td>
<td>9.18 (4.14)</td>
<td>15.14 (16.96)</td>
<td>7.78 (9.79)</td>
</tr>
<tr>
<td>m102 (isotope of hexenal)</td>
<td>−0.65 (3.37)</td>
<td>0.20 (2.56)</td>
<td>9.93 (2.18)</td>
<td>0.97 (2.73)</td>
</tr>
<tr>
<td>m115 (heptanal)</td>
<td>−4.78 (4.08)</td>
<td>4.93 (3.69)</td>
<td>7.60 (3.91)</td>
<td>4.53 (6.45)</td>
</tr>
<tr>
<td>m116</td>
<td>−0.24 (2.95)</td>
<td>5.41 (1.54)</td>
<td>5.12 (6.23)</td>
<td>6.55 (6.01)</td>
</tr>
<tr>
<td>m124</td>
<td>6.84 (0.56)</td>
<td>6.34 (2.92)</td>
<td>3.09 (0.62)</td>
<td>7.41 (3.95)</td>
</tr>
<tr>
<td>m127</td>
<td>−2.77 (5.28)</td>
<td>−0.61 (3.25)</td>
<td>2.47 (0.24)</td>
<td>7.03 (3.11)</td>
</tr>
<tr>
<td>m137 (monoterpenes)</td>
<td>1.36 (2.84)</td>
<td>14.07 (3.13)</td>
<td>14.86 (2.40)</td>
<td>11.19 (4.93)</td>
</tr>
<tr>
<td>m139</td>
<td>−2.51 (3.11)</td>
<td>−1.97 (7.64)</td>
<td>1.39 (0.55)</td>
<td>−1.59 (3.49)</td>
</tr>
<tr>
<td>m143 (hexenyl acetates)</td>
<td>−1.51 (4.13)</td>
<td>7.01 (1.54)</td>
<td>7.81 (4.22)</td>
<td>11.32 (1.23)</td>
</tr>
<tr>
<td>m163</td>
<td>2.62 (3.37)</td>
<td>6.38 (1.00)</td>
<td>3.72 (6.92)</td>
<td>2.32 (8.85)</td>
</tr>
<tr>
<td>m172</td>
<td>0.0</td>
<td>1.33 (0.42)</td>
<td>1.29 (0.89)</td>
<td>0.76 (0.95)</td>
</tr>
<tr>
<td>m175</td>
<td>1.34 (1.69)</td>
<td>0.89 (1.83)</td>
<td>2.96 (0.35)</td>
<td>0.06 (1.85)</td>
</tr>
<tr>
<td>M205 (sesquiterpenes)</td>
<td>−0.22 (1.34)</td>
<td>2.94 (0.71)</td>
<td>5.94 (2.06)</td>
<td>0.12 (1.08)</td>
</tr>
</tbody>
</table>

Data are means with SEM in parenthesis; n = 3. Emission rates significantly different from control (before any feeding) (P < 0.05, *P < 0.1) are in bold type. And 0.24%, Galbally & Kirstine, 2002). This percentage was highest in attacked leaves and in the unattacked leaves of attacked plants, given that a systemic decrease in net photosynthetic rates of unattacked leaves on attacked plants was detected (Table 2), in agreement with previous studies showing that caterpillar feeding has adverse effects on photosynthesis of leaf areas beyond the leaf areas in which caterpillars removed tissue (Zangerl et al., 2002). However, other studies have found compensatory increases of photosynthetic rates (Thomson et al., 2003) or no effects (Aldea et al., 2005). Therefore, more studies are warranted to elucidate the indirect effects of insect herbivory on leaf CO₂ and water exchange.

The CO₂ and H₂O exchange explained by caterpillars alone has been taken into account in the calculations for attacked leaves. Different letters after values indicate significant differences (P < 0.05).

Table 2 The CO₂ and H₂O exchange induced by leaves in the measuring chamber

<table>
<thead>
<tr>
<th></th>
<th>Leaf before any feeding</th>
<th>Attacked leaf + caterpillars (At 1st hour)</th>
<th>Attacked leaf + caterpillars (At 24 h)</th>
<th>Unattacked leaf of attacked plant (at 24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂ (µmol m⁻² s⁻¹)</td>
<td>4.81 ± 0.29a</td>
<td>4.99 ± 0.49a</td>
<td>3.54 ± 0.25b</td>
<td>3.39 ± 0.16b</td>
</tr>
<tr>
<td>H₂O (mmol m⁻² s⁻¹)</td>
<td>1.46 ± 0.08</td>
<td>1.44 ± 0.07</td>
<td>1.06 ± 0.23</td>
<td>1.54 ± 0.19</td>
</tr>
</tbody>
</table>

The CO₂ and H₂O exchange explained by caterpillars alone has been taken into account in the calculations for attacked leaves. Different letters after values indicate significant differences (P < 0.05).
Biological functions and possible atmospheric effects

Most of these compounds emitted de novo or in greater amounts after caterpillar attack are commonly emitted by many plants as a response to both biotic (herbivory, pathogen attack) and abiotic (ozone, wounding) stressors (Turlings et al., 1990; Fall et al., 1999; Peñuelas & Llusia, 2001; van Poecke & Dicke, 2004), a fact that implies that these emissions occur as a general response in plants to stress and have connected metabolic pathways. In addition to an immediate and direct defensive function as ways of repelling or intoxicating herbivores and pathogens, they are also involved in plant-to-plant communication and act as easily detectable and distinctive chemical cues for the predators and parasitoids of the herbivores feeding on the plants (i.e. they serve as indirect defenses) (Turlings et al., 1990; Peñuelas & Llusia, 2001; van Poecke & Dicke, 2004).

The most significant result of this study was that the attacked leaves emitted far greater amounts of methanol (20 nmol m\(^{-2}\) s\(^{-1}\)) than unattacked leaves (Fig. 2). There is ample evidence to suggest that methanol is produced from pectin demethylation in the cell walls (Gaffe et al., 1994; Fall & Benson, 1996; Galbally & Kirstine, 2002) and since this process occurs in the apoplast, methanol proves to be a common constituent of the transpiration stream in plants (Fall & Benson, 1996), which is consistent with an expected release from cut leaves. Our results show that methanol emission started to slightly but not significantly increase after the caterpillars started to feed, and that the greatest increase in emissions occurred most intensively 1 d after the attack started (Fig. 2). Unfortunately, because we did not analyse each leaf continuously for a whole day, we cannot know whether a peak of methanol or any other volatile emission occurred between the first hour and 24 h after feeding started.

The lifespan of methanol is several days in the boundary layer and a few weeks in the upper troposphere (i.e. much longer than the chemical lifetime of other common biogenic VOCs such as isoprene or monoterpenes, which are far more reactive). The up to eight-times higher foliar methanol emissions described here, together with their relatively long lifespan, might have considerable impact on tropospheric oxidants if these increased emissions were found to be case in other plant species. For example, despite the poor reactivity and large solubility of methanol in water (properties that make deposition more likely to occur in this compound than in most reactive VOCs), if increased methanol emissions were generalized in response to herbivores, they might significantly increase the 1–2% of O\(_3\) tropospheric concentrations, on average, accounted for by methanol (Tie et al., 2003).

The increased emissions of the other volatiles reported here, such as those of the lipoxygenase-derived volatiles and the monoterpenes, might also significantly affect local oxidative tropospheric chemistry and play an important role in perturbing local ozone dynamics (Litvak et al., 1999; Heiden et al., 2003), if they were generalized and occurred in other more abundant plants. Furthermore, LOX volatiles might actually be emitted at higher rates a few hours after the stress (Heiden et al., 2003; Wildt et al., 2003). This possibility was hinted at by the continuously increasing emission rates after the caterpillar attack (Fig. 1), but could not be monitored by

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**Fig. 2** Herbivore-induced emissions of foliar volatiles. The figures show huge *Succisa pratensis* foliar emissions of methanol (a), significant increases of lipoxygenase-derived (LOX) volatile compounds such as hexenols, hexanal, and hexenyl acetate (b) and monoterpenes (c) immediately after attack by *Euphydryas aurinia* caterpillars, after 1 d of continuous attack, and systemically (i.e. emitted by unattacked leaves of attacked plants). +, P < 0.10; *, P < 0.05; **, P < 0.01 (Student t-test comparison with control leaves from plants before the attack).
our experimental set-up, which only monitored the first hour and 24 h after the caterpillars started feeding.

In any case, to actually have a significant effect on the atmosphere, the responses found in *S. pratensis* should be generalizable to many other species. Further studies are thus warranted by the results reported here. Moreover, the actual amounts of volatiles emitted by this (and other possible) species, and therefore their overall atmospheric effects, depend on the performance of the herbivore populations, which vary from season to season. For example, *E. aurinia* populations experience dramatic yearly changes of abundance, mainly as a result of variations in the rates of parasitic attacks by specialist braconid parasitoids *Cotesia melitaerum* and *Cotesia bignellii* (Ford & Ford, 1930; Kankare et al., 2005). Thus, following a year of low parasitism, larval densities can attain very high levels and inflict serious damage to *S. pratensis* populations. However, the impact of VOC emissions by *S. pratensis* as a result of *E. aurinia* attack seems to be of limited scope. Thus, although noticeable defoliation might occur in years of high larval density, this happens at a reduced scale, for which reason noticeable defoliation might occur in years of high larval infestation.

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**References**


