Contrasting responses of silver birch VOC emissions to short- and long-term herbivory

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There is a need to incorporate the effects of herbivore damage into future models of plant volatile organic compound (VOC) emissions at leaf or canopy levels. Short-term (a few seconds to 48 h) changes in shoot VOC emissions of silver birch (Betula pendula Roth) in response to feeding by geometrid moths (Erannis defoliaria Hübner) were monitored online by proton transfer reaction time-of-flight mass spectrometry (PTR-TOF-MS). In addition, two separate field experiments were established to study the effects of long-term foliage herbivory (FH, 30–32 days of feeding by geometrids Agriopis aurantiaria (Clerck) and E. defoliaria in two consecutive years) and bark herbivory (BH, 21 days of feeding by the pine weevil (Hyllobius abietis L.) in the first year) on shoot and rhizosphere VOC emissions of three silver birch genotypes (gt14, gt15 and Hausjärvi provenance).

Online monitoring of VOCs emitted from foliage damaged by geometrid larvae showed rapid bursts of green leaf volatiles (GLVs) immediately after feeding activity, whereas terpenoid emissions had a tendency to gradually increase during the monitoring period. Long-term FH caused transient increases in total monoterpene (MT) emissions from gt14 and sesquiterpene (SQT) emissions from Hausjärvi provenance, mainly in the last experimental season. In the BH experiment, genotype effects were detected, with gt14 trees having significantly higher total MT emissions compared with other genotypes. Only MTs were detected in the rhizosphere samples of both field experiments, but their emission rates were unaffected by genotype or herbivory. The results suggest that silver birch shows a rapid VOC emission response to short-term foliage herbivory, whereas the response to long-term foliage herbivory and bark herbivory is less pronounced and variable at different time points.

Keywords: bark herbivory, foliage herbivory, genotype, geometrid moth, pine weevil, proton-transfer mass spectrometry, volatile organic compounds.

Introduction

Boreal forests emit a large quantity of non-methane volatile organic compounds (VOCs) into the atmosphere (Guenther et al. 1995, Laurila et al. 1997, Hakola et al. 1998, 2003, Tarvainen et al. 2007). Although conifers contribute a large share, deciduous trees such as birches (Betula spp.) make a substantial contribution during late spring and summer (Hakola et al. 2001). Birches are low isoprene emitters (Lindfors and Laurila 2000, Haapanala et al. 2009, Ghirardo et al. 2010), but the emission rates of monoterpenes (MTs) (Hakola et al. 1998), sesquiterpenes (SQTs) (Zhang et al. 1999, Mäntylä et al. 2008) and oxygenated VOCs can be substantial, especially from young leaves (Hakola et al. 2001). Various studies have indicated that insect herbivory triggers pathways regulating the synthesis of some VOCs, which are generally known as herbivore-induced plant volatiles (HIPVs) (Dicke 2009, Hare 2011). For example, herbivory by the autumnal moth (Epirrita autumnata) has been shown to induce emissions...
of (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (E)-β-ocimene, methyl salicylate (Vuorinen et al. 2007), linalool and (E,E)-α-farnesene (Blande et al. 2010) from silver birch trees. Thus, biotic factors such as herbivore damage can regulate the biochemical pathways associated with the synthesis and emission of some VOCs by plants (Peñuelas and Llusia 1998, Pare and Turnblom 1999). Plant VOC emission in response to insect herbivory has an adaptive nature (Dicke and Baldwin 2010), and thus different genotypes may show variability in this adaptive trait (Staudt and Lhoutellier 2007). The magnitude of VOC emissions in response to herbivore damage may also vary in different genotypes or cultivars (Coleman and Jones 1991, Gouinguené et al. 2001).

Many VOCs emitted by plants can directly deter herbivores (De Moraes et al. 2001) or attract natural enemies of herbivores (Dicke and Baldwin 2010). Herbivore-induced VOCs can affect the physical and chemical properties of the atmosphere (Atkinson and Arey 2003), influencing air quality through the production and degradation of tropospheric ozone (Pinto et al. 2010), and also contributing to climate-relevant secondary aerosol formation (Virtanen et al. 2010, Kulmala et al. 2013). Herbivorous insects often cause periodic disturbances in boreal forest ecosystems (Cooke and Lorenzetti 2006); recent studies indicate that some of these insects are shifting their distribution range in response to global warming (Parmesan et al. 1999, Ammunit et al. 2012). In the near future, potentially rapid changes in the distribution of insects may lead to intense and more frequent outbreaks in boreal forests (Battisti et al. 2005, Neuvonen et al. 2005, Jepsen et al. 2008) and thereby cause more deforestation of forest trees and emission of HIPVs. For instance, geometrid moths have caused extensive damage to birch forests in Finland (Neuvonen et al. 2005) and their range expansion might exacerbate the extent of defoliation in the future (Parmesan et al. 1999, Battisti et al. 2006, Jepsen et al. 2009, 2011, Ammunit et al. 2012). Severe defoliation can affect growth, development and defence capabilities of birch trees (Ruohomäki et al. 2000, Neuvonen et al. 2005, Jepsen et al. 2009, Novriyanti et al. 2010). In addition, pine weevil (Hyllobius abietis L.), a serious pest of conifer seedlings (Heijari et al. 2011), is an insect that occasionally feeds on the bark of birch saplings (Toivonen and Virri 2006) and also uses VOCs emitted from fresh wood and logging residues for long-distance orientation (Wibe and Mustaparta 1996). Clearcutting, deforestation and other human activities increase the breeding and growing conditions of H. abietis, which will probably increase the damage in birch forests in the future.

Silver birch (Betula pendula (Roth.)) is an ecologically and economically important tree species in Finland. It occasionally suffers from increased insect herbivore pressure due to cyclic population fluctuations (Neuvonen et al. 2005). There is a high probability of more frequent outbreaks of geometrid moths due to global warming (Jepsen et al. 2008, Ammunit et al. 2012), and thus the risk of large-scale defoliation of silver birch is high. However, the changes in VOC emissions of silver birch trees in response to damage caused by geometrid moths and bark-feeding weevils are not well understood.

The main purpose of this study was to determine whether the damage caused by geometrid moths (Eranthis defoliaria (Hübner) and Agriopis aurantiaria (Clerck)) and a bark-feeding weevil (H. abietis) affects the emission rates and profiles of VOCs from different genotypes of silver birch under field conditions. We were particularly interested in determining how herbivore-induced VOCs emitted from silver birch foliage after 30- to 32-day feeding periods differed from those emitted after shorter (2–7 days) feeding periods, such as those used in Vuorinen et al. (2007). This information will better allow the inclusion of herbivory into biogenic VOC emission models (Grote and Niinemets 2008, Arneth and Niinemets 2010) and serve as a step towards incorporating effects of herbivore damage into future VOC models at leaf or canopy levels (Grote et al. 2013). In the foliage herbivory (FH) experiment, three Finnish silver birch genotypes (genotypes 14 (gt14), 15 (gt15) and Hausjärvi provenance) were infested with moth larvae during the summers of 2011 and 2012. In another field experiment, the effects of bark herbivory (BH) on the same silver birch genotypes were studied by allowing two adult pine weevils to feed on the stem bark inside a clip cage. In addition, the short-term effects of foliage herbivory on Hausjarvi trees were monitored online by proton transfer reaction time-of-flight mass spectrometry (PTR-TOF-MS). The advantage of the PTR-TOF-MS methodology is that very rapid changes, even the ones occurring within a few seconds, can be observed. Our hypotheses were that (i) foliage herbivory by geometrid moth species could activate systemic defence and may thus increase the emission rates of above- and below-ground VOCs from silver birch, (ii) bark damage by pine weevils may also affect the systemic emission of VOCs from the shoots and rhizosphere of silver birch and (iii) different genotypes may display variability in VOC emissions in response to foliage or bark herbivory.

Materials and methods

Plant material

Two silver birch genotypes (gt14 and gt15) were selected from a naturally regenerated mixed birch stand (Punkaharju, 61°48′N, 29°18′E) and micropropagated at the Haapastensyrjä research station of the Finnish Forest Research Institute in January 2009. The plantlets were taken to the University of Eastern Finland (UEF) on 15 April 2009 and transplanted into growth medium on 16 and 17 April 2009. Trees of the third silver birch genotype were 3-year-old saplings (seed origin: Hausjärvi, 60°48′N, 24°01′E) acquired from Fin Forelia Oy nursery (Tuusniemi, Finland). Hausjärvi provenance trees were grown outdoors in pots at the UEF Research Garden, Kuopio.
At the end of May 2009, all micropropagated saplings were transplanted into 7-l pots filled with a mixture of Sphagnum peat and refined sand (granule size 0.5–1.2 mm, Maxit Oy Ab Hiekkaatuote) (2:1, v/v), and the pots were transferred to outdoor conditions at the Research Garden. During the growth period, the saplings were fertilized with slow release Superex (9:3:5:5 N: P: K, Kekkilä, Finland) and watered daily.

**Insect feeding experiments in the field**

Two separate field experiments were performed during the growing seasons of 2011 and 2012. For the FH experiment, two north invading (Ammunét et al. 2012) geometrid moth species (E. defoliaria and A. aurantia) were reared on young leaves of American ash (Fraxinus americana L) at room temperature (+20 °C) in early summer. Eggs to establish the cultures were acquired from mated female moths of A. aurantia and E. defoliaria that were collected from a forest patch near the Botanical Garden of the University of Turku in SW Finland in October 2009. Thereafter, the stocks were reared under laboratory conditions.

In 2011, the FH experiment started on 26 June with two second instar caterpillars placed on the side branches of five randomly selected trees per genotype. In this experiment, the leading shoot was kept intact, and the branches that larvae were allowed to feed on are from now on referred to as damaged shoots. During the 32-day feeding period (26 June to 28 July 2011), moth larvae were enclosed in mesh bags. Control trees (abbreviated to C14, C15 and CHH for gt14, gt15 and Hausjärvi provenance control trees, respectively) in the FH experiment (n = 5 per herbivory treatment per genotype) had empty mesh bags attached to branches in a similar way to herbivore-infested plants (abbreviated to FH14, FH15 and FHH for gt14, gt15 and Hausjärvi provenance foliage herbivory trees, respectively). Foliage herbivory was repeated with overwintered saplings in 2012 with six second instar larvae of E. defoliaria and A. aurantia placed on two branches. The feeding started on 29 May 2012 and lasted 30 days (29 May to 29 June 2012).

In the BH experiment, four randomly selected trees per genotype were infested with adult pine weevils (H. abietis L.). The feeding commenced on 26 July 2011 with two adult pine weevils placed in a clip cage attached to the stem, just above the root collar. Feeding continued for a total of 21 days (treatments are abbreviated to BH14, BH15 and BHH for gt14, gt15 and Hausjärvi provenance bark herbivory trees, respectively, and CH14, CH15 and CHH for the corresponding control trees). The pine weevils were collected from a saw dust storage heap of a sawmill (ilsveden Saha, Suonenjoki, Central Finland) and stored in a cold room (+8 °C) with pine twigs as food until the start of the BH experiment.

**Online VOC monitoring with PTR-TOF-MS**

A high-resolution proton-transfer reaction time-of-flight mass spectrometer (PTR-TOF 8000, Ionicon Analytik, Innsbruck, Austria) was used to track the emission of VOCs from plants of the Hausjärvi provenance during defoliation by E. defoliaria caterpillars in the summer of 2012. The monitoring was conducted under laboratory conditions with the same Hausjärvi origin seedlings as in the field experiments. In brief, the system consisted of a pre-cleaned PET bag (25 x 55 cm), which was fastened around a birch branch, enclosing both branch and E. defoliaria larvae for 24–48 h. Additional light, at a level of ~300–350 μmol m⁻² s⁻¹, was provided throughout the monitoring period by two light sources (Lival Shuttle Plus, Lival Oy, Sipoo, Finland with Osram Delux F, 24-W fluorescent lamps, Osram AG, Munich, Germany) positioned above the branch. The bag was flushed with purified air at a flow rate of 215 ml min⁻¹ and VOCs inside the bag were monitored by PTR-TOF-MS. Sample air from the chamber was introduced into the PTR drift tube via a 1.5 m length (outside diameter 1/16 inch) of heated (60 °C) PEKE tubing at a flow rate of 200 ml min⁻¹. The PTR-TOF mass spectrometer was operated under controlled conditions (2.3 mbar drift tube pressure, 600 V drift tube voltage and 60 °C temperature). The raw PTR-TOF data were post-processed with the PTR-MS Viewer 3.0.0.99 program (Ionicon Analytik). Concentrations were calculated by the program using a standard reaction rate constant of 2 x 10⁻¹⁷ cm³ s⁻¹ molecule⁻¹.

**VOC sampling in FH and BH experiments**

Volatile organic compounds were sampled from shoots using a dynamic bag enclosure method during the summers of 2011 and 2012 and analysed by gas chromatography-mass spectrometry (GC-MS). The top 40–50 cm section of the plants and the Teflon air-inlet tube were enclosed in an oven-cleaned (1 h, at 120 °C) 25 x 55 cm PET bag with the bag opening fastened around the stem of the plant with a garden wire. Air filtered through charcoal and a MnO₂ scrubber was pumped into empty bags through Teflon tubing at a flow rate of 600 ml min⁻¹ for 10 min to flush the bags. A sampling line with a Tenax TA tube attached was inserted into the PET bags at a corner and fastened with another garden wire. The air inflow rate was then reduced to 300 ml min⁻¹ and air was pulled through stainless steel tubes filled with a 150-mg Tenax TA adsorbent (Supelco, Bellefonte, PA, USA) for 15 min at a rate of ~200 ml min⁻¹ with a vacuum pump (Thomas 5002 12 V DC). Sample tubes were sealed with Teflon-coated brass caps immediately after collection, kept in a cold box and stored in a refrigerator until analysis. Temperature, relative humidity and photosynthetically active radiation were monitored during VOC sampling with a Hobo Micro Station (Onset Computer Corporation, Bourne, MA, USA).

Since the same trees were used throughout the FH and BH experiments, the shoot VOC sampling was performed in a non-destructive way.

In the FH experiment, the sampling of VOCs was conducted four times for the intact leading shoots over the growing seasons 2011–2012 (i.e., on 28 July 2011, from 12 to 14 June
2012, on 23 and 24 June 2012 and from 18 to 24 August 2012. The larvae fed on the side branches of the trees while VOC measurements were conducted from the leading shoot. In addition, VOCs were sampled directly from damaged branches on 29 June 2012 after removing the bags with some larvae and pupae half an hour before sampling. In the BH experiment, shoot VOC collections were performed three times over the growing seasons 2011–2012 (i.e., on 8 August 2011, on 23 and 24 June 2012 and from 18 to 24 August 2012). In addition to shoot VOCs, rhizosphere VOCs were sampled once on 23 and 24 June 2012. Prior to rhizosphere VOC collection, pots were weeded and the whole root system and pot were enclosed in oven-cleaned PET bags (45 × 55 cm). Rhizosphere VOCs were collected for 20 min, but otherwise the collection method was the same as for the above shoot VOC collections.

Gas chromatography-mass spectrometry procedure

Shoot and rhizosphere VOC samples were analysed by GC-MS (GC type 6890, MSD 5073: Hewlett Packard; Wilmington, DE, USA) according to Blande et al. (2010). Identification of compounds was made by comparing the mass spectra with those compounds in the Wiley library and with pure standards. Monoterpene data were calculated by using α-pinene as a reference, whereas trans-β-caryophyllene was used as a reference for SQTs. To calculate shoot VOC emission rates in nanograms of compound per square metre of leaf material per second (ng m⁻² s⁻¹), the leaf area was determined from the shoots used in VOC collections for each sampling season. Immediately after VOC collection, leaves of the enclosed shoot were photographed with millimetre marked paper as a background for scaling purposes. The ImageJ program (version 1.46r) was used to calculate the leaf areas from the photos. Rhizosphere VOC data were expressed as emission rates per pot surface area, unit used was nanograms per square metre per second (ng m⁻² s⁻¹).

Statistical analysis

As the temperature ranged from 22 to 29 °C during the field VOC measurements, the emission rates of shoot VOCs were adjusted to a standard temperature of 30 °C using the equation of Guenther et al. (1993), and then tested statistically. In addition to temperature standardization, any emissions found in blank samples were deducted from those found in the plant samples to determine the actual emission from the plant material. Shoot VOC data (total amount of DMNT, MTs, green leaf volatiles (GLVs), SQTs and all VOCs) were tested by linear mixed models where the fixed factors were genotype, herbivory treatment and measurement time, and tree identity was used as a random factor. In addition, the shoot VOC profiles were subjected to principal component analysis (PCA). Before extraction of loadings and scores, data were centred and unit variance scaling was performed. Principal component analysis was performed with SIMCA-P 11.5 (Umetrics AB, Umeå, Sweden), and extracted scores were further tested with linear mixed models using tree genotype and herbivory treatment as fixed factors and tree identity as a random factor. The differences between means were considered to be statistically significant at $P < 0.05$ and marginally statistically significant at $P < 0.1$. Natural logarithmic transformation for VOC data was performed before tests when necessary. All statistical analyses were performed with IBM SPSS Statistics 19 for Windows (International Business Machines Corp., Armonk, NY, USA).

Results

Short-term VOC responses to foliage herbivory

Online monitoring by PTR-TOF-MS indicated that herbivory by a geometrid moth under laboratory conditions caused a rapid induction of GLVs. Immediately after herbivory began, there was a substantial burst of m/z 81.070 equivalent to a dehydrated fragment of the C₆ aldehydes (e.g., cis-3-hexenal + trans-3-hexenal), whereas delayed emissions of m/z 83.086 equivalent to a dehydrated fragment of the C₇ alcohols (e.g., cis-3-hexen-1-ol + trans-3-hexen-1-ol) and m/z 143.107 representative of cis-3-hexenyl acetate were observed (Figure 1a and b). The moth larvae enclosed inside the bag showed a clear pattern in feeding activity with most of them feeding simultaneously and then resting at approximately the same time. The feeding pattern was reflected by oscillation in the VOC emissions, especially of the GLVs (Figure 1a and b). The emission rates showed gradual increases during the feeding period (Figure 1c). The diurnal pattern of VOC emissions from intact branches over 48-h monitoring periods (Figure 1d and e) showed that the same compounds were emitted from intact branches as herbivore-damaged branches, but, in contrast to damaged branches, the compounds had either relatively constant emission rates or a steady decline in emissions during the day. Methanol was the dominant VOC detected during the monitoring of both damaged and control plants, but it did not show any feeding-dependent response (Figure 1f).

Long-term VOC responses to foliage herbivory in the field

A statistically significant genotype × herbivory interaction effect was observed for the emission of total MTs by shoots in the FH experiment over the two experimental seasons (Table 1, Figure 2a). Thus, for gt14, herbivory treatment increased the total MT emissions by 81%, whereas herbivory caused 26 and 41% reductions in total MT emission by the gt15 genotype and Hausjärvi provenance, respectively. However, the genotype-dependent differences in herbivore-induced MTs varied substantially over time (a statistically significant time × genotype × herbivory interaction, Table 1). Hence, the clearest increase in herbivore-induced total MTs in gt14 was observed in 2011, but the response decreased in 2012.
Figure 1. Time course of VOCs emitted by silver birch (B. pendula) while geometrid moth larvae (E. defoliaria) were feeding on the foliage. Panel (a) shows 2-h and panels (b) and (c) show 24-h VOC emission trends from damaged foliage, respectively, whereas panels (d) and (e) show 48-h monitoring of VOCs from intact shoots while larvae were feeding on side branches. Panel (f) shows 24- and 48-h monitoring of methanol from both intact and damaged plants. Different colours represent different mass ions: panels (a), (b) and (d) present trends in the following ion masses: m/z 81.070 (corresponding to C<sub>6</sub> aldehydes, e.g., cis-3-hexenal, trans-3-hexenal and trans-2-hexenal), m/z 81.070 is a dehydration reaction product of the protonated molecular ion of m/z 99.080; m/z 83.086 (corresponding to C<sub>6</sub> alcohols, e.g., cis-3-hexenol, trans-3-hexenol, trans-2-hexenol); m/z 83.086 is a dehydration reaction product of the protonated molecular ion of m/z 101.096; m/z 143.107 (corresponding to hexenyl acetates, e.g., cis-3-hexenyl acetate, trans-3-hexenyl acetate and trans-2-hexenyl acetate). Panels (c) and (e) present trends in the following ion masses: m/z 137.133 is typical of the protonated molecular ion for all MTs (C<sub>10</sub>H<sub>16</sub>), whereas m/z 205.195 is typical for all SQTs (C<sub>15</sub>H<sub>24</sub>); m/z 151.148 (C<sub>11</sub>H<sub>19</sub>OH<sup>-</sup>) protonated DMNT; m/z 153.055 (C<sub>11</sub>H<sub>19</sub>O<sub>3</sub>) protonated methyl salicylate; m/z 155.143 (C<sub>11</sub>H<sub>19</sub>O<sub>3</sub>) is defined as protonated terpene alcohols (e.g., linalool or borneol). Panel (f) presents trends in: m/z 33.034 (CH<sub>3</sub>OH<sup>+</sup>) corresponding to the protonated molecular ion for methanol.
FH experiment

<table>
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<tr>
<th>Genotype</th>
<th>DMNT P-values</th>
<th>MTs P-values</th>
<th>SQTs P-values</th>
<th>GLVs P-values</th>
<th>Total VOCs P-values</th>
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BH experiment

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<th>GLVs P-values</th>
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Table 1. Linear mixed model results (P-values only) of total VOC emissions for main effects of herbivory, genotype and time and their interactions in foliage herbivory (FH) and bark herbivory (BH) experiments. DMNT=(E)-4,8-dimethyl-1,3,7-nonatriene; MTs= monoterpenes; SQTs= sesquiterpenes; GLVs= green leaf volatiles. P-values <0.1 are shown in bold font.

FH14 trees were separated from their control counterparts as emitted more of the SQTs cis more GLVs, such as data variation). In this measurement, Hausjärvi trees emitted (Table a), there was a statistically significant interactive effect of time × genotype × herbivory on total shoot VOC emissions (Table 1, Figure b–e), but this trend was not consistent. Volatile organic compound emissions (MTs, SQTs, GLVs, total VOCs) of damaged branches did not significantly vary with genotype, herbivory or their interaction (data not shown).

Principal component analysis showed that Hausjärvi trees were usually separated from the micropropagated trees on the basis of their shoot VOC profiles during the experimental seasons (Table 2). For instance, in 2011, Hausjärvi trees had higher α-pinene and cis-3-hexenyl butyrate emissions than gt14 and gt15 trees, whereas micropropagated genotypes emitted more myrcene, (E)-β-ocimene, neo allo-ocimene and α-amorphene (data not shown). In the second measurement series of 2012 (Figure 3a), there was a statistically significant genotype main effect on shoot VOC profiles (P<0.05) (Table 2, PC1 for the second measurement explained 34% of data variation). In this measurement, Hausjärvi trees emitted more GLVs, such as cis-3-hexen-1-ol, cis-3-hexenyl acetate, cis-3-hexenyl butyrate and methyl salicylate, and more α-pinene and trans-β-caryophyllene, than gt14 trees, which emitted more of the SQTs β-bourbonene and γ-cadinene. FH14 trees were separated from their control counterparts as C14 trees had higher emissions of 3-carene and (E)-β-ocimene than damaged gt14 trees, which in turn emitted more cis-3-hexenyl acetate and myrcene. FH15 trees instead emitted more β-bourbonene, α-humulene and δ-cadinene than C15 trees. On the last measurement date, VOC profiles from the intact shoots of Hausjärvi trees were again separated from both micropropagated genotypes, with gt14 and gt15 trees having higher emissions of α-pinene and methyl salicylate than trees of the Hausjärvi origin. For the damaged shoot data, there was a marginally statistically significant difference in the VOC profiles due to a genotype × herbivory interaction effect (P=0.066 for PC1). Hence, PC1 (explaining 24% of total variation in the data) showed that only C14 trees were separated from FH14 trees, whereas for other genotypes herbivory effects were not seen (data not shown).

Only five MTs (α-pinene 8.33×10⁻⁵–2.56×10⁻² ng m⁻² s⁻¹, camphene 0–4.72×10⁻² ng m⁻² s⁻¹, myrcene 0–7.78×10⁻⁴ ng m⁻² s⁻¹, 3-carene 0–5.56×10⁻³ ng m⁻² s⁻¹ and limonene 0–8.33×10⁻³ ng m⁻² s⁻¹) were detected in the rhizosphere samples of the FH experiment. Neither these compounds nor the ratios of above-ground to below-ground total MTs showed statistically significant differences due to genotype, herbivory or their interaction.

Long-term VOC responses to bark herbivory in the field

In the BH experiment, there was a statistically significant genotype main effect on both total MT and total VOC emission rates over the two growing seasons (Table 1, Figure 4a). In general, gt14 emitted 40% more MTs and 62% more total VOCs than Hausjärvi provenance trees, whereas gt15 emitted 43% more
MTs and 42% more total VOCs than Hausjärvi provenance trees. There was also a statistically significant time × genotype interaction ($P = 0.008$, Table 1) and a marginally significant time × herbivory interaction ($P = 0.065$, Table 1) effect on the emission rates of GLVs. Hence, in 2011 both micropropagated trees emitted more GLVs than Hausjärvi provenance trees (Figure 4b), but on the first measurement date of 2012 only gt14 trees emitted more GLVs than Hausjärvi provenance trees (Figure 4c), and on the last measurement date of 2012 the pattern was again changed, as the Hausjärvi provenance trees emitted more GLVs than micropropagated genotypes (Figure 4d). BH14 trees emitted less GLVs than C14 trees at all times, whereas BH15 trees emitted 57% more GLVs than C15 trees in 2011, but thereafter the herbivory effect disappeared (Figure 4b–d). In trees of the Hausjärvi provenance, the herbivory treatment only caused an increase in GLV emissions (89%) at the end of the experiment (Figure 4d).

Principal component analysis combined with linear mixed ANOVA showed that there was a statistically significant ($P < 0.05$) genotype main effect on shoot VOC profiles for the first year (2011) measurements, as gt14 trees differed from Hausjärvi provenance trees in general (Table 2). The main reason for this was that gt14 trees seemed to have higher emission rates of some GLVs (cis-3-hexen-1-ol and cis-3-hexenyl acetate) and SQTs (α-humulene, germacrene and γ-cadinene) than the Hausjärvi trees. On the first measurement date in 2012, PC1 explained 26% and PC2, 23% of the total variation in shoot VOC profiles (Figure 3b). The ANOVA test also showed a statistically significant genotype × herbivory interaction (Table 2, $P = 0.046$ for PC2). Thus, C14 trees emitted more GLVs (cis-3-hexen-1-ol, cis-3-hexenyl butyrate, cis-3-hexenyl acetate and 1-hexanol) than BH14 trees, but herbivory effects were not seen for the profiles of other genotypes (Figure 3b). On the last measurement date, PC2 indicated that there was a marginally significant difference in data due to herbivory; BHH trees emitted slightly more MTs such as 3-carene and α-pinene than CH trees (Table 2).

Seven MTs (tricyclene, α-pinene, camphene, sabinene, myrcene, 3-carene, (E)-β-ocimene, eucalyptol) were detected in rhizosphere VOC samples of the BH experiment. All of the compounds were emitted in very low rates ranging from $0$ to $5.83 \times 10^{-3}$ ng m$^{-2}$ s$^{-1}$, and none of these compounds showed significant differences due to herbivory, genotype or interaction effects. Moreover, the ratios of above-ground to below-ground total MTs did not show significant difference due to genotype, herbivory or their interactive effects.

**Discussion**

**Short-term VOC emissions follow herbivore activity**

The online monitoring revealed interesting emission patterns of VOCs from branches of silver birch in response to herbivore feeding. The emission of GLVs peaked immediately after herbivore began and decreased when the larvae were not eating; GLV emissions thus showed a strong dependence on the feeding activity of insects. This was expected as GLVs are usually emitted in response to cell wall damage, wound-related
Table 2. Linear mixed model results (P-values) for shoot VOC profile data in the foliage herbivory (FH) and bark herbivory (BH) experiments. P-values for main effects (genotype and herbivory) and the interaction effect (genotype × herbivory) are given. P-values < 0.1 are shown in bold font.

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Figure 3. Principal component analysis biplot diagrams showing the loading plot of shoot VOCs (indicated by x symbols and names of compounds) superimposed on the score plot of control and herbivore-damaged trees: (a) FH trees sampled on 23–24 June 2012 (b) BH trees sampled on 23–24 June 2012. C14, gt14 control trees; C15, gt15 control trees; CH, Hausjärvi control trees; FH14, gt14 foliage herbivory trees; FH15, gt15 foliage herbivory trees; FHH, Hausjärvi foliage herbivory trees. BH14, gt14 bark herbivory trees; BH15, gt15 bark herbivory trees; BHH, Hausjärvi bark herbivory trees (n = 3–5 per treatment per genotype in FH and n = 3–4 per treatment per genotype in BH).
breakdown of membrane fatty acids and elicitation of stress signalling pathways (Pare and Tumlinson 1999, Loreto et al. 2006, Grote et al. 2013), and their emission rates are proportional to the extent of damage (Copolovici et al. 2011). A similar trend in the emission of GLVs was reported for hybrid aspen during a 20-h feeding period by autumnal moth larvae under field conditions (Schaub et al. 2010). Moreover, there is a striking similarity in the delayed emission of alcohols and acetates in our online monitoring to the results observed in wounded aspen leaves (Fall et al. 1999). Terpenoids and other VOCs that increased in this experiment (DMNT, methyl salicylate, MT, SQT) had similarities in their emission patterns to those observed previously in silver birch, grey poplar and hybrid aspen analysed by traditional GC-MS (Blande et al. 2007), and also monitored by PTR-MS (Graus et al. 2004, Schaub et al. 2010). The terpenoid emission did not closely follow the feeding activity, but the emission rates tended to gradually increase due to extended herbivory stress as reported earlier in black alder (Copolovici et al. 2011). Schaub et al. (2010) also reported induction of terpenes, particularly at 16 h after herbivory began, thus exemplifying a delayed induction. Only very small emissions of isoprene (0.7 nmol m$^{-2}$ s$^{-1}$) have been detected in online measurements of silver birch (Ghirardo et al. 2010). We observed increasing emission rates of an isoprene-indicating mass (m/z 69.070) during feeding and frass accumulation. However, isoprene was not detected in the GC-MS analysis of VOC samples from birch trees in the course of the field experiments nor from samples collected from larvae and frass (unpublished data). Folivorous larvae feeding on deciduous trees and their frass are considered to be non-emitters of VOCs (Staudt and Lhoutellier 2007). However, on resin-storing tree species larval VOC emissions can be substantial (Ghimire et al. 2013). There was also substantial methanol emissions during the feeding hours, but these emissions were not influenced by herbivory, which is not in agreement with data obtained from herbaceous plants (Peñuelas et al. 2005, von Dahl et al. 2006). On the other hand, methanol may also be induced by handling of plants or it can be emitted from young leaves during the growth process (Galbally and Kirstine 2002).

**Long-term FH effects are less pronounced**

In the FH experiment, the emission rates of total MTs increased in response to herbivory, but this only occurred transiently in one genotype. The changes in MT (an 81% increase in gt14 trees) and SQT (27–270% in different genotypes) emissions in our FH experiment were weaker than in other studies where up to 453% increases in MTs + DMNT and 413% increases in SQT emissions have been observed in silver birch in response to FH (Vuorinen et al. 2007). However, in Vuorinen et al. (2007) only micropropagated genotypes were studied and feeding lasted for a relatively short period (3 days), whereas in our study three different genotypes (including seed origin) were used and herbivory lasted for a longer period. The variability in VOC emission throughout the two growing seasons could be attributed to many factors, including the developmental stage of leaves (Hartikainen et al. 2012), relative humidity and light intensity. Moreover, variation in VOC emissions might be associated with a damage threshold for each plant as multiple and intense damage events may induce emissions (Girling et al. 2011). It could also be associated with the time between the start of herbivory and the VOC sampling, as VOC emissions from damaged parts usually decrease some
days after damage (Staudt and Lhoutellier 2007). The larvae used in our study were not very active immediately after infestation of trees in the field, probably because of their lower preference for birch in their natural diet, compared with, for example, E. autumnata. Various abiotic factors also have the potential to alter plant–herbivore interactions, which can result in decreased insect herbivory in the field (Valkama et al. 2007). Furthermore, during the open field experiment, it was not possible to totally exclude the natural background herbivory, as trees were growing without a net covering the whole tree.

Silver birch has large genetic variation and therefore it is probable that the responses to herbivory differ widely between different genotypes (Prittinen et al. 2003). In a previous warming and ozone exposure experiment (Hartikainen et al. 2012) gt14 trees had a higher gas exchange efficiency and SQT emissions than gt15 trees, but in this study the highest SQT emissions were observed from seed origin trees (Hausjärvi et al. 2009).

**Bark herbivory did not cause clear VOC responses in birch shoots**

Pine weevil feeding is capable of inducing the emission of MTs and SQTs from damaged bark and intact shoots of resin-storing coniferous Scots pine (Heijari et al. 2011) and Norway spruce (Blande et al. 2009) trees. In Norway spruce, pine weevil damage caused an approximately eightfold increase in MT and a 45-fold increase in SQT emissions (Blande et al. 2009). The GLV emission from Norway spruce trees also increased by approximately fourfold due to pine weevil feeding compared with intact plants (Blande et al. 2009). In this study, there was no evidence of systemic induction of VOCs from foliage due to bark damage. This could be partly explained by the fact that birches do not store terpenes as they lack resin ducts in their stem (Haapanala et al. 2009), whereas conifers are able to store large amounts of terpene-based resins in their stems (Martin et al. 2002). Interestingly, in the BH experiment GLVs dominated the emission profiles of the gt14 trees, even though the damage was occurring in the bark, but this could also be due to natural foliage herbivory in the field.

**Rhizosphere MT emissions were not affected by herbivory**

In both FH and BH experiments, there was no significant change in the emission rates or profiles of rhizosphere VOCs. In agreement with our findings in the FH experiment, a previous study using foliar feeding by beet armyworm (Spodoptera exigua (Hubner)) on cotton (Gossypium herbaceum L.) showed no significant change in rhizosphere VOC emissions related to foliage herbivory (Bezemer et al. 2004). Rhizosphere VOC emissions were also not significantly affected by artificial defoliation in maize (Collantes et al. 1999) or shoot hormone application in wild Brassica spp. (van Dam et al. 2004). However, in terpene-storing Scots pine, late season foliar herbivory resulted in substantial reduction in rhizosphere VOC emissions, probably as a result of reduced carbon allocation to below-ground resin storage (Ghimire et al. 2013).

**Conclusions**

This study provides quantitative information of systemic and damage-related VOC emissions from silver birch genotypes in response to foliar herbivory by geometrid moth species and bark herbivory by pine weevils. Online monitoring by PTR-TOF-MS showed the transient nature of foliar herbivory-related VOC emissions, especially GLVs, which closely followed bursts of larval feeding activity. The VOC emissions following long-term foliar feeding periods in the field indicated that there was not a continuous increase of VOC emissions from silver birch; thus the response to long-term herbivory was weaker than we expected. Bark herbivory only had transient effects on shoot VOCs and no clear effect on below-ground VOCs. There was also some intraspecific variability in the VOC emissions and profiles of silver birch genotypes, suggesting that the genotypes differ in their resistance to geometrid moths and weevils.

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**Conflict of interest**

None declared.

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Populus, following root and leaf injury.


