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# Foliar methyl salicylate emissions indicate prolonged aphid infestation on silver birch and black alder

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**Summary** It is well documented that when plants are damaged by insects they respond by emitting a range of volatile organic compounds (VOCs). While there have been numerous reports concerning VOCs induced by chewing herbivores, there are relatively few studies detailing the VOCs induced by aphid feeding. The effects of aphid feeding on VOCs emitted by boreal forest trees have been particularly neglected. Herbivore-induced VOCs have relevance to direct and indirect plant defence and atmospheric chemistry. In this study, we analysed the VOCs emitted by *Betula pendula* (Roth) and *Alnus glutinosa* (L.) (Gaertn.) infested by specialist aphid species under laboratory conditions. We also complemented this by collecting VOCs from leaf beetle-damaged saplings under field conditions. In addition to induction of some inducible terpenes, we detected substantial aphid-induced emissions of methyl salicylate (MeSA) in both *B. pendula* and *A. glutinosa*. MeSA emission intensity depended on the length of aphid infestation. Feeding by beetles induced emission of (*E*)-DMNT in both tree species and (*E*)-β-ocimene in *A. glutinosa* but had no effect on MeSA emissions. MeSA has been shown to have aphid-repellent qualities and has been shown recently to have impact on formation of secondary organic aerosols in the atmosphere. We discuss our results in relation to these two phenomena.

*Keywords: Agelastica alni, Euceraphis betulae, monoterpenes, sesquiterpenes, volatile.*

## Introduction

Plants produce and emit a range of volatile organic compounds (VOCs) both constitutively and as an induced response to herbivore feeding. Herbivore-induced plant volatiles (HIPV) mediate complex interactions between plants and organisms of higher trophic levels (Dicke and van Loon 2000, Dicke et al. 2003, van Poecke and Dicke 2004, D'Alessandro and Turlings 2006, Dicke 2009), other plants, both conspecific (Heil and Silva Bueno 2007) and heterospecific (Karban et al. 2003, 2004) and microorganisms (e.g., Shulaev et al. 1997). Individual compounds may be responsible for some of these processes; for example, methyl salicylate emitted by tobacco mosaic virus-infected plants activates plant virus defence in neighbouring plants (Shulaev et al. 1997), and (*Z*)-3-hexenyl acetate has been assigned particular importance in the mediation of plant–plant communication in Lima bean (Kost and Heil 2006) and hybrid aspen (Frost et al. 2008). However, some processes are facilitated by complex volatile mixtures, as observed for the foraging behaviour of predators and parasitoids (Hoballah et al. 2002, Pareja et al. 2009).

Mechanical damage facilitates the rapid emission of preformed compounds stored in cellular structures (D'Auria et al. 2007), while sustained damage by herbivores can result in emissions that include a range of de novo synthesized compounds. The mode of herbivore feeding has considerable impact on the quality and quantity of induced emissions, which can provide olfactory cues to the location of specific herbivore species. Leaf-chewing herbivores create clear mechanical damage through tissue removal, but additional induction of volatiles is facilitated by factors in the insect oral secretion (Turlings et al. 1990; reviewed by Arimura et al. 2009). Interestingly, progressive mechanical damage may induce similar volatile blends (Mithöfer et al. 2005) and parasitoid responses (Connor et al. 2007) to herbivore-damaged plant material.

Feeding by aphids is extremely subtle compared to feeding by chewing herbivores; aphids use stylets to navigate the cuticle, epidermis and mesophyll and establish feeding sites in the phloem. During probing, aphids puncture virtually all mesophyll cells on their path to a major vein of the phloem (Walling 2008). Despite the subtlety underlying their feeding methods, there are several studies detailing the effects of aphid feeding on volatile emissions from plants (Du et al. 1998, Zhu and Park 2005, Girling et al. 2006, Pareja et al. 2007, Gosset et al. 2009), and it is well known that other piercing sucking insects induce volatile blends that differ from those induced by chewing insects (Leitner et al. 2005, Delphia et al. 2007). In addition, there is evidence that saliva from single-cell-feeding plant bugs is important in eliciting volatile emissions (Williams et al. 2005). However, there is currently very limited information about the effects of aphid feeding on VOCs emitted by forest trees (Holopainen 2008).

Black alder, *Alnus glutinosa* (L.) Gaertn, and silver birch, *Betula pendula* (Roth.), are two common species of the Betulaceae family in Europe. *Betula pendula* also forms a significant component of boreal forests, which is the largest terrestrial biome in the world. These species are both subject to significant infestation by aphids, which are more diverse and abundant in boreal forest ecosystems than at more southern latitudes (Dixon et al. 1987). As well as playing integral roles in a number of biological processes, VOCs emitted by boreal forest trees have a significant effect on the production of secondary organic aerosols (SOA) in the atmosphere (Bonn and Moortgat 2003, VanReken et al. 2006, Boy et al. 2008, Spracklen et al. 2008, Mentel et al. 2009). Therefore, significant induction of VOCs by insect feeding could have a large impact on SOA formation. In turn, this SOA formation could influence the global radiative cooling effect as described by Kulmala et al. (2004).

In this study, we investigated the effects of feeding by two specialist aphid species that do not host-alternate on emissions from *B. pendula* and *A. glutinosa*. We conducted experiments in the laboratory to avoid interference by naturally occurring aphid populations. We used *Euceraphis betulae* Koch (Homoptera: Drepanosiphinae), a *B. pendula* specialist that feeds mainly on the underside of leaves, and *Pterocallis alni* deGeer (Homoptera: Drepanosiphinae), an *Alnus* spp. specialist (Heie 1982) that also feeds on the underside of leaves in colonies with scattered distribution. Our aim was to elucidate the effects of aphid feeding on the VOC blends emitted by deciduous forest trees. In order to compare aphid-induced emissions with those induced by chewing herbivores, we collected VOCs from beetle-infested saplings in late season in the field; we also compared our results with literature-reported emissions from *B. pendula* and *A. glutinosa* (Table 1). Our study was divided into four experiments with the following aims: (i) to assess the effect of aphid feeding on volatile emissions of *B. pendula* plantlets, (ii) to assess the direct contribution of aphids and their honeydew to emissions, (iii) to assess the effect of aphid feeding and length of infestation on volatile emissions from 1-year-old *B. pendula* and *A. glutinosa* saplings and (iv) to assess the effect of beetle feeding on emissions from *B. pendula* and *A. glutinosa* saplings in the field. We discuss our results in terms of significance to signalling between and within trophic levels and to atmospheric composition.

Table 1. Summary of previous VOC collections from *B. pendula* and *A. glutinosa*.

| Authors (year) | Tree species | Field/lab | Treatment | Replicates (n) | MeSA | MeSA (approximate percentage of total emission) |
|---|---|---|---|---|---|---|
| Tscharntke et al. (2001) | *A. glutinosa* | F | Control | 1 | No emission | – |
| | | | *Agelastica alni* | 1 | Emitted but not quantified | – |
| Scutareanu et al. (2003) | *A. glutinosa* | F | *Agelastica alni* | 1 | 48 GC area units $h^{-1}$ $g^{-1}$ leaf | 1.8 |
| Vuorinen et al. (2005) | *B. pendula* | L | Control, | 4 | *† 4–77 ng $gFW^{-1}$ $h^{-1}$ | 0.2–1.7 |
| | | | Ozone, | 4 | *† 7–45 ng $gFW^{-1}$ $h^{-1}$ | 0.2–0.6 |
| | | | CO2, | 4 | *† 7–142 ng $gFW^{-1}$ $h^{-1}$ | 0.1–1.5 |
| | | | Combination. | 4 | *† 5–43 ng $gFW^{-1}$ $h^{-1}$ | 0.1–0.6 |
| | | | Control | 4 | * 1–3 ng $gFW^{-1}$ $h^{-1}$ | 0.3–0.6 |
| | | | Detached branch | 4 | * 3–10 ng $gFW^{-1}$ $h^{-1}$ | 0.1–0.2 |
| Vuorinen et al. (2007) | *B. pendula* | L | Control | 6 | * § 0 ng $gDW^{-1}$ $h^{-1}$ | 0 |
| | | | *Epirrita autumnata* | 6 | * § 170 ng $gDW^{-1}$ $h^{-1}$ | 21.45 |
| | | | Fungal pathogen | 10 | * § 0 ng $gDW^{-1}$ $h^{-1}$ | 0 |
| Hakola et al. (2001) | *B. pendula* | F | Undamaged | 15 | Not recorded | – |
| Zhang et al. (1999) | *B. pendula* | F | Undamaged | 6 (Jun) | 26.3 ± 22 ng $gDW^{-1}$ $h^{-1}$ Jun | 5.0 |
| | | | | 4 (Aug) | 1.2 ± 1.7 ng $gDW^{-1}$ $h^{-1}$ Aug | 0.4 |

Footnote: * denotes that the study incorporated two tree clones. † denotes that the study incorporated two sampling dates (July and August). § denotes that detached branches were used in the study.

## Materials and methods

### *Plants*

Experiments 1 and 2 were conducted with micropropagated plantlets of *B. pendula* (Roth.) (silver birch) obtained from the Finnish Forest Research Institute (Haapastensyrjä, Loppi, Finland). Plantlets were potted in a mixture of peat and sand (3:1) and nurtured under greenhouse conditions until they had reached heights of approximately 25 cm. The young plants were then moved to controlled environment chambers (Weiss Bio 1300, Weiss Umwelttechnik Gmbh, Reiskirchen-Lindenstruth, Germany) in the laboratory.

Experiments 3 and 4 were conducted with 1-year-old *B. pendula* saplings (seed origin: Hausjärvi, 60°48′ N, 24°01′ E) obtained from Fin Forelia Oy nursery (Tuusniemi, Finland) or 1-year-old micropropagated black alder, *A. glutinosa* (L.) Gaertn., var. Carelica (origin: Punkaharju, Finland) saplings obtained from Taimityllilä Oy (Mäntyharju, Finland). After acquisition, saplings were grown outdoors in pots at the University of Kuopio Research Garden. Saplings were moved to the laboratory and grown in controlled environment chambers for Experiment 3 and were distributed throughout the Ruohoniemi experimental field site at the Research Garden for Experiment 4.

### *Insects and infestation procedures*

*Euceraphis betulae* (Koch.) aphids were collected from naturally occurring infestations of *B. pendula* trees a few days before the start of Experiment 1 and reared on *B. pendula* plantlets for the duration of the study. *Pterocallis alni* (De Geer) aphids were collected from naturally occurring infestation of black alder trees a few days before the start of Experiment 3 and used directly from the collected leaves. Adult alder leaf beetles (*Agelastica alni* L.) were collected from naturally occurring infestations of grey alder (*Alnus incana*) and stored in plastic containers with fresh leaf material before use in Experiment 4.

Clonal *B. pendula* plantlets were treated with the following protocol. Seedlings were placed into plastic-framed insect-rearing cages (60 × 33 × 33 cm, external dimensions) in groups of four. Two rearing cages were placed into each of two controlled environment chambers (Weiss Bio 1300, Weiss Umwelttechnik Gmbh, Reiskirchen-Lindenstruth, Germany). Cages containing plants to be infested with aphids were kept in one controlled environment chamber, while those containing non-infested control plants were kept in a second chamber. As *E. betulae* viviparae are all alate, infestations were made by adding 100 alatae to each cage. This procedure was used for Experiments 1 and 2. In addition, for Experiment 1 approximately 25 nymphs were added to each plant.

For Experiment 3, *B. pendula* and *A. glutinosa* saplings were placed in four growth chambers containing the following isolated treatments: six non-infested control *A. glutinosa*, six *P. alni* infested *A. glutinosa*, six non-infested control *B. pendula* and six *E. betulae* infested *B. pendula*. All saplings were fitted with a fine-mesh bag fastened around the top portion of the foliage, covering approximately half of the foliage of *A. glutinosa* saplings and a third of the foliage of *B. pendula* saplings. Aphid infestation was made directly into each bag. Each *B. pendula* sapling was infested with 25 *E. betulae* alatae and 25 nymphs; each alder sapling was infested with approximately 200 *P. alni* mixed instar nymphs, apterae and alatae.

For Experiment 4, 16 alder and 16 *B. pendula* saplings were distributed among four ambient air plots of the Ruohoniemi Free Air Concentration Enrichment (FACE) facility for ozone enhancement (Blande et al. 2007). In each plot, two *B. pendula* and two *A. glutinosa* saplings were infested with five *A. alni* beetles, while matching controls were non-infested. *Agelastica alni* beetles were enclosed in fine-mesh bags attached to the top foliage of alder saplings and to single breast-level branches of *B. pendula* saplings.

*Experiment 1* Sampling of volatile organic compounds emitted by *E. betulae* infested and non-infested *B. pendula* plantlets were conducted at two time intervals (4 and 10 days) after the addition of aphids to cages. At 4 days after aphid infestation, the six plantlets with the heaviest aphid infestations were selected for sampling. Sampling was conducted in two groupings, both consisting of three infested plants and three controls. Prior to sampling, the alatae were removed from the plantlets and returned to the cage. After sampling, the plantlets were returned to their respective cages and to the controlled environment chambers where they were re-colonized by alatae. VOC sampling was repeated at 10 days following the infestation, after which the plantlets were harvested for calculation of leaf dry masses. Dry masses were calculated by placing the excised leaves in paper bags, which were placed in an incubator maintained at 60 °C for 3 days, and then weighed with a microbalance. The average dry mass of leaves per control plant was 1.14 g; 1 g of dry mass corresponded with a leaf area of 264.57 $cm^2$. The average dry mass of leaves per aphid-infested plant was 0.99 g; 1 g of dry mass corresponded with a leaf area of 286.33 $cm^2$.

*Experiment 2* In this experiment, VOC sampling from *B. pendula* was conducted 10 days after the infestation with *E. betulae* aphids, in four rounds. The first round included six non-infested control plantlets, the second round consisted of six aphid-infested plantlets with alatae removed (but nymphs produced during the experiment still present), the third round consisted of the same six plantlets with all nymphs produced during the experiment removed and the final round consisted of the same six plantlets with the aphid honeydew washed away. Aphids were carefully removed with a paintbrush with efforts made not to damage the plants. Honeydew was washed with distilled water, which was applied using a 500-ml bottle fitted with a fine nozzle. The plants were left for 30 min to dry before sampling. After this final round of sampling, the leaves of all plants were harvested and dry masses obtained. The average dry mass of leaves per control plant was 1.80 g; 1 g DW = 270.62 $cm^2$ (leaf area). The average dry mass of leaves per infested plant was 1.69 g; 1 g DW = 285.14 $cm^2$ (leaf area).

*Experiment 3* Sampling of VOCs was made from the saplings at two time points, 7 (29 August 2008) and 21 (12 September 2008) days after infestation. Sampling was conducted in four rounds. The first round consisted of three control and three aphid-infested *B. pendula* saplings; the second round consisted of the remaining *B. pendula* saplings, three for each treatment. The third round consisted of three controls and three aphid-infested *A. glutinosa* sap-

lings, and the final round consisted of the remaining *A. glutinosa* saplings, three for each treatment. In all cases, alatae were removed prior to sampling. Saplings were returned to the growth chambers between the two sampling points, and dry weights were determined at the finish. The average dry masses and leaf areas corresponding with 1 g DW were: control birch 1.73 g, 1 g = 117.24 $cm^2$; infested birch 1.56 g, 1 g = 107.39 $cm^2$; control alder 2.61 g, 1 g = 147.68 $cm^2$; infested alder 3.57 g, 1 g = 153.18 $cm^2$.

*Experiment 4* Sampling of VOCs was conducted a week after infestation with adults of the leaf beetle *A. alni*. Infested and control saplings were sampled in pairs concurrently, with pairs of *B. pendula* and *A. glutinosa* saplings sampled alternately. Leaves were harvested for dry weights upon completion of sampling. The average dry masses and leaf areas corresponding with 1 g DW were: control birch 1.00 g, 1 g = 123.25 $cm^2$; infested birch 1.04 g, 1 g = 127.41 $cm^2$; control alder 2.31 g, 1 g = 135.78 $cm^2$; infested alder 2.30 g, 1 g = 133.98 $cm^2$.

### *Volatile organic compound collection protocols*

*Laboratory method* VOC samples (Experiments 1–3) were collected at bench level with constant temperature (23 °C) and light. In Experiments 1 and 2, Teflon covers were attached to the pots to cover the soil and prevent VOCs passing from the soil to the headspace. Polyethylene terephthalate (PET) bags (35 × 43 cm: Look, Terinex Ltd., Bedford, UK) were used to enclose the whole plantlets and were fastened to the pots with elastic bands; after expansion, the volume of bags was approximately 3 l. For sampling from saplings, the PET bags enclosed the foliage and were fastened to the stem with wire tags. One of the top corners of each bag was cut, and an air inlet tube was inserted. Clean air was pumped into bags at a rate of 220 ml $min^{-1}$ for 15 min before sampling began; the total volume of air flushed through the system was 3.3 l. After flushing with clean air, stainless steel tubes containing approximately 150 mg of Tenax TA adsorbent (Supelco, mesh 60/80) were inserted into the remaining top corner of each bag and fastened in position. Air inflow was maintained at 220 ml $min^{-1}$ throughout collection. Air was pulled through the Tenax tube at a rate of 200 ml $min^{-1}$ for 30 min; the total volume of air sampled was 6 l. Flow rates were calibrated using an M-5 bubble flowmeter (A.P. Buck, Orlando, FL, USA).

*Field method* Collection of volatile samples under field conditions (Experiment 4) was conducted using the protocol described by Mäntylä et al. (2008), in which the VOC collection system was installed into a plastic toolbox, including battery-operated inlet and outlet pumps (Rietschle Thomas, Puchheim, Germany), air filters, rechargeable battery and HOBO Micro Station Data Logger (MicroDAQ.com Ltd, Contoocook, NH, USA). PET bags were used to enclose the foliage, and sampling lines were inserted as above. The HOBO data logger was used to log temperature and photosynthetically active radiation (PAR). A temperature sensor was inserted into the bag at the point where it was attached to the branch or stem and secured using wire tags. A PAR sensor was enclosed in a PET bag and positioned close to the branches being sampled. Prior to sampling, the bags were flushed with filtered air for 10 min at a rate of 600 ml $min^{-1}$; the total volume of air flushed through the system was 6 l. After flushing, the flow rate was reduced to 220 ml $min^{-1}$ and maintained at this level throughout the experiment. Samples were collected onto Tenax TA at a rate of 200 ml $min^{-1}$ for 1 h; the total volume of air sampled was 12 l.

### *Gas chromatography-mass spectrometry procedure*

The VOC samples were analysed by gas chromatography-mass spectrometry (Hewlett-Packard GC 6890, MSD 5973) (see Mäntylä et al. 2008). Trapped compounds were desorbed with a thermal desorption unit (Perkin-Elmer ATD400 Automatic Thermal Desorption system) at 250 °C for 10 min, cryofocused at −30 °C and injected onto a HP-5 capillary column (50 m × 0.2 mm i.d. × 0.5 μm film thickness, Hewlett-Packard) with helium as a carrier gas. The oven temperature programme was held at 40 °C for 1 min and then raised to 210 °C at a rate of 5 °C $min^{-1}$ and finally further to 250 °C at a rate of 20 °C $min^{-1}$. The compounds (mono-, homo- and sesquiterpenes and green-leaf volatiles, GLVs) were identified by comparing their mass spectra with the Wiley library and with pure standards. Emissions are presented in nanograms per gram dry weight per hour.

### *Statistical analyses*

All data were analysed using the statistical package SPSS 14.0 for windows. Due to differences in the growth histories of the different tree species, they were analysed separately. Experiments 1 and 3 were analysed for time by treatment effects using repeated measurements analysis with herbivore feeding as the between-subjects factor. Experiments 2 and 4 were both analysed by one-way ANOVA with the Waller–Duncan post hoc test used to indicate significant differences between treatments.

## Results

### *Experiment 1*

VOC emissions from *B. pendula* plantlets after 4 (Figure 1A) and 10 days (Figure 1B) of aphid feeding were analysed with repeated measurements analysis. There was a significant effect of sample date on emissions of camphene ($P = 0.037$), myrcene ($P = 0.042$), 3-carene ($P = 0.005$), (*E*)-β-ocimene ($P = 0.007$), (*E*)-4,8-dimethyl-1,3,7-nonatriene ((*E*)-DMNT) ($P = 0.019$) and methyl salicylate (MeSA) ($P = 0.004$). There was a significant effect of feeding damage on emissions of (*E*)-β-ocimene ($P = 0.011$), (*E*)-DMNT ($P = 0.003$) and MeSA ($P = 0.001$). Additionally, there was an interactive

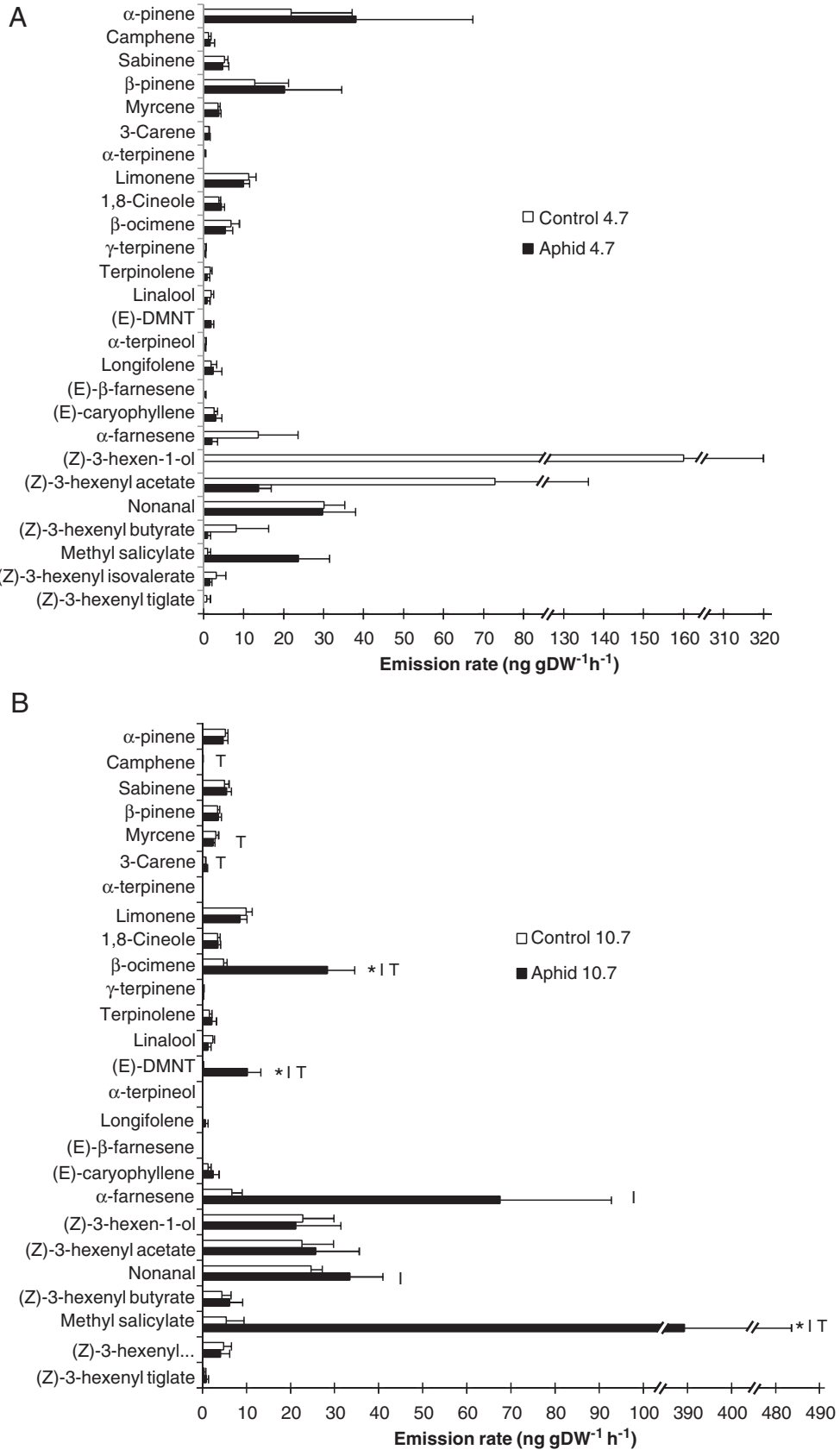


Figure 1. Emissions of VOCs from aphid-infested and control *B. pendula* plantlets collected on 04 July 2008 (A) and 10 July 2008 (B) 4 and 10 days after first exposure to aphids, respectively (Experiment 1). $n = 6$ for each treatment. Significant time effects (T), herbivore effects (asterisks) and interactive effects (I) are denoted in B.

Table 2. Aphid-induced VOC emissions (ng $gDW^{-1}$ $h^{-1}$) from *B. pendula* plantlets after 10 days infestation.

| Compound groups and compounds | Control | Plant–aphid complex | Aphids removed | Plants washed |
|---|---|---|---|---|
| *Monoterpenes* | | | | |
| α-Pinene | 3.41 ± 0.48 | 5.14 ± 1.17 | 6.15 ± 1.91 | 4.48 ± 1.20 |
| Camphene | 0.06 ± 0.06 | 0.26 ± 0.13 | 0.19 ± 0.12 | 0 ± 0 |
| Sabinene | 1.88 ± 0.76 | 5.51 ± 2.08 | 8.97 ± 3.62 | 7.69 ± 3.08 |
| β-Pinene | 3.61 ± 0.51 | 6.00 ± 1.47 | 6.90 ± 2.08 | 6.31 ± 1.60 |
| Myrcene | 2.38 ± 0.82 a | 2.62 ± 0.62 a | 5.57 ± 1.10 b | 3.48 ± 0.78 ab |
| 3-Carene | 0.48 ± 0.15 a | 0.17 ± 0.17 ab | 0.10 ± 0.10 b | 0 ± 0 b |
| α-Terpinene | 0.21 ± 0.21 | 1.49 ± 0.53 | 1.33 ± 0.68 | 0.85 ± 0.44 |
| Limonene | 2.38 ± 1.08 a | 10.56 ± 2.87 b | 17.56 ± 3.34 b | 15.25 ± 2.80 b |
| 1,8-Cineole | 1.29 ± 0.48 a | 3.92 ± 1.43 ab | 6.75 ± 2.65 b | 2.49 ± 0.97 ab |
| (*E*)-β-Ocimene | 13.29 ± 7.00 a | 50.73 ± 17.08 ab | 89.53 ± 26.34 b | 75.47 ± 15.40 b |
| γ-Terpinene | 0.09 ± 0.09 | 0.57 ± 0.23 | 0.84 ± 0.45 | 0.58 ± 0.31 |
| Terpinolene | 0.33 ± 0.33 a | 1.53 ± 0.70 ab | 2.77 ± 1.36 ab | 4.29 ± 1.42 b |
| Linalool | 0.27 ± 0.18 a | 0.69 ± 0.44 a | 2.93 ± 0.73 b | 4.68 ± 0.76 c |
| p-Mentha-1,5,8-triene | 1.58 ± 0.57 a | 7.03 ± 0.97 bc | 9.61 ± 1.34 b | 5.54 ± 0.77 c |
| α-Terpineol | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0.58 ± 0.58 |
| Allo-ocimene | 0.84 ± 0.39 a | 2.29 ± 0.77 ab | 3.91 ± 0.43 b | 2.91 ± 0.62 b |
| *Homoterpene* | | | | |
| (*E*)-DMNT | 2.03 ± 1.04 a | 19.54 ± 5.15 b | 27.11 ± 6.20 b | 20.62 ± 4.30 b |
| *Sesquiterpenes* | | | | |
| Longifolene | 0 ± 0 | 0.22 ± 0.22 | 0.22 ± 0.22 | 0 ± 0 |
| (*E*)-Caryophyllene | 0 ± 0 a | 3.69 ± 0.39 b | 5.12 ± 0.61 c | 5.07 ± 0.62 bc |
| α-Farnesene | 41.51 ± 22.27 a | 127.63 ± 50.85 ab | 246.37 ± 65.47 b | 243.26 ± 74.19 b |
| *Green-leaf volatiles* | | | | |
| (*Z*)-3-Hexen-1-ol | 247.08 ± 172.86 | 27.18 ± 6.67 | 16.95 ± 6.79 | 218.05 ± 110.23 |
| (*Z*)-3-Hexenyl acetate | 41.18 ± 20.87 | 8.96 ± 1.61 | 6.90 ± 2.38 | 34.89 ± 13.66 |
| Nonanal | 12.94 ± 0.95 a | 17.57 ± 1.78 ab | 23.50 ± 3.06 bc | 25.12 ± 2.53 c |
| (*Z*)-3-Hexenyl butyrate | 12.84 ± 8.86 | 0.48 ± 0.32 | 0.37 ± 0.37 | 4.45 ± 1.71 |
| Methyl salicylate | 25.45 ± 10.08 a | 370.82 ± 99.35 b | 551.32 ± 121.44 b | 387.82 ± 77.14 b |
| (*Z*)-3-Hexenyl isovalerate | 0.46 ± 0.46 a | 0.30 ± 0.30 a | 0 ± 0 a | 5.50 ± 1.70 b |
| (*Z*)-3-Hexenyl tiglate | 1.21 ± 0.54 a | 0.13 ± 0.13 b | 0 ± 0 b | 0 ± 0 b |

A breakdown of VOC sources in the plant–aphid complex (Experiment 2). The complete plant–aphid complex, complex with aphids removed and the host plant after washing to remove honeydew are presented. Numbers ± SE are presented. Numbers not followed by the same letter are significantly different. $n = 6$ for each treatment.

effect of sample date and herbivore feeding for emissions of (*E*)-β-ocimene ($P = 0.003$), (*E*)-DMNT ($P = 0.022$), α-farnesene ($P = 0.021$), nonanal ($P = 0.05$) and MeSA ($P = 0.004$). In Figure 3A, the summed emissions of monoterpenes, sesquiterpenes, homoterpenes and GLVs are presented, with MeSA depicted alone. The most notable effect of aphid feeding is the scale of MeSA induction, which comprises almost two-thirds of the total emission from plantlets subjected to aphid stress for 10 days whilst representing a negligible emission from controls.

### *Experiment 2*

Aphid-infested *B. pendula* plantlets emitted significantly more limonene ($P < 0.05$), p-mentha-1,5,8-triene ($P < 0.01$), (*E*)-DMNT ($P < 0.05$), (*E*)-caryophyllene ($P < 0.001$) and MeSA ($P < 0.05$) than their comparative controls. Control plants emitted significantly more of the GLV (*Z*)-3-hexenyl tiglate ($P < 0.05$). After removing all the aphids, the formerly infested plants emitted significantly more myrcene ($P < 0.05$), 1,8-cineole ($P < 0.05$), (*E*)-β-ocimene ($P < 0.01$), linalool ($P < 0.01$), allo-ocimene ($P < 0.01$), α-farnesene ($P < 0.05$) and nonanal ($P < 0.01$) as well as all of the previously induced compounds. 3-Carene ($P < 0.05$) and (*Z*)-3-hexenyl tiglate ($P < 0.01$) were both emitted in significantly lower amounts than from controls. Plantlets that were washed to remove honeydew emitted significantly more terpinolene ($P < 0.05$) and (*Z*)-3-hexenyl isovalerate ($P < 0.01$) than controls as well as all the previously induced compounds except for myrcene and 1,8-cineole. Overall, the action of removing all aphids and washing plants significantly induced a number of VOC emissions (Table 2). This is probably due to the disturbance of plants during these processes. In Figure 3B, the significance of MeSA as an aphid-induced emission is emphasized, with summed GLV emissions more dominant in control plants than aphid-infested plants. An increase in GLV emissions following washing indicates a possible physical disturbance during this process.

Table 3. Aphid-induced VOC emissions (ng $gDW^{-1}$ $h^{-1}$) from *B. pendula* saplings at two time points.

| Compound groups and compounds | Control, 29 August 2008 | Aphid infested, 29 August 2008 | Control, 12 September 2008 | Aphid infested, 12 September 2008 | *P* values | | |
|---|---|---|---|---|---|---|---|
| | | | | | H | T | I |
| *Monoterpenes* | | | | | | | |
| α-Pinene | 1.88 ± 0.69 | 1.67 ± 0.53 | 1.43 ± 0.37 | 0.85 ± 0.10 | | | |
| Camphene | 0.18 ± 0.12 | 0.14 ± 0.10 | 0.05 ± 0.05 | 0 ± 0 | | | |
| Sabinene | 0.43 ± 0.27 | 0.18 ± 0.18 | 0.11 ± 0.11 | 0.13 ± 0.13 | | | |
| β-Pinene | 0.35 ± 0.22 | 0.91 ± 0.74 | 0 ± 0 | 0.10 ± 0.10 | | | |
| Myrcene | 0.97 ± 0.08 | 0.90 ± 0.21 | 0.48 ± 0.35 | 0.55 ± 0.25 | | | |
| 3-Carene | 0.04 ± 0.04 | 0.21 ± 0.21 | 0 ± 0 | 0 ± 0 | | | |
| Limonene | 6.60 ± 0.79 | 5.47 ± 1.42 | 1.12 ± 0.71 | 17.05 ± 4.23 | 0.026 | | <0.001 |
| (*E*)-β-Ocimene | 42.77 ± 10.60 | 65.60 ± 16.82 | 35.76 ± 9.56 | 48.19 ± 12.82 | | | |
| γ-Terpinene | 0.05 ± 0.05 | 0 ± 0 | 0 ± 0 | 0 ± 0 | | | |
| Terpinolene | 0.15 ± 0.15 | 0.11 ± 0.11 | 0 ± 0 | 0 ± 0 | | | |
| p-Mentha-1,5,8-triene | 3.25 ± 0.64 | 3.68 ± 0.85 | 0.82 ± 0.40 | 0.35 ± 0.35 | | 0.002 | |
| Allo-ocimene | 2.10 ± 0.40 | 1.60 ± 0.40 | 0.67 ± 0.41 | 0 ± 0 | | 0.001 | |
| *Homoterpene* | | | | | | | |
| (*E*)-DMNT | 14.61 ± 2.03 | 33.82 ± 11.66 | 24.10 ± 8.93 | 40.71 ± 7.56 | | | |
| *Sesquiterpenes* | | | | | | | |
| α-Humulene | 0.06 ± 0.06 | 0 ± 0 | 0 ± 0 | 0 ± 0 | | | |
| (*E*)-Caryophyllene | 10.16 ± 1.15 | 13.26 ± 2.59 | 5.10 ± 1.78 | 8.78 ± 3.05 | | | |
| α-Farnesene | 31.59 ± 11.50 | 100.03 ± 43.52 | 19.50 ± 5.62 | 77.42 ± 12.27 | 0.033 | | |
| *Green-leaf volatiles* | | | | | | | |
| (*Z*)-3-Hexen-1-ol | 213.04 ± 95.23 | 74.32 ± 37.05 | 43.22 ± 14.97 | 190.16 ± 47.64 | 0.026 | | 0.026 |
| 1-Hexanol | 0.99 ± 0.99 | 0 ± 0 | 1.13 ± 1.13 | 0 ± 0 | | | |
| (*Z*)-3-Hexenyl acetate | 115.55 ± 40.78 | 49.05 ± 19.88 | 15.99 ± 5.08 | 62.38 ± 16.54 | | | 0.033 |
| Nonanal | 8.19 ± 1.06 | 8.56 ± 1.62 | 6.51 ± 1.61 | 3.94 ± 2.76 | | | |
| (*Z*)-3-Hexenyl butyrate | 11.17 ± 6.78 | 4.20 ± 2.45 | 0.19 ± 0.19 | 2.42 ± 1.04 | | | |
| Methyl Salicylate | 23.63 ± 16.51 | 384.20 ± 189.37 | 172.09 ± 120.15 | 721.63 ± 204.91 | 0.035 | 0.047 | |
| (*Z*)-3-Hexenyl isovalerate | 2.26 ± 1.07 | 1.53 ± 1.09 | 0.49 ± 0.49 | 0 ± 0 | | 0.045 | |
| (*Z*)-3-Hexenyl tiglate | 0.83 ± 0.44 | 0.66 ± 0.66 | 0 ± 0 | 0 ± 0 | | | |

Emissions collected on 29 August 2008 and 12 September 2008, 7 and 21 days after infestation, respectively (Experiment 3). Numbers ± SE are presented. $n = 6$ for each treatment. Where significant differences exist, the relevant *P* values are provided; H, herbivore feeding effect; T, time (date) effect; I, interactive effect.

Table 4. Aphid-induced VOC emissions (ng $gDW^{-1}$ $h^{-1}$) from *A. glutinosa* saplings at two time points.

| Compound groups and compounds | Control, 29 August 2008 | Aphid infested, 29 August 2008 | Control, 12 September 2008 | Aphid infested, 12 September 2008 | *P* values | | |
|---|---|---|---|---|---|---|---|
| | | | | | H | T | I |
| Monoterpenes | | | | | | | |
| α-Pinene | 0.37 ± 0.02 | 0.29 ± 0.01 | 0.36 ± 0.03 | 0.25 ± 0.01 | 0.001 | | |
| Myrcene | 0 ± 0 | 0.29 ± 0.29 | 0 ± 0 | 0 ± 0 | | | |
| Limonene | 0.14 ± 0.14 | 0.69 ± 0.50 | 5.86 ± 0.40 | 4.10 ± 2.17 | | 0.004 | |
| (*E*)-β-Ocimene | 0.57 ± 0.36 | 10.23 ± 7.49 | 0 ± 0 | 7.21 ± 3.57 | 0.041 | | |
| p-Mentha-1,5,8-triene | 0 ± 0 | 1.27 ± 0.78 | 0 ± 0 | 0.24 ± 0.24 | | | |
| Allo-ocimene | 0 ± 0 | 0.23 ± 0.23 | 0 ± 0 | 0 ± 0 | | | |
| *Homoterpene* | | | | | | | |
| (*E*)-DMNT | 0 ± 0 | 2.80 ± 2.60 | 0 ± 0 | 0.23 ± 0.23 | | | |
| *Sesquiterpenes* | | | | | | | |
| (*E*)-α-Bergamotene | 0 ± 0 | 0 ± 0 | 0.12 ± 0.12 | 1.69 ± 0.43 | 0.006 | 0.002 | 0.006 |
| (*E*)-Caryophyllene | 0 ± 0 | 0.68 ± 0.35 | 0 ± 0 | 0 ± 0 | | | |
| α-Farnesene | 1.81 ± 0.45 | 1.91 ± 0.71 | 0 ± 0 | 0.63 ± 0.22 | | 0.009 | |
| *Green-leaf volatiles* | | | | | | | |
| (*Z*)-3-Hexen-1-ol | 14.79 ± 8.74 | 14.24 ± 2.90 | 14.42 ± 4.63 | 57.58 ± 31.53 | | | |
| (*Z*)-3-Hexenyl acetate | 22.92 ± 11.49 | 22.47 ± 4.51 | 21.44 ± 6.11 | 43.91 ± 20.52 | | | |
| Nonanal | 3.45 ± 0.75 | 2.21 ± 0.25 | 3.92 ± 0.87 | 0.79 ± 0.50 | 0.017 | | |
| Methyl salicylate | 0 ± 0 | 1.09 ± 0.77 | 1.39 ± 1.39 | 137.98 ± 45.16 | 0.012 | 0.012 | 0.014 |

Emissions collected on 29 August 2008 and 12 September 2008, 7 and 21 days after infestation, respectively (Experiment 3). Numbers ± SE are presented. Where significant differences exist, the relevant *P* values are provided; H, herbivore feeding effect; T, time (date) effect; I, interactive effect. $n = 6$ for each treatment.

*Experiment 3*

Aphid-infested *B. pendula* saplings were sampled after 7 and 21 days of infestation. There was a significant effect of sample date on emissions of p-mentha-1,5,8-triene ($P = 0.002$), allo-ocimene ($P = 0.001$), MeSA ($P = 0.047$) and (*Z*)-3-hexenyl isovalerate ($P = 0.045$). There was a significant effect of herbivore feeding on emissions of limonene ($P = 0.026$), α-farnesene ($P = 0.033$), (*Z*)-3-hexen-1-ol ($P = 0.026$) and MeSA ($P = 0.035$). Additionally, there was an interactive effect of sample date and herbivore feeding for emissions of limonene ($P < 0.001$), (*Z*)-3-hexen-1-ol ($P = 0.026$) and (*Z*)-3-hexenyl acetate ($P = 0.033$) (Table 3). MeSA comprised more than half of the total emission both at 7 and 21 days of infestation (Figure 3C).

*Alnus glutinosa* saplings were sampled according to the same time frame. There was a significant effect of sample date on emissions of limonene ($P = 0.004$), (*E*)-α-bergamotene ($P = 0.002$), α-farnesene ($P = 0.009$) and MeSA ($P = 0.012$). There was a significant effect of treatment on emissions of α-pinene ($P = 0.001$), (*E*)-β-ocimene ($P = 0.041$), (*E*)-α-bergamotene ($P = 0.006$), nonanal ($P = 0.017$) and MeSA ($P = 0.012$). Additionally, there was an interactive effect of sample date and herbivore feeding for emissions of (*E*)-α-bergamotene ($P = 0.006$) and MeSA ($P = 0.014$) (Table 4). After 21 days of aphid feeding, MeSA emissions comprised over half of the total emission (Figure 3D).

*Experiment 4*

Feeding by August-emerging second-generation *A. alni* beetles under field conditions induced emission of (*E*)-DMNT ($P < 0.05$) from *B. pendula* saplings (Figure 2A) and emissions of (*E*)-β-ocimene ($P < 0.01$) and (*E*)-DMNT ($P < 0.01$) from alder saplings (Figure 2B). There were no detected emissions of MeSA from *A. glutinosa*; while beetle-damaged *B. pendula* emitted trace amounts only. The total emission of monoterpenes and GLVs were moderately increased in beetle-damaged individuals of both tree species, although sesquiterpene emissions were lower in damaged *B. pendula* (Figure 3E and F). There were no sesquiterpene emissions detected in *A. glutinosa* samples.

## Discussion

Our results demonstrate clear induction of VOC emissions from leaves of *B. pendula* and *A. glutinosa* in response to phloem feeding by specialist aphids. We identified MeSA as the most distinctive indicator of aphid feeding in the induced VOC blend of both tree species in Experiments 1, 2 and 3. This is in agreement with Zhu and Park (2005), who found MeSA emissions to be a good indicator of aphid feeding on soybean leaves, and Pareja et al. (2009) showing MeSA to be induced by aphid feeding on alfalfa. Here we report for the first time the aphid-induced emission of MeSA from boreal forest trees. Ear-

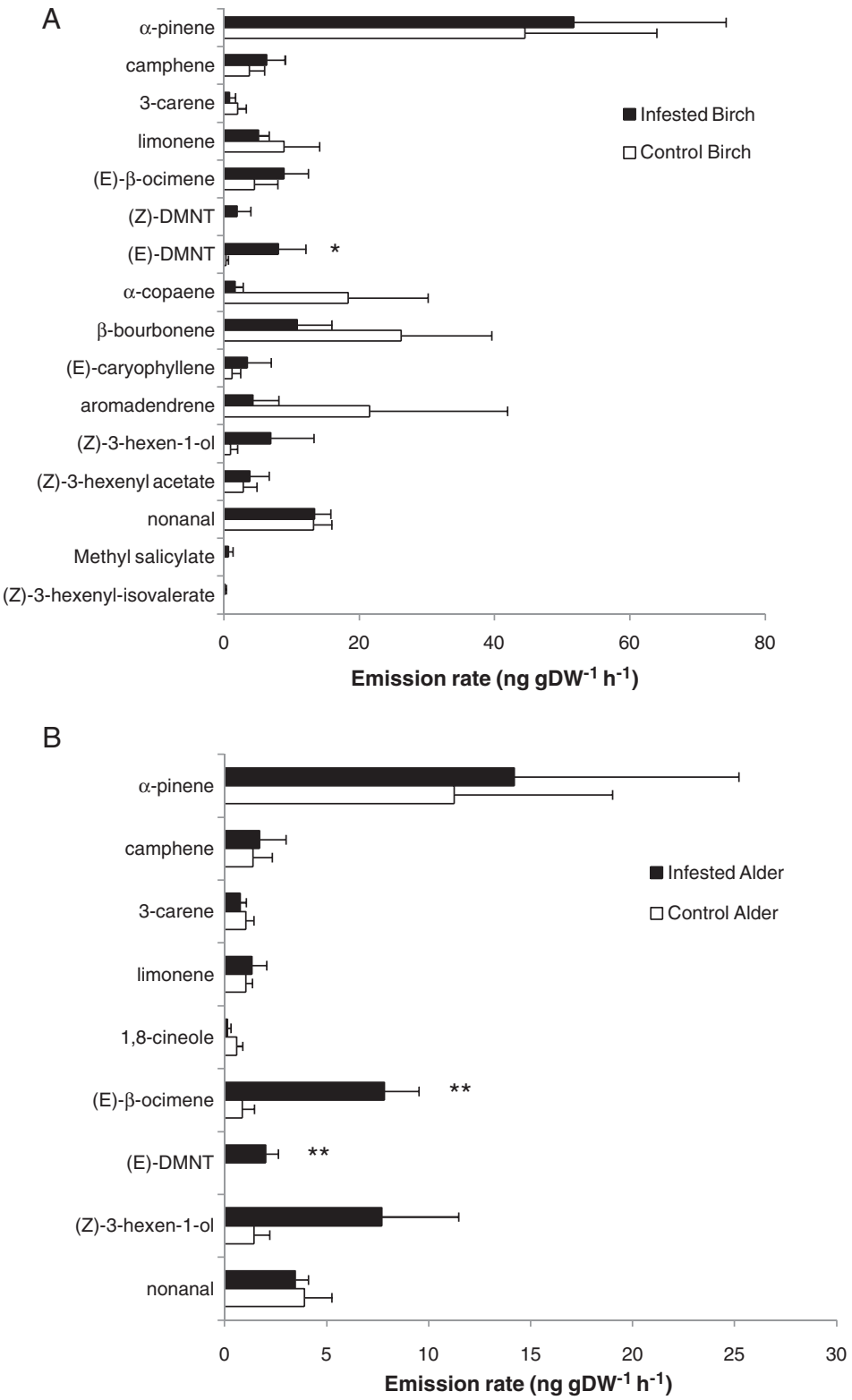


Figure 2. Emissions of VOCs from leaf beetle-infested *B. pendula* (A) and *A. glutinosa* (B) saplings (Experiment 4). Significant differences between control and infested plants are denoted by $*P < 0.05$ and $**P < 0.01$. $n = 8$ for each treatment.

lier, Pettersson et al. (1994) presented data showing that MeSA is a constitutive component of detached branch emissions of *Prunus padi* and that MeSA will repel migrating individuals of the first generation of *Rhopalosiphon padi* aphids.

When aphids feed, they excrete a sugar-rich by-product called honeydew which contaminates plant surfaces. In Experiment 2, we dissected the components of the plant–aphid complex responsible for the induced emissions and found

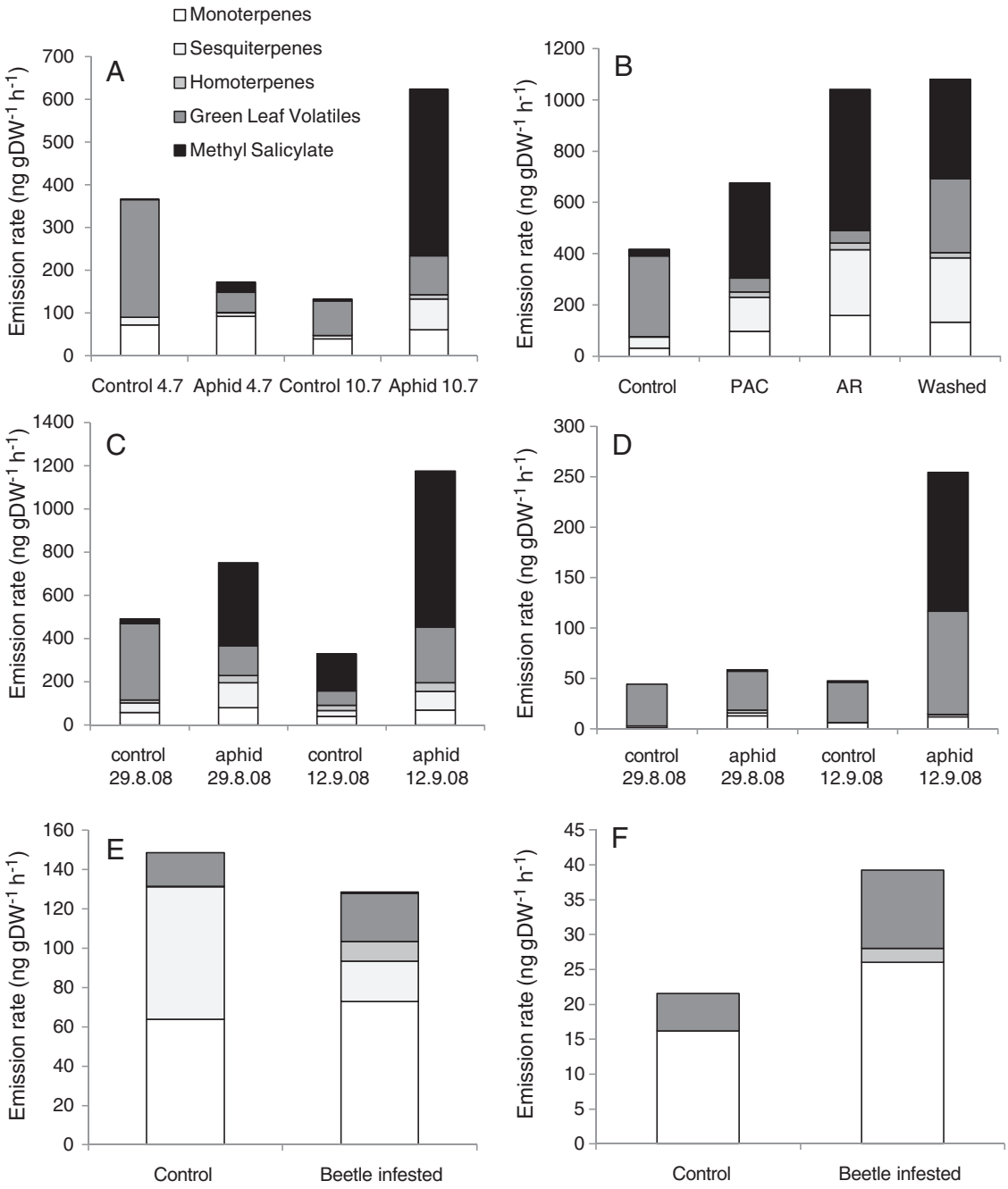


Figure 3. Emissions of total monoterpenes, total sesquiterpenes, total green-leaf volatiles, (*E*)-DMNT and methyl salicylate for each experiment. (A) *B. pendula* plantlets with aphids (Experiment 1) $n = 6$; (B) *B. pendula* plantlets with aphids (Experiment 2) $n = 6$; (C) *B. pendula* saplings with aphids (Experiment 3) $n = 6$; (D) *A. glutinosa* with aphids (Experiment 3) $n = 6$; (E) *B. pendula* with leaf beetle (Experiment 4) $n = 8$; (F) *A. glutinosa* with leaf beetle (Experiment 4) $n = 8$. PAC, plant–aphid complex; AR, aphids removed.

that removal of aphids and honeydew actually resulted in greater emissions of several compounds. The explanation for this could be that physical disturbance of the plant caused an increase in emissions or that honeydew accumulation had covered the surface of the leaf, blocked stomata and reduced emission rates. Emissions from the aphids did not appear to make a significant contribution to the volatile blends.

The composition of emissions from aphid-infested *B. pendula* showed distinct quantitative differences to emissions induced by leaf-chewing beetles in this study and leaf-chewing moth larvae in a study by Vuorinen et al. (2007). The main difference is in the scale of MeSA emissions, contributing up to 50% of emissions from aphid-infested plants, while Vuorinen et al. (2007) reported induction of a greater quantity of monoterpenes and sesquiterpenes. The composition of emis-

sions induced by aphid feeding on *A. glutinosa* also differed from that induced by beetle feeding, and again MeSA constituted the main difference between the two emission profiles. In our experiments, a direct comparison of emissions from the aphid-damaged plants and the beetle-damaged plants is not possible due to the different experimental conditions, with aphid-damaged plants sampled in the laboratory and beetle-damaged plants sampled under field conditions. However, comparison with other studies documenting VOC emissions in *B. pendula* and *A. glutinosa* (Table 1) suggests that aphid feeding has a particularly strong impact on MeSA emissions compared to other biotic and abiotic stimuli, and our data are in agreement with this. However, there was some commonality in the induced terpenoid emissions. (*E*)-DMNT was induced by both aphid and beetle feeding in both tree species. (*E*)-β-ocimene was also induced by aphid and beetle feeding in *A. glutinosa*. However, the most abundant terpenoid emitted by aphid-infested *B. pendula* was α-farnesene, which was not emitted by beetle-damaged plants.

Walling (2000) and Kempena et al. (2007) have suggested that phloem-feeding aphids could induce gene sets similar to those activated by fungal or bacterial pathogens and have drawn parallels between the intimate and long-lasting interaction of the aphid stylet with plant cells during feeding and the pathogen–plant cell interactions during infection. Indeed, aphid attack on *Arabidopsis thaliana* induces the transcription of genes associated with salicylic acid-dependent responses to pathogens such as PR-1 and BGL2 (Moran and Thompson 2001, De Vos et al. 2005, Thompson and Goggin 2006), while the role of salicylic acid in defence against chewing herbivores is comparatively minor (Wu and Baldwin 2009). Recently, Girling et al. (2008) showed that activation of both octadecanoid and salicylic acid pathways is involved in aphid-induced volatile production in Arabidopsis, which is consistent with the aphid-induced emission of MeSA reported here. Interestingly, Vuorinen et al. (2007) found MeSA induction in *B. pendula* seedlings damaged by moth larvae but not in seedlings damaged by a fungal pathogen. However, in this case the MeSA emissions induced by moth larvae could be an artefact caused by using detached branches in experiments (Vuorinen et al. 2007).

In all our experiments, it is clear that before aphid feeding induces volatile emission the feeding pressure must exceed a threshold level. Pareja et al. (2007) showed that aphid density is important for the odour of aphid-infested *Centaurea nigra* (L) plants to become more attractive than the odour of undamaged plants to an aphid parasitoid. In the case of *Myzus persicae* feeding on herbaceous *A. thaliana*, feeding by 100 individuals for 3 days was sufficient to induce a volatile bouquet that attracts aphid parasitoids (Girling et al. 2008). In our first experiment, we showed that after 4 days of feeding there were no induced emissions in the woody plant *B. pendula* but that there were significantly induced terpenoids and MeSA after 10 days of feeding. Since the density of aphids increased throughout our experiment, it is not possible to state whether it is the duration of feeding or the density of aphids feeding that is the decisive factor or if it is a combination of the two. However, it is clear that there is a damage threshold, after which emissions are induced.

MeSA appears to be an important compound in defence of plants to aphid attack, acting as both an aphid repellent (Glinwood and Pettersson 2000) and an attractor of foraging predators and parasitoids, which have been shown to orientate toward MeSA, both as simple formulations in the field (James and Price 2004, Zhu and Park 2005) and as components of a complicated blend (Pareja et al. 2009). This twofold defensive function of MeSA has potential in the development of biological control programmes.

In addition to the function of MeSA in structuring interactions within plant–herbivore–natural enemy systems, there are recent data showing that MeSA from agroforest canopies is involved in significant formation of secondary organic aerosols (Karl et al. 2008). As a semi-volatile compound, MeSA can partition into the particle phase directly without prior oxidation in the atmosphere (Karl et al. 2008). MeSA was shown to be a dominant VOC constituent of ambient air in a Juglans orchard, with the average MeSA flux during the course of the study comparable to the monoterpene flux. Abiotic stresses were implicated as elicitors of across-ecosystem increases in MeSA, but the effects of insect feeding have not been suitably addressed. In our study, we have shown such large increases in MeSA following aphid damage that we may expect aphid outbreaks to have significant impact on SOA formation above boreal forests.

In this study, we have added weight to the accumulating evidence that MeSA is an important component of direct and indirect defence against aphids. We have also shown that aphids, which have been largely ignored in boreal forest systems, have the potential to significantly enhance emissions of MeSA and some reactive VOCs which may lead to SOA formation. We suggest that infestation by phloem-sucking insects together with leaf-chewing insects should be considered in future models of biogenic VOC emissions from forests.

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