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Volatile-Mediated within-Plant Signaling in Hybrid Aspen: Required for Systemic Responses

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Abstract Plant volatiles play crucial roles in signaling between plants and their associated community members, but their role in within-plant signaling remains largely unexplored, particularly under field conditions. Using a system comprising the hybrid aspen (*Populus tremula* x *tremuloides*) and the specialized herbivorous leaf beetle (*Phratora laticollis*) and, combining field, greenhouse and laboratory experiments, we examined whether local damage triggered systemic responses in undamaged branches that lack vascular connection to the damaged branches, and to what extent this was caused by airborne volatile signals versus internal signals. An experiment tracing dye through the vasculature of saplings revealed no downward movement of the dye from upper to lower branches, suggesting a lack of vascular connectivity among branches. However, we found under both field and laboratory conditions that herbivore feeding on upper branches elicited volatile emissions by undamaged lower branches. Greenhouse experiments manipulating air contact between damaged and undamaged branches showed that systemic induction of volatiles was almost eliminated when air contact was interrupted. Our findings clearly demonstrate that herbivore-induced volatiles overcome vascular constraints and mediate within-plant signaling. Further, we found that volatile signaling led to induction of different classes of volatiles under field and environment controlled conditions, with a weaker response observed in the field. This difference not only reflects the dose- and time-dependent nature of volatile signaling, but also points out that future studies should focus more on field observations to better understand the ecological role of volatile-mediated within-plant signaling.

Keywords Defense induction · *Phratora laticollis* · Plant volatiles · *Populus* · Priming · Within-plant signaling

Introduction

Plants can respond to herbivore attack with phenotypic changes that may reduce herbivore feeding. These induced responses consist of direct defences such as production of defensive secondary metabolites and proteins that instantly affect the herbivore's physiology (Agrawal 2011), and indirect defences such as the release of complex blends of volatile organic compounds (VOC) that guide predators to their prey (Heil 2014). Furthermore, plants can also prime their defense responses (Balmer et al. 2015). In this context, plants are altered in response to an initial contact with the attacking herbivore and respond more quickly and/or strongly the second time that they encounter it.

Defense induction and priming are not necessarily restricted to the injured plant parts, but extend to distant, as yet undamaged areas of the injured plant, that is, herbivore attack may trigger both local and systemic defense responses. Mechanistically, the systemic response to localised damage is usually regarded as resulting from internal signals such as the phytohormone jasmonic acid and the polypeptide systemin that are generated

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at the damage site and transported to neighbouring undamaged sites through the vascular system (Heil and Ton 2008; Heil 2010; Park et al. 2007). However, the movement of these internal signals is relatively slow over long distances and constrained by both the vascular connectivity among tissues and the source-sink gradient (e.g., Ferrieri et al. 2015; Orians et al. 2000). Consequently, systemic responses exhibit a high degree of spatial and temporal variation within a plant and may take hours to days to occur (Heil and Ton 2008; Orians et al. 2000). Intriguingly, recent research has provided several lines of evidence showing that airborne VOC signals can act in concert with internal signals in eliciting systemic responses, and are particularly essential where undamaged and damaged tissues are vascularly disconnected (Heil 2010). For instance, exposure of undamaged leaves or branches to herbivore-induced VOCs emitted from adjacent branches on the same plants have been shown to induce and/prime a wide set of defense responses, including up-regulation of defense-related genes, enzymes and phytohormone signaling pathways (Frost et al. 2007, 2008), enhanced secretion of extrafloral nectar (Heil and Silva Bueno 2007), augmented emission of VOCs (Erb et al. 2015; Frost et al. 2007; Girón-Calva et al. 2014; Rodriguez-Saona et al. 2009), and reduced leaf damage (Karban et al. 2006; Rodriguez-Saona et al. 2009). However, when air contact is interrupted by, for example, sealing damaged parts in a plastic bag, these responses disappear.

From an evolutionary perspective, VOC-mediated within-plant signaling is thought to precede VOC-mediated between-plant signaling in that the former is more likely to benefit the signal emitter (Heil and Ton 2008). To date, however, the phenomenon of within-plant signaling via VOCs has been described only in seven plant species – sagebrush (*Artemisia tridentata*) (Karban et al. 2006), lima bean (*Phaseolus lunatus*) (Heil and Silva Bueno 2007), hybrid poplar (*Populus deltoides* x *nigra*) (Frost et al. 2007), California mugwort (*Artemisia douglasiana*) (Shiojiri and Karban 2008), blueberry (*Vaccinium corymbosum*) (Rodriguez-Saona et al. 2009), birch (*Betula* spp.) (Girón-Calva et al. 2014), and maize (*Zea mays*) (Erb et al. 2015), which is substantially lower than the increasing body of work on between-plant signaling that has documented compelling evidence in over 35 plant species spanning 16 families (Karban et al. 2014). Moreover, among the aforementioned studies on within-plant signaling, only studies with sagebrush, California mugwort and lima bean were conducted under natural conditions. Although volatile signals, unlike vascular signals, travel rapidly and can be detected by all leaves that have air contact with the damaged parts of a plant, airborne signaling comes at a price of limited controllability by the plant. The transport of volatiles through air is influenced to varying extents by many environmental factors such as wind, humidity, temperature, and atmospheric chemical composition (Blande et al. 2014), which in turn may affect the efficiency of within-plant VOC signaling. Indeed, a recent meta-analysis has revealed stronger induced resistance mediated through between-plant signaling under laboratory or greenhouse conditions than under field conditions (Karban et al. 2014). The limited number of studies on within-plant signaling, in particular under natural conditions, still constrain our understanding of the ecological and evolutionary role within-plant signaling plays in VOC-mediated processes, and call for more research in this area.

In the present study, we investigated within-plant signaling via volatiles in *Populus* under field, laboratory and greenhouse conditions using a system comprising the hybrid aspen (*P. tremula* x *tremuloides*) and the specialist-feeding herbivorous leaf beetle (*Phratora laticollis*). VOC-mediated within-plant signaling in *Populus* has previously been observed in hybrid poplar (*P. deltoides* x *nigra*) (Frost et al. 2007), in which undamaged leaves exposed to VOCs emitted from adjacent leaves damaged by larvae of the generalist-feeding gypsy moth were primed for augmented emissions of several terpenoids upon subsequent larval damage. In addition, an early study on hybrid poplar (*Populus* x *euroamericana*) (Baldwin and Schultz 1983), and our recent study on hybrid aspen (*P. tremula* x *tremuloides*) (Li et al. 2012) have also disclosed the occurrence of VOC-mediated between-plant signalling in *Populus*, in which plants that were exposed to VOCs emitted from neighbouring plants damaged either by artificial tearing of leaf lamina or infested with generalist herbivores showed a strong induction of direct or indirect defences. While these studies together have provided important insights into whether and how *Populus* species perceive and respond to volatile signals, they were all conducted in the laboratory and employed either generalist herbivores or artificial wounding. Consequently, the ecological relevance remains open to debate, particularly considering that plants may respond differently to environmental signals under field and controlled environment conditions (Karban et al. 2014), and that mechanical wounding and damage by specialist and generalist herbivores can induce different volatile responses in many plant species (Ali and Agrawal 2012), including *Populus* (Arimura et al. 2004).

Specifically, we aimed to answer the following questions: 1) to what extent are leaves within and between branches vascularly connected in hybrid aspen? 2) Do VOC signals mediate signaling between different branches under both field and laboratory conditions and if so, is there any difference between them? 3) To what extent do VOC signals contribute to within-plant signaling relative to internal vascular signals?

Materials and Methods

Plants and Insects All experiments were conducted with 1.5 or 4-year-old saplings of hybrid aspen (*P. tremula* L. × *P. tremuloides* Michx.) clone 55. Plantlets were micropropagated in the laboratory (c. 300 μmol m^{-2} s^{-1}), potted in a mixture of

peat and sand (3:1) and nurtured under greenhouse conditions until they were moved to the laboratory or the Ruohoniemi field site at the University of Eastern Finland Research Garden. In late May 2011, 80 three-week-old seedlings were distributed throughout four field plots (see Blande et al. 2007 for more details on experimental site). From 2012 to 2015, every year in early May before bud break began, plants were cut back to 30–50 cm above the ground which promoted the development of five to ten new branches. The field study was conducted in 2012 and 2014.

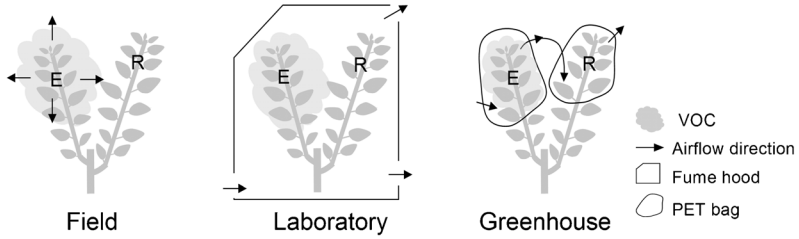
For both the laboratory and greenhouse experiments, saplings were grown in the greenhouse and overwintered in a cold room. The next spring they were moved back to regular growth conditions, and were cut back at the start of bud growth to promote the growth of new branches. By the beginning of the experiments, plants were approximately 1.5 years old and had three to five branches on the main shoot.

Phratora laticollis larvae and adults were collected from naturally occurring infestations of aspen trees at the experimental site of the Finnish Forest Research Institute in Suonenjoki, Finland, and were reared for the duration of the study on hybrid aspen plants in the laboratory at room temperatures and humidities under natural light and room lighting but out of direct sunlight. We used 25 larvae or 20 adults to inflict damage, which is comparable to previous studies on volatile-mediated plant signaling in *Populus* (Frost et al. 2007, 2008; Li et al. 2012). In addition, *P. laticollis* larvae were observed to feed close together in groups of up to 45 larvae (personal observation), a well-known feeding habit of Chrysomelina leaf beetles (Gross et al. 2008).

Vascular Connectivity between Branches To determine the degree of vascular connectivity between leaves within a branch and on different branches, we conducted a Rhodamine-B (Sigma-Aldrich, St. Louis, MO, USA) dye assay following the method described previously by Orians et al. (2000). The assay was carried out in August 2012 at the Ruohoniemi field site. Two middle leaves on an upper branch of a sapling (n = 13) were excised under water with a razor blade, and the cut petioles attached to the branch were each inserted into a 1.5-ml Eppendorf tube filled with a 0.25% (w/v) dye solution. A small hole (ca. 2 mm diameter) at the center of the tube lid provided entry for the cut petiole. Tubes were refilled periodically to ensure constant submergence of the petioles. Movement of the dye through the plant was monitored daily over 6 days. The percentage of stained leaf area (0%, 1–25%, 26–50%, 51–75%, and 76–100%) was visually scored for leaves from the following positions of the saplings: all leaves (about 10 leaves) located both in front and behind the dye-fed petioles on the branch containing the dye, and all leaves (about 25 leaves) on branches immediately above and below the branch containing the dye.

Systemic VOC Emission Following Exposure to Herbivore-Induced VOCs in Field Conditions The experiment was conducted at the Ruohoniemi field site from July 7 to 14, 2014. Four saplings that were located at least 1 m apart from each other and had minimal visible damage by natural herbivores were selected from each of the four plots. Half of the saplings in each plot were haphazardly assigned to a herbivory treatment with the other half left as a control. On each sapling, two branches situated on different shoots were tagged, one branch was randomly designated as the VOC-emitting branch and the other as the recipient branch (Fig. 1). We did not control the orientation of the branches with respect to wind direction; distances between branches ranged from 20 to 50 cm. On 7 July, we enclosed the upper 13 leaves of the emitter branch in a polyester mesh bag for each of the eight saplings in the herbivore treatment, and infested them by placing 25 mixed-instar larvae in the bag. Emitter branches in the control group were bagged similarly but without herbivores added. Approximately 5.5 days later, we removed bags and/or larvae from emitter branches and measured VOC emissions from receiver branches to investigate whether VOCs emitted from infested emitters directly activated defense responses in systemic receiver branches. The duration of exposure was initially set to be three days, but was prolonged to 5.5 days due to intermittent rain. To investigate whether VOCs from emitter branches also prime neighboring receiver branches for faster and/or stronger defense responses, we then infested receiver branches by enclosing 13 upper leaves of the receiver branch into a mesh bag, along with 25 larvae. After one and two days, VOCs were again collected from receiver branches; larvae were removed before the first collection and not returned.

Systemic VOC Emission in Response to Herbivore-Induced VOCs in Laboratory Conditions The experiment followed a paired design and was conducted in well ventilated fume hoods at room temperature and humidity with supplementary lighting (ca. 300 $\mu mol\ m^{-2}\ s^{-1}$) (Fig. 1). Greenhouse-grown saplings were allowed to acclimate to their new environment in the fume hoods for one week. Afterwards, we selected five pairs of saplings of similar size and with an equal number of branches on the main shoot. One sapling in each pair was haphazardly selected for the herbivory treatment and the other was untreated to serve as a control. Since there was no vascular connection from upper to lower branches (see Results), we designated the uppermost branch as the emitter and the branch immediately below as the receiver. We induced emitter branches in the same way as in the field study by adding 25 larvae onto the bagged branches. Infested and control plants were placed in separated fume hoods, with the receiver branch of each plant oriented downwind. After exposure for three days, bags and larvae were removed from emitter branches and



	Emitter branch (E)		Receiver branch (R)			
	Damage agent	Damage duration (d)	Infestation agent	Infestation duration (h)	VOC sampling time dpe	hpi
Field	25 larvae	5.5	25 larvae	20	5.5	24; 48
Laboratory	25 larvae	3	25 larvae	20	3	6; 21; 28; 35; 43; 59
Greenhouse	20 adults	2	20 adults	20	1; 2	24; 48

Fig. 1 Schematic diagram of experimental designs to test for volatile-mediated within-plant signaling under field, laboratory and greenhouse conditions. *Phratora laticollis* first wounded the emitter branch (E) for a certain period of time, during which the receiver branch (R) was exposed to VOCs from the emitter branch by natural wind (field), by orienting the receiver branch downwind of the emitter branch in a ventilated fume hood (laboratory), or by bagging the emitter and receiver branches and manipulating air flow via Teflon tubing (greenhouse). After exposure, the emitter branch was infested with *P. laticollis* to test for the potential priming effects of VOC exposure. VOC emissions from the receiver branch were measured at different time points during exposure and infestation. The table below depicts the timeline for each experiment (d: day; h: hour; dpe: days post exposure; hpi: hours post infestation). For a detailed description of the methodology see the Materials and Methods section

VOCs collected from receiver branches. Immediately after that, receiver branches were challenged in the same way as above by adding 25 larvae onto the bagged branches, followed by VOC collections at several time points. To increase the likelihood of detecting a priming effect, VOC measurements in the laboratory were taken earlier and more frequently than in the field, namely at 6, 21, 28, 35, 43 and 59 h post challenge. For the first two VOC collections, larvae were removed from the branches before collection and added back after the first collection, but not after the second collection, such that larval feeding on receiver branches lasted approximately 20 hr. To account for the potential spatial effects due to plants from the control and herbivory treatments being located in different fume hoods, plants from these two treatments were switched between fume hoods daily throughout the experiment.

Systemic VOC Emission in Response to Herbivore-Induced VOCs in Greenhouse Conditions To explicitly investigate the role that airborne volatile signals have in within-plant signaling and disentangle it from the effect of internal signals, we employed an open flow system to either promote or block air contact between branches. This experiment was performed in a greenhouse under natural lights in July, 2014. Four saplings of similar size and with the same number of branches on the main shoot were selected, and the uppermost branch of each sapling was designated as the emitter and the branch below as the receiver. For both the receiver and emitter branches of each sapling, the upper 14 leaves were enclosed in a transparent plastic bag, and air filtered through a desiccant dryer and an activated carbon filter was continuously pumped (1.5 L/min) in and out of the bag from the two opposite corners of the bag via Teflon tubing (Fig. 1). Temperature and humidity inside the bags were monitored and found to be similar to the outside because of the continuous air flow (data not shown). The receiver branch of each of the four saplings was then randomly subjected to one of the following treatments: 1) exposure to herbivore-induced VOCs from the adjacent infested emitter branch (iVOC); 2) exposure to iVOC disturbed (iVOC-dis), 3) exposure to constitutive VOCs from the adjacent non-infested emitter branch (cVOC), and 4) exposure to clean air (control). In treatments iVOC and cVOC, the air leaving the emitter branch was directed to the receiver branch through Teflon tubing while air was channeled away in the control and iVOC-dis treatments. To induce emitter branches in treatments iVOC and iVOC-dis, we added 20 adults into the bag instead of using larvae, due to their shortage. VOC exposure lasted two days, during which VOC collections were made at day 1 and 2 after the start of the exposure. Following VOC exposure, we disconnected the Teflon tubes between receiver and emitter branches, elicited receiver branches by placing 20 adults in the bags, and collected VOCs at 24 and 48 hr after herbivore addition to investigate the possible priming effects. Herbivorous adults were removed before the first VOC collection and not put back afterwards. Throughout the experiment, both emitter and receiver branches remained inside the bags and continuous airflow was maintained. The experiment was repeated four times.

VOC Collection and Analysis VOCs were collected using a previously described dynamic headspace sampling system that consists of battery-operated inlet and outlet pumps and

air filters (Blande et al. 2007). In brief, polyethylene terephthalate (PET) bags (25 × 55 cm; Look, Terinex Ltd., Bedford, UK) were used to enclose the foliage and were fastened to the stem with wire tags. At one corner of each bag, pressurized air purified over a charcoal filter was pushed through Teflon tubing into the bag at a flow rate of 230 ml min^{-1}. A stainless steel tube filled with 150 mg of Tenax TA and 150 mg of Carbopack B (Markes International, Llantrisant, RCT, UK) was inserted at the second corner of the bag, and headspace was pulled out at 200 ml min^{-1} through the tube using a vacuum pump [Model (N022AN.18), KNF Neuberger, Freiburg, Germany]. In all experiments, VOCs were collected from the outer 13 leaves for 30 min, and plants from different treatments were sampled concurrently. For field and greenhouse studies, temperature and humidity inside the bags were measured during collection using temperature/humidity data loggers (DS1923, iButton Hygrochron, Maxim Integrated, San Jose, CA, USA), and the photosynthetically active radiation (PAR) was recorded with a PAR sensor that was enclosed in a PET bag and positioned close to the plants being sampled.

VOC samples were analyzed by GC-MS (Agilent 7890A GC and 5975C VL MSD; New York, USA). Trapped compounds were thermally desorbed (TD100; Markes International, Llantrisant, RCT, UK) at 250 °C for 10 min, cryofocused at −30 °C and injected onto an HP-5 capillary column (50 m × 0.2 mm; film thickness 0.5 μm) with helium as a carrier gas. The column temperature was held at 40 °C for 1 min, then ramped at 5 °C min^{-1} to 210 °C, and ramped again at 20 °C min^{-1} to 250 °C. Individual VOCs were tentatively identified by comparing mass spectra with those in Wiley and NIST spectral libraries, verified by chromatography with authentic standards where available, and quantified based on characteristic quantifier ions as well as external calibration curves generated with authentic standards. No standards are available for one monoterpene [(*E*)-β-ocimene], seven sesquiterpenes [α-cubebene, β-bourbonene, (*E*)-α-bergamotene, germacrene D, (*E,E*)-α-farnesene and two unknown compounds], and four benzenoids (benzaldehyde, benzeneethanol, benzeneacetonitrile, and 1H–indole), so quantification of these compounds was assessed relative to (*Z*)-ocimene, (*E*)-β-farnesene and methyl salicylate, respectively. Emission rates of individual compounds are expressed in ng $g(DW)^{-1}$ h^{-1}.

Statistical Analysis Data from the field study were analyzed with two-way ANOVA. Treatment was included as the fixed factor and plot as a random factor. In cases of no significant interaction found between treatment and plot, the interaction term was excluded from the model. Wherever the assumption of normality and homoscedasticity was violated, log(x + 1)-transformed data were analyzed. To account for the potential effects of temperature and PAR on terpenoid emissions, normalized emission rates were also analyzed. The emission rates of isoprene were adjusted to standard conditions of temperature and PAR, with values of 30 °C and 1000 μmol m^{-2} s^{-1}, respectively. Likewise, the emission rates of monoterpenes, sesquiterpenes and homoterpenes were adjusted to a standard temperature of 30 °C, but not to standard PAR due to relatively little available information on the correlation of these compounds with PAR. Algorithms and formulae developed by Guenther et al. (1993) were used for these corrections. For the laboratory study, data were analyzed with Wilcoxon signed rank tests. For the greenhouse study, the experiment was repeated on different days, causing high variation in the emission rates among replicates. Therefore, data were presented as percentage change relative to the control and analyzed with one-sample t test. Percent change was conservatively set to 200, 50 or 100% when compounds were detected only in treated samples, in control samples, or not detected in both control and treated samples, respectively. To further visualize and characterize differences in the VOC blends of differently treated plants, the VOC profiles for each plant were subjected to a Partial Least Squares-Discriminant Analysis (PLS-DA) (SIMCAP 13.0; Umetrics, Umeå, Sweden). To avoid compounds that were rarely present in samples disproportionably affecting the outcome, only those compounds that were found at least five (field experiment) or three (laboratory and greenhouse experiments) times in a treatment group were retained for the PLS-DA analyses.

Results

Vascular Connectivity The appearance of the red dye varied heavily depending on leaf position. When Rhodamine-B dye was fed via two middle leaves of a branch, it moved rapidly to the outer leaves on the same branch in the first 4 hr after dye application and accumulated in almost all these leaves after 1 day (Fig. 2). By comparison, it moved inward less rapidly along the branch, and after 1 day a variable amount of staining occurred on approximately 38% of leaves in from the point of dye injection, which increased to 45% after 6 days. Weak staining was observed on the inner leaves of the branch located immediately above the branch containing the dye, which only occurred in two out of the 13 studied plants, and no dye loading was observed on branches below the dye-containing branch. These observations point to a high degree of vascular connectivity between leaves within a branch, and little or no connectivity between leaves from different branches.

Herbivore-Induced VOCs Induce Systemic VOC Emission in Field Conditions Exposure to *Phratora laticollis*-induced VOCs (iVOC) triggered systemic induction of VOCs under open field conditions. A PLS-DA analysis of the VOC blends emitted by receiver branches at approximately 5.5 days post exposure (dpe) generated a model with two

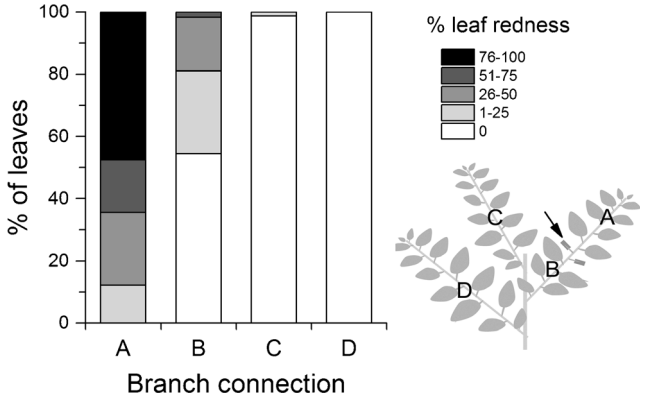


Fig. 2 Vascular connectivity among leaves within a branch, and from different branches within hybrid aspen plants. (*Insert*) Schematic representation of experimental design. Rhodamine-B dye was fed via the petioles of two middle leaves on a branch (arrow), then the amount of red staining was visually assessed daily over 6 days for leaves from each of the following four locations of the plants (n = 13): leaves above (**a**) and below (**b**) the dye-fed leaves on the dye-fed branch, and leaves on a branch directly above (**c**) and below (**d**) the dye-fed branch. After one week, percent of leaves with different amounts of red staining was determined

significant principal components (R^2X = 0.676, R^2Y = 0.151, Q^2 = 0.056 and R^2X = 0.097, R^2Y = 0.225, Q^2 = 0.076), which distinguished the receiver branches exposed to iVOC from those exposed to constitutive VOCs (cVOC), although with a certain degree of overlap (Fig. 3a). VOCs that were most influential in the separation of iVOC- and cVOC-exposed receiver branches were the hemiterpene isoprene and four monoterpenes (*E*)-β-ocimene, α-pinene, (*Z*)-ocimene and β-pinene. The ANOVA analysis further revealed that they were all emitted in significantly greater amounts by receiver branches exposed to iVOC [isoprene: $F_{(1,11)}$ = 14.39, P = 0.003; (*E*)-β-ocimene: $F_{(1,11)}$ = 13.79, P = 0.003; (*Z*)-ocimene: $F_{(1,11)}$ = 10.20, P = 0.009; α-pinene: $F_{(1,11)}$ = 15.21, P = 0.002; β-pinene: $F_{(1,11)}$ = 6.35, P = 0.028] (Fig. 3b; Table S1). The total VOC emissions were also significantly higher in iVOC-exposed branches compared to cVOC-exposed branches ($F_{(1,11)}$ = 17.87, P = 0.001). The results remained the same even when temperature/PAR-standardised emissions were analyzed (Table S1).

To assess the potential priming effects of iVOC exposure, receiver branches were subsequently subjected to continuous feeding by *P. laticollis* larvae for about 20 hr. This feeding period caused statistically similar levels of damage to plants of each treatment (Table 1), and induced VOC emissions (Fig. 3b; Table S1) in iVOC- and cVOC-exposed receiver branches. Compared to cVOC-exposed branches, however, iVOC-exposed ones did not display augmented VOC emissions at either 24 or 48 hr post infestation (hpi) (Fig. 3b; Table S1). This was confirmed by the PLS-DA analyses, which did not show any significant components that could separate cVOC- and iVOC-exposed receiver branches (Fig. S1).

Herbivore-Induced VOCs Induce Systemic VOC Emission in Laboratory Conditions As in the field, iVOC-induced systemic VOC emissions were observed in the laboratory. The PLS-DA analysis of the VOC profiles emitted from each receiver branch at 3 dpe resulted in a model with two significant principal components (R^2X = 0.372, R^2Y = 0.549, Q^2 = 0.195 and R^2X = 0.231, R^2Y = 0.273, Q^2 = 0.209; Fig. 4a), which clearly separated cVOC-exposed receiver branches from iVOC-exposed receiver branches. The compounds responsible for such separation were sabinene, (*E*)-β-ocimene, (*E*)-β-caryophyllene, α-humulene, germacrene D, (*E*,*E*)-α-farnesene, (*Z*)-3-hexenyl acetate, (*E*)-DMNT, methyl salicylate and 1H–indole. Among them, sabinene (Wilcoxon test: Z = −1.75, P = 0.08), (*E*)-β-ocimene (Z = −2.02, P = 0.043), (*E*)-β-caryophyllene (Z = −2.02, P = 0.043), α-humulene (Z = −2.02, P = 0.043), germacrene D (Z = −1.83, P = 0.068) and (*Z*)-3-hexenyl acetate (Z = −2.02, P = 0.043) were emitted in significantly or marginally significantly higher quantities by iVOC-exposed branches than cVOC-exposed branches, whilst 1H–indole was emitted in the opposite manner (Z = −1.82, P = 0.068) (Fig. 4b; Table S2).

Since the field experiment did not show a priming effect on induced VOC emissions at the two measurement time points (i.e. 24 hr and 48 hr) after herbivore challenge, in the laboratory experiment we monitored induced VOC emissions earlier and more frequently at shorter intervals after herbivore addition. Again, we found similar levels of feeding damage on iVOC and cVOC-exposed branches (Table 1), and detected no primed responses of VOCs as early as at 6 hpi or at other time points (Fig. 4b). At 6 hpi VOC-exposed branches were clearly separated from cVOC-exposed branches on the PLS-DA scores plot (Fig. S2), with the former releasing greater quantities of (*E*)-β-ocimene (Z = −1.75, P = 0.08), (*Z*)-ocimene (Z = −1.75, P = 0.08) and (*E*)-β-caryophyllene (Z = −2.02, P = 0.043). These results were similar to those observed at 3 dpe, and were presumably due to a carryover effect of iVOC exposure rather than a priming effect in that herbivore challenge following iVOC exposure did not lead to a further increase in VOC emissions. While the PLS-DA analysis detected one significant component when comparing iVOC- and cVOC exposed branches at both 35 and 43 hpi (Fig. S2), the emission rates of the compounds underlying this significant component were not statistically different between iVOC- and cVOC-exposed branches (Table S2).

Herbivore-Induced VOCs Induce Systemic VOC Emission in Greenhouse Conditions To further elucidate whether volatile cues are required for systemic induction of VOCs observed under both field and laboratory conditions, both the emitter and receiver branches were bagged and air flow in between was manipulated in the greenhouse. The PLS-

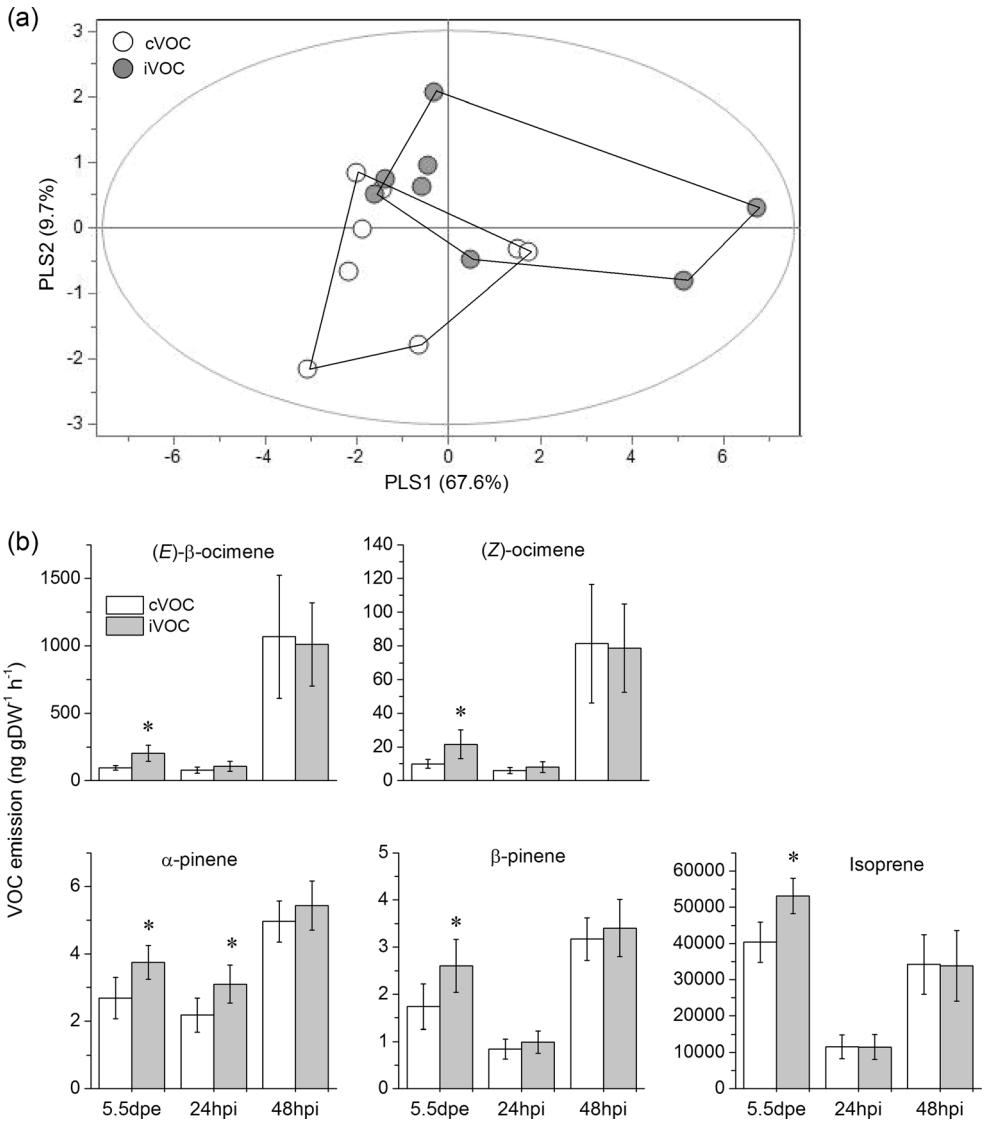


Fig. 3 Induction and priming of hybrid aspen VOCs by herbivore-induced VOCs in the field. Branches were exposed for 5.5 days to constitutive or herbivore-induced VOCs (cVOC and iVOC, respectively) emitted from adjacent branches within a plant, after which they were subjected to herbivore infestation for approximately 20 hr. VOCs were collected at 5.5 days post exposure (dpe) and at 24 and 48 hr post infestation (hpi). **a** Partial Least Squares-Discriminant Analysis (PLS-DA) scores plot of the VOC profiles of cVOC- and iVOC-exposed branches at 5.5 dpe. **b** Individual VOCs that were induced by iVOC exposure. Asterisks indicate significant differences determined by ANOVA (mean ± se; $n = 8$). VOC emissions are reported here as actual (non-normalized) emission rates, and temperature/PAR-normalized VOC emissions are presented in Table S1 showing similar results

Table 1 Leaf area consumed by *Phratora laticollis* on receive branches

	Feeding period (h)	Treatment	Leaf area consumed	
			cm^2	%
Field	20	cVOC	3.78 ± 0.50	0.80 ± 0.12
		iVOC	4.63 ± 0.57	1.05 ± 0.13
Laboratory	5	cVOC	4.87 ± 0.68	2.37 ± 0.28
		iVOC	4.13 ± 0.56	2.11 ± 0.49
	20	cVOC	18.35 ± 1.06	9.06 ± 0.85
		iVOC	16.69 ± 1.47	8.45 ± 1.59
Greenhouse	20	Control	9.49 ± 1.35	4.02 ± 0.49
		cVOC	8.41 ± 0.80	3.49 ± 0.20
		iVOC	8.51 ± 1.42	3.32 ± 0.53
		iVOC-dis	7.63 ± 0.98	3.33 ± 0.44

Data are mean (± SE) of 4–8 samples. There were no statistically significant differences in damage levels among treatments within either experiment

DA analyses showed that at 1 dpe, there was no clear distinction between treatments (Fig. S3), although both iVOC- and cVOC-exposed receiver branches released significantly higher amounts of (*E*,*E*)-α-farnesene than control branches exposed to clean air (one-sample t test: $t = 3.2$, $P = 0.049$ for iVOC; $t = 3.48$, $P = 0.04$ for cVOC; Fig. 5; Table S3). At 2 dpe, there was a significant separation of iVOC-exposed receiver branches from both control branches and cVOC-exposed branches ($R^2X = 0.343$, $R^2Y = 0.213$, $Q^2 = 0.132$), which were not significantly separated from each other (Fig. 5b). The compounds that contributed most to this discrimination were (*E*)-β-caryophyllene, α-humulene, germacrene D, δ-cadinene and benzeneacetonitrile. One-sample t tests showed that compared to control branches, iVOC-exposed branches released markedly higher amounts of (*E*)-β-caryophyllene ($t = 4.29$, $P = 0.023$), α-humulene ($t = 9.73$, $P = 0.002$), (*E*)-α-bergamotene ($t = 4.38$, $P = 0.022$), (*E*,*E*)-α-farnesene

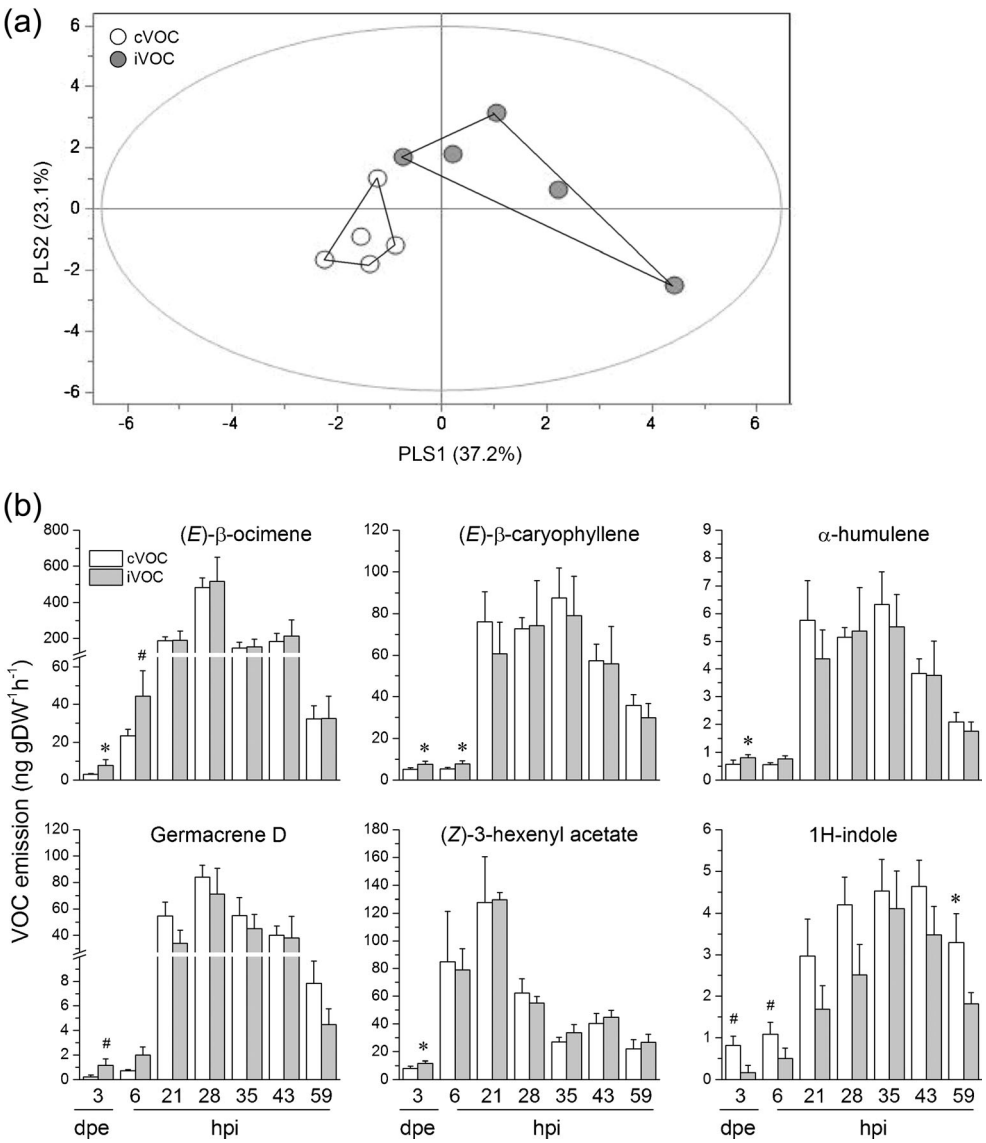


Fig. 4 Induction and priming of hybrid aspen VOCs by herbivore-induced VOCs in the laboratory. Branches were exposed for 3 days to constitutive or herbivore-induced VOCs (cVOC and iVOC, respectively) emitted from adjacent branches within a plant, after which they were infested by herbivores for approximately 20 hr. VOCs were collected at 3 days post exposure (dpe) and at different hours post infestation (hpi). **a** Partial Least Squares-Discriminant Analysis (PLS-DA) scores plot of the VOC profiles of cVOC- and iVOC-exposed branches at 3 dpe. **b** Individual VOCs that were induced by iVOC exposure. Data (mean + se; $n = 5$) at each time point were analyzed with Wilcoxon signed rank tests (# $P < 0.1$; * $P < 0.05$). For other compounds, see Table S2

($t = 4.09$, $P = 0.026$), germacrene D ($t = 2.67$, $P = 0.075$) and δ-cadinene ($t = 3.0$, $P = 0.058$) (Fig. 5c). However, when VOC exposure was interrupted, the levels of direct VOC induction significantly decreased, as evidenced by the finding that only (*E*)-β-caryophyllene ($t = 2.53$, $P = 0.085$) and α-humulene ($t = 3.23$, $P = 0.048$) exhibited a weak induction. These results indicate that effective systemic induction of VOCs depends on VOC exposure.

Again, no systemic priming of VOC emissions was observed at either 24 or 48 hpi. Regardless of previous VOC exposure, all receiver branches had similar levels of leaf damage (Table 1) and emitted comparable amounts of VOCs (Fig. 5c; Table S3). While iVOC-exposed receiver branches appeared to emit slightly, but significantly, lower amounts of benzeneacetonitrile ($t = -3.9$, $P = 0.030$), their VOC blends were not distinguished from the VOC blends of any other treatments (Fig. S3).

Discussion

Overall, we observed under both field and environment controlled conditions that local feeding caused systemic induction of VOC emission in branches that are lacking vascular connection to damaged branches. Through manipulating air contact between damaged and undamaged branches, we further showed that the systemic VOC induction was mainly caused by volatile signals from damaged branches, while internal signals contributed little if anything. Within-plant VOC signaling has been thought to be crucial for coordinating systemic responses as they can overcome restrictions in vascular signaling. Within-plant signaling has so far been demonstrated in seven plant species (e.g., Erb et al. 2015; Frost et al. 2007), and our study adds hybrid aspen to the list.

It has been shown that many VOCs can adsorb onto waxy plant surfaces and be released back to the atmosphere

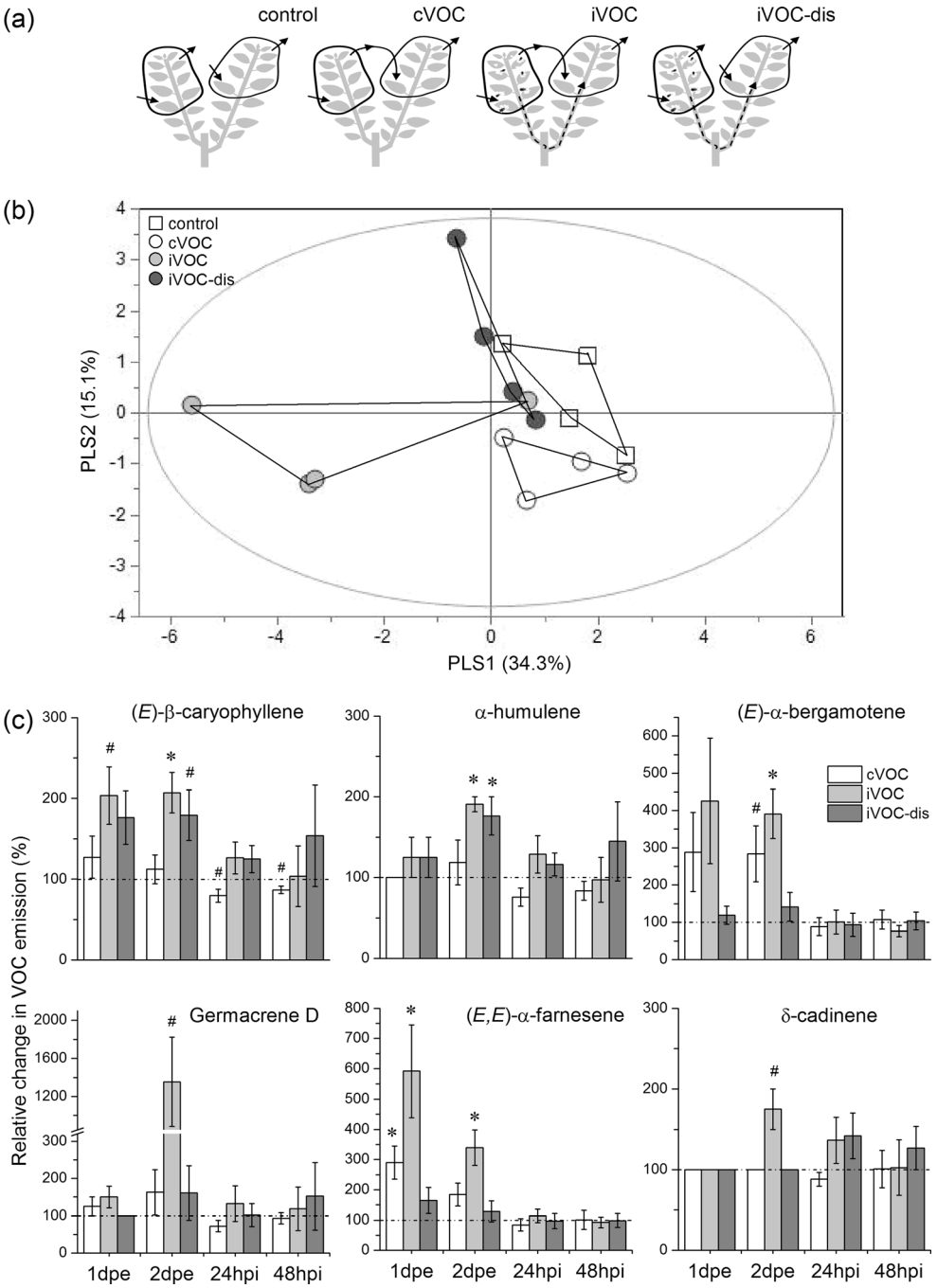


Fig. 5 Induction and priming of hybrid aspen VOCs by herbivore-induced VOCs in the greenhouse. **a** Schematic diagram of the experimental setting. Solid arrows stand for the direction of flow of the airstream carrying VOC signals, and dashed arrows indicate possible involvement of internal signals. Branches were either exposed to clean air (control), constitutive VOCs (cVOC) or herbivore-induced VOCs (iVOC), or exposure to iVOC was disturbed (iVOC-dis). The exposure lasted 2 days, after which receiver branches were infested by herbivores for approximately 20 hr. VOCs were collected at different time points throughout the experiment. **b** Partial Least Squares-Discriminant Analysis (PLS-DA) scores plot of the VOC profiles of differently treated receiver branches at 2 dpe. **c** Percentage change (mean ± se; $n = 4$) in VOC emission relative to the control (dashed line, set to 100%). Data were analyzed with one-sample t tests (# $P < 0.1$; * $P < 0.05$). For actual emission rates see Table S3

(eg., Li and Blande 2015). Systemic VOC induction observed here is most likely not due to adsorption/re-release of VOCs from damaged branches, though our experimental design cannot fully rule out this possibility. This is because undamaged branches of damaged plants did not consistently emit greater amounts of the dominant constituents of herbivore-induced VOC blends compared to undamaged branches of control plants. Under field conditions, for instance, sesquiterpenes, which have lower volatility than many of the other plant volatiles identified, and should adsorb better to plant surfaces (Schaub et al. 2010), were not released at much higher amounts from branches exposed to herbivore-induced VOCs.

While we found clear evidence of within-plant signaling under field, greenhouse and laboratory conditions, we also observed some clear disparities under these conditions with regard to which compounds and to what extent they were responsive to volatile signals. In the field, VOC induction was found exclusively for several monoterpenes and the hemiterpene isoprene – which originate from the plastidic methylerythritol phosphate (MEP) pathway (Dudareva et al. 2013), whereas under environment controlled laboratory and

greenhouse conditions, particularly in the greenhouse, the induced VOC blends were dominated by sesquiterpenes – which stem primarily from the cytosolic mevalonic acid (MVA) pathway (Dudareva et al. 2013). Furthermore, the greenhouse yielded the strongest induction, followed by the laboratory and then the field. For example, germacrene D, the most responsive compound in both the greenhouse and laboratory conditions, exhibited a 13.5 and 5-fold change, respectively, as opposed to a 2.2-fold change for the most responsive compound (*E*)-β-ocimene in the field. These discrepancies are most likely to be explained by the difference in the exposure regime among these experiments. In the greenhouse, exposure presumably had the highest levels of VOCs concentrated in the small open flow-through exposure system, compared to the intermediate levels in the large well-ventilated enclosures in the laboratory and the lowest levels in the field where exposure hinges on prevailing winds. Experimental variation in the dose and duration of VOC exposure may result in selective induction, and in some cases even suppression, of different volatile compounds as well as affecting the strength of such plant responses (Erb et al. 2015; Farag and Paré 2002; Girón-Calva et al. 2012; Shiojiri et al. 2012). Furthermore, in contrast to a single herbivore stress under laboratory and greenhouse conditions, plants growing in the field face multiple abiotic and biotic stresses. These factors could potentially affect the dynamics of VOC emission by damaged tissues and the perception of these signals by the receiver. Nevertheless, previous studies on volatile-mediated multitrophic interactions, including between-plant communication have found weaker responses in the field than in the laboratory and greenhouse (Karban et al. 2014).

Unexpectedly, we did not observe systemic priming of VOC emissions under all experimental conditions studied. This disagrees with earlier studies, including studies with *Populus*, which have shown that herbivore-induced VOC blends prime VOC emissions in either systemic organs of the same plant (Erb et al. 2015; Frost et al. 2007; Girón-Calva et al. 2014) or in other neighboring plants (Engelberth et al. 2004; Li et al. 2012; Li and Blande 2015; Muroi et al. 2011; Ton et al. 2007). This inconsistency most likely reflects the fact that defense induction and priming is dose- and time-dependent. Studies on plant disease resistance have convincingly demonstrated that pretreatment with pathogens or disease-resistance-inducing chemicals at high doses directly activate defense expression, whilst pretreatment at low doses mainly primes for augmented defense responses upon subsequent challenge by pathogens or defense elicitors (Katz et al. 1998; Kohler et al. 2002; van Hulten et al. 2006). Some of these studies have further shown that the doses of elicitors applied upon pretreatment can also influence the priming effects, with more apparent priming effects appearing at low doses that alone cause only faint defense responses compared to high doses that greatly induce defense responses (Katz et al. 1998). These principles may also hold true for VOC-mediated defense induction and priming, as evidenced by two recent studies on VOC-mediated signaling (Girón-Calva et al. 2012; Shiojiri et al. 2012), though there remains little information on the amount of VOCs or the duration of VOC exposure that is required for a recipient plant organ to trigger a defense response. In *Arabidopsis*, for instance, intermittent exposure for three weeks to trace amounts of green leaf volatiles has been shown to prime receiver plants for enhanced attraction of parasitoid wasps, but not after only two exposure events in a single week (Shiojiri et al. 2012).

Perhaps, priming may have been missed due to our experimental approach. In the present study, exposure lasted two to five days before subsequent herbivore challenge, compared to most previous studies that used an exposure duration ranging from less than one day to a maximum of three days (e.g., Erb et al. 2015). Such prolonged exposure may have directly induced, rather than primed, VOC emission, as in our case. In addition, we subjected branches to continuous feeding for 6 to 22 hr before onset of VOC monitoring. While the feeding period and the damage inflicted over that period fall within the range observed in previous studies of VOC-mediated priming (Frost et al. 2007, 2008; Li et al. 2012), they may have greatly induced VOC emissions, thus overwhelming VOC-mediated priming. To better capture snapshots of VOC-mediated priming, more extensive time-course measurements are required. Adoption of PTR-TOF-MS (Proton-Transfer-Reaction Time-of-Flight Mass Spectrometry), which has high time resolution and allows high throughput measurements of VOC emission in real time (e.g., Brilli et al. 2011), would enhance the ability to detect priming of VOC emission and to assess its dose/time-dependent nature, and is certainly worth pursuing in future research.

Nonetheless, our finding that volatile signals are strictly required for an effective systemic response among branches of hybrid aspen that have very limited vascular connection agree with early studies documenting the importance of volatile signals in both within- and between-plant signaling in *Populus* species (Baldwin and Schultz 1983; Frost et al. 2007; Li et al. 2012). Since our study used a specialist herbivore while others employed either generalist herbivores or mechanical wounding, some commonly emitted volatile compounds in the induced VOC blends may dictate the content of the messages in volatile signaling of *Populus*. Although the emission of most VOCs vary with plant and herbivore species (Ali and Agrawal 2012), green leaf volatiles (GLVs), which are widespread in the plant kingdom and whose emission is not specific to herbivore induction, are likely to be the actual messengers in this context. Indeed, numerous studies have demonstrated the capacity of GLVs to induce and/or prime plant defense responses in various plant species (e.g., Engelberth et al. 2004; Frost et al. 2008; Kost and Heil 2006). In hybrid poplar, for example, (*Z*)-3-hexenyl acetate, the most

dominant GLV found in the present study, has been reported to prime terpenoid emissions (Frost et al. 2008). However, other candidate volatiles may also be implicated in within- and between-plant signaling in *Populus*, including terpenoids such as (*E*)-DMNT [(3*E*)-4,8-diemthyl-1,3,7-nonatriene], (*E*)-β-ocimene, (*E,E*)-α-farnesene, and (*E*)-β-caryophyllene, as well as benzenoids such as methyl salicylate and 1H–indole. These compounds have been shown in many studies including the present study to be greatly induced following mechanical wounding or herbivore attack, and have been shown to trigger defense responses in other plant species (e.g., Erb et al. 2015; Godard et al. 2008).

Apart from airborne volatile signals, our study indicates that some internal signals may have also contributed to the observed systemic responses. This is supported by the greenhouse observation that two days after local damage, systemic undamaged branches released significantly higher levels of (*E*)-β-caryophyllene and α-humulene than control plants even when air contact between damaged and undamaged branches was blocked, albeit to a lesser extent compared to the situation where air contact was allowed. Volatile signals have been suggested to prime for enhanced defense expression upon perception of internal signals (Heil and Ton 2008; Heil 2010).

While functional elucidation of VOC emissions by systemic branches via volatile signaling is beyond the scope of the present investigation, it is likely that induced VOC emissions would strengthen direct and/or indirect defences by repelling herbivores and/or attracting their natural enemies. A few studies have shown that volatiles play a crucial role in structuring plant-insect interactions in *Populus* (Clavijo McCormick et al. 2014; Havill and Raffa 2000; Kendrick and Raffa 2006). For example, herbivore-induced VOCs emitted from gypsy moth larvae infested poplar leaves (*P. nigra*) were found to attract *Glyptapanteles flavicoxis*, a gregarious parasitoid of gypsy moth larvae (Havill and Raffa 2000). However, the potential effects on herbivore behaviors need to be assessed as we recently observed that *Phratora laticollis*, as with other specialists (Kendrick and Raffa 2006), utilize plant volatiles in search for host plants (Li and Blande, unpublished data). In addition, induced VOC emissions may serve other biological functions which have not been considered before in the context of within- and between-plant signaling. It is known that terpenoids can function as antioxidants (Loreto and Schnitzler 2010) and that both biotic and abiotic stresses cause oxidative stress (Kerchev et al. 2012). The induced release of isoprene and several monoterpenes observed in our field study may protect plants from oxidative stress.

In summary, we demonstrate under both field and environment controlled conditions that systemic responses can occur among branches lacking vascular connections and that volatiles are essential in mediating this process. Our findings along with previous work on hybrid poplar (Baldwin and Schultz 1983; Frost et al. 2007) and hybrid aspen (Li et al. 2012), provide strong evidence that within- and between-plant signaling via herbivore-induced volatiles may be a common and ecologically important phenomenon in *Populus*. Future studies are needed to identify active components in the induced VOC blends responsible for volatile signaling in *Populus* and to elucidate the ecological significance.

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