Within-plant signalling via volatiles overcomes vascular constraints on systemic signalling and primes responses against herbivores

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Abstract
Plant volatiles play important roles in signalling between plants and insects, but their role in communication among plants remains controversial. Previous research on plant–plant communication has focused on interactions between neighbouring plants, largely overlooking the possibility that volatiles function as signals within plants. Here, we show that volatiles released by herbivore-wounded leaves of hybrid poplar (Populus deltoides × nigra) prime defences in adjacent leaves with little or no vascular connection to the wounded leaves. Undamaged leaves exposed to volatiles from wounded leaves on the same stem had elevated defensive responses to feeding by gypsy moth larvae (Lymantria dispar L.) compared with leaves that did not receive volatiles. Volatile signals may facilitate systemic responses to localized herbivory even when the transmission of internal signals is constrained by vascular connectivity. Self-signalling via volatiles is consistent with the short distances over which plant response to airborne cues has been observed to occur and has apparent benefits for emitting plants, suggesting that within-plant signalling may have equal or greater ecological significance than signalling between plants.

Keywords
Lymantria dispar, plant communication, plant volatiles, Populus, priming, terpenes.


INTRODUCTION
Plant volatiles are known to mediate a diverse array of interactions between plants and insects (e.g. Turlings et al. 1990; De Moraes et al. 1998; Thaler 1999; De Moraes et al. 2001; Hoballah & Turlings 2001). The role of volatiles in communication between plants is less clear and remains one of the most fascinating and controversial topics in modern ecology (Agrawal 2000; Baldwin et al. 2006; Dudareva et al. 2006). While some parasitic plants have been shown to exploit volatiles from neighbouring plants for host location (Runyon et al. 2006), the class of plant–plant interactions where volatile cues have received the most attention is in signalling the presence of herbivores. Early reports that damage-induced airborne volatiles mediate plant defensive responses in neighbouring plants (e.g. Baldwin & Schultz 1983; Rhoades 1982) sparked debate about possible interpretations of the results obtained and about the ecological significance of volatile-mediated between-plant signalling (e.g. Fowler & Lawton 1985; Shonle & Bergelson 1995; Karban & Baldwin 1997; Baldwin et al. 2002). This controversy stimulated additional research to gain further supporting evidence from field and laboratory experiments (Farmer & Ryan 1990; Bruin et al. 1992; Agrawal 2000; Bruin & Dicke 2001; Karban et al. 2003). While the hypothesis that plant volatiles can transmit information between plants is appealing, serious questions remain about whether between-plant volatile communication can operate over sufficient distances to have ecological significance in most cases.

For an herbivore-induced plant volatile (HIPV) to act as a signal, it must be produced and released by an ‘emitter’, transmitted from the emitter to a ‘receiver’, then detected by the receiver (Baldwin et al. 2006). There is now overwhelming evidence that plants produce HIPVs (De Moraes et al. 1998, 2001; Kessler & Baldwin 2001; Janssen et al. 2002; Fatouros et al. 2005), and enhanced defensive phenotypes in plants exposed to HIPVs demonstrate that volatiles can be
detected (Arimura et al. 2000; Engelberth et al. 2004). However, studies in nature suggest that receiver plants located beyond c. 20 cm of wounded emitter plants show limited responsiveness (Karban et al. 2003). This short transmission distance is the most significant challenge to the suggestion that HIPVs play an ecologically important role in mediating defence reactions in neighbouring plants. One possibility that has been largely overlooked is that HIPVs may serve as wound signals within individual plants, rather than between neighbours. The short distances over which HIPVs have been shown to induce responses are consistent with the spatial separation among leaves on a stem or closely positioned branches within a plant.

Systemic wound signalling within plants follows assimilate transport and occurs via vascular connections (Davis et al. 1991; Zhang & Baldwin 1997; Stratmann 2003). However, wound signals transported via vascular traces do not reach systemic regions uniformly: orthostichous (connected) regions receive vascular wound signals from herbivore-damaged leaves, while non-orthostichous (unconnected) regions do not (Watson & Casper 1984; van Dam et al. 2001). Isolated vascular traces constrain systemic responses to herbivores in space and time, which causes variability in the systemic induction of foliar defences (Davis et al. 1991; Shulaev et al. 1995; Rhodes et al. 1996, 1999; Orians et al. 2000; Arnold & Schultz 2002; Schittko & Baldwin 2003) even among leaves of basal rosettes (Kiefer & Slusarenko 2003). Systemic regions orthostichous to sites of herbivore damage can therefore be better defended against herbivores compared with non-orthostichous regions (Jones et al. 1993; Viswanathan & Thaler 2004; Anderson & Agrell 2005). The ecological importance of systemic defence signalling suggests that mechanisms may exist to overcome vascular constraints. Combining the distance limitations observed for airborne HIPV transport with vascular constraints on systemic induction suggests the hypothesis that HIPVs act as wound signals to non-orthostichous systemic leaves within a plant (Farmer 2001; Orians 2005).

This hypothesis has some support from mechanical wounding experiments with sagebrush (Karban et al. 2006), but has not been explored with herbivores. In this paper, we describe two experiments conducted using hybrid poplar saplings (Populus deltoides × nigra) and gypsy moth larvae (Lymantria dispar L.). By using volatile collection chambers that enclose individual leaves, we were able to isolate poplar leaves positioned close to one another (within 10 cm) and to manipulate air connections between them. In one experiment, we established dilute air contact between ‘treatment’ leaves on which L. dispar larvae were allowed to feed and undamaged non-orthostichous systemic ‘target’ leaves, and then measured the response of the target leaves to later herbivore damage. In a second experiment, we tested for systemic wound signalling via vasculature to confirm that non-orthostichous leaves are less responsive to herbivores than are orthostichous leaves following initial localized herbivore damage.

Our results show that leaves of individual poplar saplings, separated by only centimetres, can transmit and receive wound information via airborne volatiles that prepare (‘prime’) the recipient leaves for herbivore attack. This demonstrates that HIPVs can function in systemic anti-herbivore defence by self-priming leaves adjacent to damaged leaves that receive little or no vascular wound signals. Our results offer a novel perspective on the ecological function of HIPVs as a mechanism for within-plant defence signalling via priming of systemic defences.

**MATERIALS AND METHODS**

**Experimental system**

All experiments were conducted between April and September 2006 in a walk-in growth chamber maintained at 25 °C with a 16 : 8 h (L : D) photoperiod. Poplar saplings (Populus deltoides × nigra, clone ‘OGY’) were grown from cuttings in an adjacent, herbivore-free growth chamber with identical environmental conditions. Cuttings were obtained from a clonal group of trees maintained in our laboratory greenhouse specifically for that purpose. They were planted in 5-gallon pots in commercial potting soil (MetroMix 250; SunGro, Bellevue, WA, USA) and watered as necessary. Based on supplements in the soil, fertilization was not required and poplars grew to experimental heights of c. 1.5 m in 12–15 weeks. Two to five saplings were assayed at once because of space limitations in the growth chamber.

Lymantria dispar third or fourth instar larvae were used for all experiments. Egg masses were obtained from APHIS (Otis Plant Protection Lab, Cape Cod, MA, USA) and reared to third instar on artificial diet. Before experiments, larvae were transferred to fresh poplar leaves for 24 h and then starved for 24 h before being applied to the experimental leaves.

We designed and built Teflon/glass chambers that allowed us to isolate individual leaves, measure their volatile production and, most importantly, control the chemical composition of the air for each leaf (Fig. S1). These chambers measured 15.2 × 13.3 cm with a volume of c. 260 cm³ and had two side ports for connecting push and pull airflow systems.

**Leaf selection**

We chose fully expanded source leaves to avoid confounding source–sink relationships that occur with developing...
Experimental manipulations

The general protocol for both experiments was to first provide an initial herbivore treatment to specific leaves followed by a secondary herbivore treatment to experimental target leaves. After a 24-h pre-treatment collection period (Table 1, day 1), the initial herbivore treatment for both experiments consisted of three third instar *L. dispar* larvae added to each LPI$_{n+5}$ leaf and allowed to feed for 24 h (Table 1, day 2). This feeding period generated statistically similar levels of damage across treatments (Table 2). We then used a different set of larvae to elicit local responses to the target leaves (LPI$_{n}$ or LPI$_{n+1}$); this secondary treatment occurred from a single fourth instar *L. dispar* larva on each leaf. These larvae were allowed to feed for 48 h (Table 1, days 4–5), producing average damage levels that were also not significantly different in area or mass consumed within or among treatments (Table 2).

In experiment 1, we investigated volatile self-signalling by connecting Teflon air tubes between LPI$_{n+5}$ treatment leaves challenged by *L. dispar* and non-orthostichous LPI$_{n+1}$ target leaves (Fig. 1a). We randomly determined which saplings would either receive the air tube treatment or remain as a control. As each trial was conducted with at least two saplings, at least one control and one experimental sapling were assayed for each trial. Trials were repeated until ten and nine replicates were collected of the experimental and control plants, respectively.

In experiment 2, no air contact occurred among the three experimental leaves. The only air source for each leaf was the charcoal-filtered air supplied to the chamber, and positive pressure prevented any outside air from entering the chambers. As a result, any differences in responses between the LPI$_{n}$ and LPI$_{n+1}$ leaves are presumably the result of vascular signal transduction from the wounded LPI$_{n+5}$ leaves. The experiment was repeated until 10 independent replicates (i.e. 10 poplar saplings) were assayed.

Volatile collection, measurement, and analysis

To address our hypothesis, we looked for evidence of treatment-based differential induction over time. The most accessible, non-destructive metric of induction was the quantification of terpene volatiles from the target leaves during the course of the experiments. Volatile emissions were our sole metrics for induction. Terpene volatile emissions correlate directly with terpene synthase activity and terpene accumulation in leaf tissue (Kollner et al. 2004) and the expression of terpene synthases in response to insect herbivores correlates with the expression of polyphenol oxidase and proteinase inhibitors, both of which are direct defences in the arsenal employed by poplar (Arimura et al. 2004; Ralph et al. 2006). Terpene volatiles are therefore good proxies for overall induction responses over time. Although poplars emit an array of terpene and green-leaf volatiles (GLVs) following herbivore damage, we focused on the five most abundant terpene volatiles produced in...
these experiments and on total overall volatile production (Fig. 2).

For all chambers, charcoal-filtered clean air was added at the rate of 2.5 L min$^{-1}$ through Teflon tubing, and chamber air was subsampled at 1.0 L min$^{-1}$ through side ports with filters containing c. 30 mg Super-Q (Alltech, Deerfield, IL, USA). Chamber air was sampled continuously during the experiments, replacing the filters every 24 h. As we were using air contact as the experimental treatment and then measuring volatile production as a response variable, care was exercised to avoid artificially concentrating the volatiles transmitted from treatment to target leaves. A connector was fitted to the inlet port of each experimental target leaf to mix a passive flow of air from the connected LPI$_{a+5}$ leaf with the incoming flow of air to the target leaf. Based on tests with concentrated, commercially available volatiles, this air tube connection regime reduced the transfer of air to c. 5–6% of the LPI$_{a+5}$ leaf chambers, which is less concentrated than would be expected in open airspace over the same distance (Harold et al. 2004). In addition, all target leaves had similar input flows at all times and experimental air tubes were disconnected 8 h before all target chambers received herbivores (Table 1).

Table 1 Timelines for herbivore additions to leaves used in experiments to test the hypothesis of within-plant wound signalling via herbivore-induced volatile emissions (experiment 1) and vascular signal transduction (experiment 2)

<table>
<thead>
<tr>
<th>Day</th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LPI$_{a+5}$</td>
<td>LPI$_{a+1}$</td>
<td>LPI$_{a+5}$</td>
</tr>
<tr>
<td>1</td>
<td>Pre-treatment</td>
<td>Pre-treatment</td>
<td>Pre-treatment</td>
</tr>
<tr>
<td>2</td>
<td><em>L. dispar</em></td>
<td>Air tube connection (randomly assigned)</td>
<td><em>L. dispar</em></td>
</tr>
<tr>
<td>3</td>
<td>No herbivores</td>
<td>Air tube removed overnight</td>
<td>No herbivores</td>
</tr>
<tr>
<td>4</td>
<td>No herbivores</td>
<td><em>L. dispar</em></td>
<td>No herbivores</td>
</tr>
<tr>
<td>5</td>
<td>No herbivores</td>
<td><em>L. dispar</em></td>
<td>No herbivores</td>
</tr>
<tr>
<td>6</td>
<td>No herbivores</td>
<td>No herbivores</td>
<td>No herbivores</td>
</tr>
<tr>
<td>7</td>
<td>No herbivores</td>
<td>No herbivores</td>
<td>No herbivores</td>
</tr>
</tbody>
</table>

The days are relative to the establishment of leaf chambers for any given experimental trial, as replicate trials of each experiment were conducted over the course of several months.

Table 2 Summary data for leaf area and consumption measurements

| Experiment 1 | | Experiment 2 | |
|--------------|---|---------------|
| LPI$_{a+1}$ (+ air tube) | LPI$_{a+1}$ (- air tube) | LPI$_{a+5}$ | LPI$_a$ | LPI$_{a+1}$ | LPI$_{a+5}$ |
| Total leaf area (cm$^2$) | 41.9 (15.3) | 38.9 (13.1) | 46.3 (12.9) | 41.6 (8.5) | 42.0 (9.3) | 46.2 (10.1) |
| Leaf mass consumed (mg) | 41.0 (37.8) | 62.9 (42.0) | 82.2 (55.2) | 43.9 (25.5) | 46.2 (26.9) | 71.9 (33.2) |
| Leaf area consumed (cm$^2$) | 6.2 (5.7) | 9.5 (6.4) | 12.7 (8.4) | 6.6 (3.7) | 7.1 (4.1) | 10.8 (5.2) |
| Leaf area consumed (%) | 13.9 (8.7) | 26.5 (20.5) | 26.4 (16.4) | 17.1 (12.2) | 18.3 (11.4) | 24.5 (11.5) |

Data are mean (± 1 SD) of 9–10 samples. There were no statistically significant differences in damage levels among treatments within either experiment. See Fig. 1 for schematic of leaf positions for each experiment.

Figure 2 Representative GC-FID chromatograms of undamaged (day 1) and gypsy moth-damaged volatile profiles. IS$_1$ = internal standard n-octane, IS$_2$ = internal standard nonyl acetate, 1 = E-β-ocimene, 2 = DMNT ([3/E]-4,8-dimethyl-1,3,7-nonatriene), 3 = β-caryophyllene, 4 = Germacrene D, 5 = α-farnesene. Compound identifications were confirmed by GC-MS.

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leaves, and data were analysed with repeated measures ANOVA (PROC GLM, SAS 8.2; SAS Institute, Cary, NC, USA) with Tukey post-hoc analysis ($\alpha = 0.05$) to determine significant differences between treatment means for each date.

RESULTS

Undamaged LPI$_{+1}$ leaves having air contact with non-orthostichous L. dispar-damaged LPI$_{+5}$ leaves were primed for defence induction (Fig. 3). In experiment 1, target leaves with prior air contact to treatment leaves responded to attack by L. dispar larvae with significantly greater terpene volatile emissions than did target leaves without such air contact ($P < 0.0001$; $F_{6,119} = 6.46$; $E$-farnesene: $F_{6,119} = 7.06$, $P < 0.0001$; DMNT: $F_{6,119} = 3.61$, $P = 0.0027$; $\beta$-caryophyllene: $F_{6,119} = 4.66$, $P = 0.0003$; Germacrene D: $F_{6,119} = 3.13$, $P = 0.0073$; $\alpha$-farnesene: $F_{6,119} = 7.34$, $P < 0.0001$). As we estimate that only c. 5–6% of the air from the treatment leaves was transmitted to the experimental target leaves, the day 4–7 increases in volatile production were not the result of volatiles carried over from the treatment leaves but rather volatiles emitted from the target leaves themselves in response to herbivore feeding. There was no indication that air contact affected systemic induction of volatiles before herbivores were physically applied to the target leaves (days 2–3), either for total volatile production or specific terpene volatiles (Fig. 3). However, all volatile compounds collected on days 2 and 3 were slightly higher in the chambers with air tubes than without, which suggests that small but detectable amounts of volatiles were transferred from the LPI$_{+5}$ treatment leaves to the experimental target leaves.

The results of the second experiment, in which there were no air connections among leaves, indicated that the saplings could and did signal via vasculature (Fig. 4). Orthostichous systemic induction (i.e. increased volatile production in systemic leaves without physical damage to the leaf) was modest but significant for total volatile production (Fig. 4a, day 3). However, the most pronounced effects of ortho-
lysis, we were interested in the systemic

\[ \text{LPI}_n \] when there was a statistical difference between \( \text{LPI}_n \) on days 4 and 5. Data units are ng cm\(^{-2}\) days, respectively, both of which received herbivores on experimental

\[ \text{LPI}_n \] asterisk (*). \( \text{LPI}_n \) mean ± SE, treatment of \( \text{L}. \text{dispar} \) observed. These results suggest that lack of vascular

interaction would indicate the response was influenced by leaf age. No effects of \( \text{LPI} \) or \( \text{LPI}* \text{orthostichy} \) were observed. Results suggest that lack of vascular connection constrained the systemic response to herbivory.

**DISCUSSION**

The goal of our experiments was to test the hypothesis that airborne volatiles mediate within-plant wound signalling. Taken together, our results provide empirical evidence that constraints on systemic signalling can be overcome by air contact with non-orthostichous herbivore-damaged leaves. While our data were collected only under laboratory conditions, the results are consistent with and provide plausible mechanisms for field observations that preventing air flow from mechanically clipped sagebrush branches results in increased herbivore damage to adjacent branches of the same plant (Karban *et al.* 2006; Shiojiri & Karban 2006). Our findings are also consistent with the recent report that lima bean HIPVs can induce extrafloral nectar production in systemic undamaged leaves (Heil & Silva Bueno 2007). Moreover, our results provide insight into the dynamics of induced responses over time and account explicitly for vascular connections among adjacent leaves (experiment 2), which was the rationale for the original within-plant signalling hypothesis (Orians 2005). Given the obvious differences in ecology and evolutionary history among the poplar, sagebrush and lima bean systems, these studies suggest collectively that within plant volatile signalling may be a widespread and ecologically important phenomenon. Indeed, the evidence for within-plant signalling mediated by volatiles is now arguably as convincing as the evidence for volatile signalling between neighbouring plants.

The mechanisms underlying within-plant signalling effects are likely the same as those for between-plant effects (e.g. Engelberth *et al.* 2004; Kessler *et al.* 2006; Ton *et al.* 2007), the only difference being that emitter and receiver leaves are on the same plant. The within-plant volatile wound signalling hypothesis accounts for the observation that distance matters for HIPV-mediated signalling. There are obvious instances where leaves on a single plant are separated by a greater distance than are adjacent plants (e.g. distant leaves of a mature tree), and those leaves would have similar distance limitations on airborne signalling. However, vascular limitations on systemic signalling to closely positioned leaves and branches provides context for within-plant mediated signalling occurring over short distances, which is what our experiments tested. Within that context, HIPVs may therefore act essentially as airborne hormones.

Herbivore-induced plant volatiles may simultaneously function as indirect defences by attracting parasitoids (Turlings *et al.* 1990; De Moraes *et al.* 1998; e.g. Hoballah & Turlings 2001) and as wound signals to systemic undamaged leaves. Both of these ecological functions appear to confer obvious fitness advantages to the emitting plant – advantages not realized by emitting plants in the case of between-plant volatile signalling. This does not exclude the possibility that volatiles also function between plants. Between-plant airborne signalling via HIPVs may comprise mainly ‘eavesdropping’, through which undamaged neighbours may benefit from detecting airborne wound signals.
and upregulating their own defences (Karban & Maron 2002). In addition, the report that parasitic plants use volatile cues for host location underscores the importance of volatiles in between-plant interactions in a broader context (Runyon et al. 2006). Overall, the within-plant volatile signalling hypothesis is entirely consistent with the between-plant signalling literature (for reviews see Baldwin et al. 2002, 2006; Dudareva et al. 2006), just operating over generally shorter distances and with clear benefits for the emitting plant.

An important aspect of our findings is the observation that HIPV-mediated wound signals can prime systemic leaves for their own responses to localized physical attack. Priming appears to be a general response of plants to a number of biotic and abiotic stresses (reviewed in Conrath et al. 2006). There is growing evidence for defence priming in plant–herbivore interactions, particularly by airborne volatiles (Engelberth et al. 2004; Heil & Kost 2006; Kessler et al. 2006; Kost & Heil 2006; Ton et al. 2007), though the molecular mechanisms and ecological significance of defence priming remain largely unknown (Dudareva et al. 2006). It is now well-established that induced plant defences have fitness costs to the plants that produce them (Gershenzon 1994; Karban & Baldwin 1997; Baldwin 1998; Redman et al. 2001; Heil & Baldwin 2002; Cipollini et al. 2003; Zavala et al. 2004), with costs even incurred by ‘eavesdropping’ plants such as wild tobacco (Karban & Maron 2002). The high cost of plant defences should select for defence strategies that are engaged only when herbivory is imminent, which is the basic evolutionary argument for the development of inducible responses to insect feeding (Karban & Baldwin 1997). Indeed, systemic induction of defensive phenotypes is wasted energy that can reduce relative fitness if herbivores do not reach systemic regions (Baldwin 1998). Priming is a logical extension of an inducible defence strategy, whereby plants or even individual leaves can respond to the recognition of a perceived threat (e.g. airborne volatiles or vascular signals) by activating preparations for defence but delaying the phenotypic deployment of defences until the threat is realized in the local environment.

A common criticism of laboratory experiments on volatile signalling is the possibility that volatiles may be artificially concentrated within enclosed experimental chambers. To avoid this problem, our leaf chambers were not airtight and the air flow was passive between treatment and target chambers. Thus, volatile transmission between the collection and target chambers was dilute. As a result, the concentrations of volatiles detected in the experimental target chambers during the treatment were not statistically distinguishable from controls, though small elevations were observed. This indicates that plants can perceive and respond to very low concentrations of volatiles. We also cannot determine from our results which compounds in the airborne blend induced priming responses in target leaves. While this is not ideal, many previous studies have also shown effects of air contact without identifying the volatile components of the air (e.g. Baldwin & Schultz 1983; Bruin et al. 1992; Karban et al. 2000, 2003, 2006; Shiojiri & Karban 2006). Both volatile terpenes and GLVs have been shown to affect defence responses (Arimura et al. 2000; Farag & Pare 2002; Farag et al. 2005), and our most recent work with poplar indicates that priming effects are observed in response to specific GLVs (Frost, unpublished data). However, we cannot discount the possibility that airborne hormones such as ethylene may play an important role (Xu et al. 1994; Voelckel et al. 2001; Ruther & Kleier 2005).

In summary, our results provide strong evidence that herbivore-induced volatiles can have a signalling function within plants and can elicit the priming of defences in undamaged leaves. This function is not incompatible with the possibility that HIPVs play a similar role in signalling between neighbouring plants. However, the short distances over which such signals have been observed to act and the apparent benefits of self-signalling for emitting plants suggest that the former function may have equal or greater ecological significance than the latter. When our findings are considered in combination with recent work on sagebrush (Karban et al. 2006) and lima bean (Heil & Silva Bueno 2007), they provide considerable evidence that within-plant signalling via HIPVs may be a common and ecologically important phenomenon.

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**SUPPLEMENTARY MATERIAL**

The following supplementary material is available for this article:

**Figure S1** (a) Leaf volatile collection chambers designed for push-pull collection with slight positive pressure inside to isolate the air space. In the current studies, 2.5 L/min charcoal-filtered air was pushed into the chamber via flexible Teflon tubing and 1.0 L/min pulled out through a SuperQ column. The Teflon frame was machined with grooves such that the bottom plate of glass is fixed in position, while the upper plate slides to open, (b) Simultaneous collection from LPI and LPI leaves. The petioles of the two samples are visibly non-orthostichous to one another.

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