

# Defense-Inducing Volatiles: In Search of the Active Motif

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**Abstract** Herbivore-induced volatile organic compounds (VOCs) are widely appreciated as an indirect defense mechanism since carnivorous arthropods use VOCs as cues for host localization and then attack herbivores. Another function of VOCs is plant–plant signaling. That VOCs elicit defensive responses in neighboring plants has been reported from various species, and different compounds have been found to be active. In order to search for a structural motif that characterizes active VOCs, we used lima bean (*Phaseolus lunatus*), which responds to VOCs released from damaged plants with an increased secretion of extrafloral nectar (EFN). We exposed lima bean to (*Z*)-3-hexenyl acetate, a substance naturally released from damaged lima bean and known to induce EFN secretion, and to several structurally related compounds. (*E*)-3-hexenyl acetate, (*E*)-2-hexenyl acetate, 5-hexenyl acetate, (*Z*)-3-hexenylisovalerate, and (*Z*)-3-hexenylbutyrate all elicited significant increases in EFN secretion, demonstrating that neither the (*Z*)-configuration nor the position of the double-bond nor the size of the acid moiety are

critical for the EFN-inducing effect. Our result is not consistent with previous concepts that postulate reactive electrophile species (Michael-acceptor-systems) for defense-induction in Arabidopsis. Instead, we postulate that physicochemical processes, including interactions with odorant binding proteins and resulting in changes in transmembrane potentials, can underlie VOCs-mediated signaling processes.

**Keywords** Herbivore-induced volatiles · Hexenyl acetate · Indirect defense · Induced defense · Plant–plant communication · Signal

## Introduction

Plants respond to herbivore damage with the release of volatile organic compounds (VOCs) that signal the presence of herbivore prey to predators and parasites and thereby serve as an indirect defense mechanism (e.g., Heil 2008). Research has demonstrated that these VOCs can also be perceived by neighboring plants or intact, systemic parts of the damaged plant (Baldwin et al. 2006; Heil 2008). Particularly, green-leaf volatiles (GLVs) have been associated with induced resistance in intact plants (Arimura et al. 2000; Engelberth et al. 2004; Farag et al. 2005; Ruther and Kleier 2005; Kost and Heil 2006;). However, little is known about the identity of VOCs that are active in this context or about a structural motif that active VOCs might have in common. In an attempt to explain the inducing activity of such VOCs on gene expression, it has been suggested that GLVs with an  $\alpha,\beta$ -unsaturated carbonyl group such as (*E*)-2-hexenal can trigger defense responses in Arabidopsis through their activity as reactive electrophile species (Almeras et al. 2003).

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In the present study, we used lima bean (*Phaseolus lunatus* L.) to search for traits that characterize defense-inducing VOCs. Lima bean responds to herbivore damage with the jasmonate-mediated production of VOCs and extrafloral nectar (EFN), an aqueous, sugar-containing secretion on nonreproductive plant organs that attracts predatory arthropods (mainly ants). The natural blend of VOCs that is released from a herbivore-damaged lima bean induces EFN secretion in intact neighboring plants (Kost and Heil 2006) and serves as a within-plant signal (Heil and Silva Bueno 2007). Among the quantitatively dominant VOCs that are released from induced lima bean, however, only (*Z*)-3-hexenyl acetate significantly induced EFN secretion when used as pure compound (Kost and Heil 2006). In the present study, we applied different structurally related esters to lima bean plants and monitored their EFN secretion.

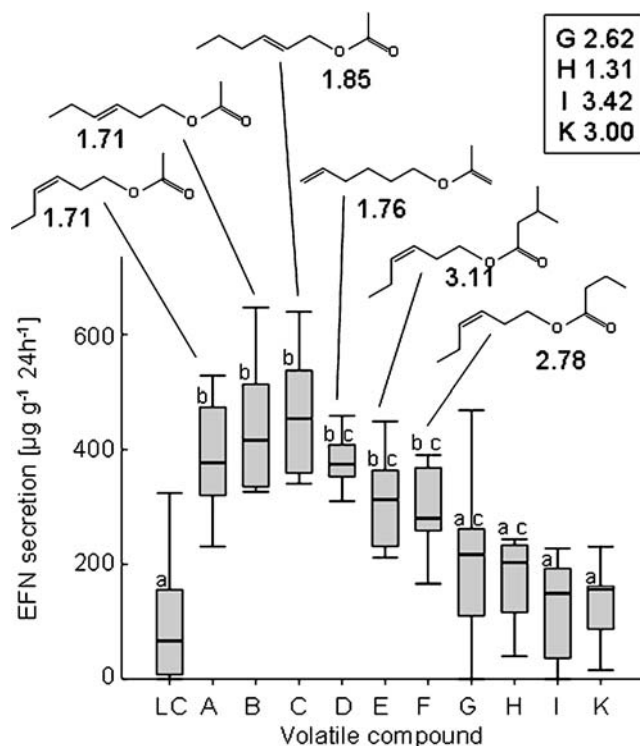
## Materials and Methods

Plants were cultivated from seeds collected in the coastal area of Puerto Escondido, Oaxaca, México (15°55.596 N and 097°09.118 W, elevation 15 m). Seedlings were cultivated under ambient conditions in 250 ml pots filled with soil from the original growing site. The plants were watered daily and fertilized 6 weeks after germination with commercial fertilizer: 10 ml per plant of a solution of 3 mg l<sup>-1</sup> of “Fertilisante foliar de alta concentración” (Grupo Bioquímico Mexicano, Aaltillo, Coah., Mexico). Experiments were conducted with plants of an age of 8–10 weeks. The following compounds were dissolved in Lanolin paste (all at 0.1 μg μl<sup>-1</sup>): (A) (*Z*)-3-hexenyl acetate, (B) (*E*)-3-hexenyl acetate, (C) (*E*)-2-hexenyl acetate, (D) 5-hexenyl acetate, (E) (*Z*)-3-hexenyl isovalerate, (F) (*Z*)-3-hexenyl butyrate, (G) 2-ethylhexanol, (H) (*E*)-2-hexanal, (I) decanal, and (K) nonanal (Lanolin paste and all compounds were purchased from Sigma Aldrich). Each 0.25 g paste per plant was applied on green plastic stripes (MAX Bindeband) attached to the plant in order to avoid direct contact of the paste with the plant. The plants were then packed in perforated PET foil bags (Bratenschlauch, Toppits, Minden, Germany) and in nets to protect them from EFN consumers. Amounts of VOCs released from the lanolin paste into the plants’ headspace under these conditions were monitored in a parallel experiment that used a closed-loop stripping system as described previously (Kost and Heil 2006). EFN secretion was quantified from the five youngest leaves 24 h later. In short, EFN concentration was measured with a portable refractometer, and nectar volume was measured with glass capillaries (Kost and Heil 2006) to calculate the total amount of soluble solids secreted, which in the case of lima bean EFN are mainly glucose, fructose, and sucrose.

Leaves were then collected and dried to calculate EFN secretion as soluble solids secreted per gram leaf dry mass and per 24 h. Plants to which Lanolin paste without any compound added had been applied served as controls (Lanolin control, LC). Per day, two groups each comprising all 11 treatments were investigated, and in total eight plants per treatment were used.

## Results and Discussion

Amounts of VOCs released from the lanolin paste into the plant’s headspace resembled those released from an induced plant, i.e., all individual VOCs were present at 80–130% of the amount of (*Z*)-3-hexenyl acetate that is released under comparable conditions from five induced leaves of lima



**Fig. 1** EFN secretion in μg soluble solids secreted per gram leaf dry mass and per 24 h is depicted. Plants were exposed for 24 h to (A) (*Z*)-3-hexenyl acetate, (B) (*E*)-3-hexenyl acetate, (C) (*E*)-2-hexenyl acetate, (D) 5-hexenyl acetate, (E) (*Z*)-3-hexenyl isovalerate, (F) (*Z*)-3-hexenyl butyrate, (G) 2-ethylhexanol, (H) (*E*)-2-hexanal, (I) decanal, and (K) nonanal dissolved in Lanolin paste. Plants to which pure Lanolin paste had been applied served as controls (LC). Lower and upper whiskers represent the 5% and the 95% percentile, lower and upper margins of boxes the 25% and the 75% percentile, the lines within boxes indicate medians. Different letters appearing above boxes mark treatment effects that differ significantly from each other ( $P < 0.05$  according to LSD posthoc analysis), and structures of compounds leading to a significant induction of EFN secretion as compared to a control are graphically presented. LogP values of all compounds were calculated with ChemDraw Ultra 6.0 and are given under the structures (compounds A–F) and in the insert (G–K)

bean (data not shown, for amounts of VOCs released from an induced plant see Kost and Heil 2006). The different VOCs to which lima bean plants had been exposed affected their EFN secretion significantly (General linear model:  $F_{77,10}=10.05$ ,  $P<0.001$ ). Compounds A–F consisted of acyl hexenols in which the configuration and position of the double bond was systematically shifted from the polar head to the aliphatic terminus of the molecule. Moreover, the importance of the size of the acyl moiety was evaluated by using acetates, butyrate, and isovalerate of (*Z*)-3-hexenol. Although only one of the tested VOCs is naturally released from lima bean, exposure to the six compounds (A–F) elicited EFN secretions that were significantly higher than those of the controls (LSD posthoc analysis:  $P<0.05$ , see Fig. 1). These compounds were (*Z*)-3-hexenyl acetate, (*E*)-3-hexenyl acetate, (*E*)-2-hexenyl acetate, 5-hexenyl acetate, (*Z*)-3-hexenyl isovalerate, and (*Z*)-3-hexenyl butyrate. Hence, EFN secretion by lima bean can be induced by VOCs that are not released naturally from this species, while several VOCs that are released from induced plants did not significantly change EFN secretion in previous experiments (Kost and Heil 2006).

How are VOCs perceived by plants, and via which mechanisms do they affect defense expression patterns? It has been suggested that molecules with an  $\alpha,\beta$ -unsaturated carbonyl group can trigger defenses in *Arabidopsis* through their activity as reactive electrophile species (Almeras et al. 2003). In principle, VOCs could also be perceived by binding to specific receptor-proteins or to odorant binding proteins with a preference for a class of compounds, similar to animal olfactory systems.

Apparently, neither the configuration nor the position of the double bond is a critical factor. Compounds reported to prime or induce gene activity or phenotypic defenses in intact corn plants comprise (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenal, and (*Z*)-3-hexenyl acetate (Engelberth et al. 2004; Farag et al. 2005; Ruther and Kleier 2005). We found that (*Z*)-3-hexenyl acetate, (*E*)-3-hexenyl acetate, (*E*)-2-hexenyl acetate, and 5-hexenyl acetate all elicited particularly high EFN secretion rates in lima bean. The majority of these substances lack an  $\alpha,\beta$ -unsaturated carbonyl group, which thus cannot be a generally required motif. In addition, a compound such as 5-hexenyl acetate cannot yield an electrophile such as (*E*)-2-hexenal via the sequence of ester hydrolysis, oxidation, and isomerization, as it is principally possible for (*E* or *Z*)-3-hexenyl acetate or (*E* or *Z*)-2-hexenyl acetate. Moreover, unlike (*Z*)-3-hexenyl acetate the potential end product of the transformation sequence, (*E*)-2-hexenal, had only a weak effect on the nectar flow. Similarly, the observation that (*Z*)-3-hexenyl acetate and (*E*)-3-hexenyl acetate elicited almost identical EFN secretion rates contradicts the involvement of classical receptor proteins, since these usually require a specific stereochem-

istry of the interacting molecule. We further calculated LogP values to check for putative importance of the compound's lipophilicity but did not find any clear relation between the EFN-inducing activity of a compound and its octanol-water partition coefficient (Fig. 1).

More studies are required to understand which VOCs induce plant defenses via which mechanism. However, our results point to a new mechanism. Changes in transmembrane potentials—occurring through modulations of ion fluxes—are involved in early signaling events in the cellular response to stress (Maffei et al. 2007), and exposition to VOCs indeed changes membrane potentials in intact lima bean leaves (M. Maffei, personal communication). It is thus tempting to speculate that the dissolving of VOCs in the membranes coupled to interactions with membrane proteins, similar to the odorant binding proteins of insects (Campanacci et al. 2001), leads to changes in transmembrane potentials and thereby induces gene activity. This hypothesis gains support from the observation that the induction of EFN secretion by VOCs appears to be a gradual one rather than a clear 'yes-or-know' response. Several of the compounds that are released naturally from lima bean showed a trend towards an increase in EFN secretion, although the difference was not significant: EFN secretion in response to Linalool was on average higher by 40% than in control plants, and DMNT ((3*E*)-4,8-dimethylnona-1,3,7-triene) increased EFN secretion by 50% (see Table 1 in Kost and Heil 2006). Similarly, plants exposed to ethylhexanol or (*E*)-2-hexenal in the present study showed a trend towards higher EFN secretions than controls (Fig. 1). EFN responds to a comparably wide variety of structures, and slight changes in the molecular structure gradually alter the induction effect. This observation is best explained by a comparably simple physicochemical process, whose detailed nature remains to be elucidated.

How can signals evolve that apparently lack chemical specificity? The answer might be simply that plants seldom are exposed to GLVs or chemically related compounds that are not released from attacked plants. In evolutionary terms, the probability of this situation was probably too low to cause any selection towards a more specific signal perception.

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