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Induced defenses of *Veronica spicata*: Variability in herbivore-induced volatile organic compounds

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ABSTRACT

Plants release volatile organic compounds (VOCs) that have many eco-physiological functions. Induction of plant VOCs is known to occur upon herbivory. Herbivore-induced VOCs are involved in the attraction of predators and parasitoids, a phenomenon known as an indirect defense of plants. We measured the VOC profiles of the wild species *Veronica spicata* with and without larval feeding and oviposition by the specialist butterfly *Melitaea cinxia*. *V. spicata* showed great plasticity when deploying indirect defences. The induction of several ubiquitous terpenoids and green leaf volatiles (GLVs) was associated with larval feeding, whereas the increase of two ketones, 6-methyl-5-hepten-2-one and t-geranylacetone and the suppression of GLVs were associated with oviposition by the butterfly.

1. Introduction

Plants emit volatile organic compounds (VOCs) that include an array of terpenoids, fatty acid derivatives including lipoxygenase pathway products, benzenoids and phenylpropanoids, C5-branched compounds, and several nitrogen and sulfur containing compounds (Dudareva et al., 2004). Plant VOCs are involved in many eco-physiological functions. They protect the plant against environmental pollution (Loreto et al., 2001), heat (Peñuelas and Llusià, 2003) and biotic stresses such as pathogens (Huang et al., 2012) and herbivorous insects (McCallum et al., 2011). They also mediate several biotic interactions, acting as cues for foraging herbivores (Bruce et al., 2005) as well as for their natural enemies (Clavijo McCormick et al., 2012), and allowing communication with conspecifics (plant–plant interactions) (Kost and Heil, 2006). In addition to being emitted constitutively, several volatile compounds, particularly terpenoids and some alcohols, aldehydes and ketones (so-called green leaf volatiles, GLVs) can be induced. Their concentrations can increase or decrease in response to environmental stimuli such as mechanical wounding (Mithöfer et al., 2005), environmental pollution (Vuorinen et al., 2004), pathogenic infection (Rostás et al., 2006), as well as herbivore feeding (Pinto et al., 2007) and egg deposition (Fatouros et al., 2012). Herbivore-induced VOCs attract natural enemies e.g. predators and parasitoids towards their prey or host, a phenomenon regarded as an indirect defense of plants (Kessler and Heil, 2011). These compounds offer reliable information for natural enemies, not only in clean air but also in conditions of environmental pollution (Pinto et al., 2007).

Spiked speedwell (*Veronica spicata* L.) (Lamiales: Plantaginaceae) is a self-compatible, protogynous and insect-pollinated perennial that contains high quantities of iridoid glycosides (Suomi et al., 2002). In Finland, it is one of the two hosts of the Glanville fritillary butterfly *Melitaea cinxia* (Linnaeus, 1758) (Lepidoptera: Nymphalidae), a Eurasian species that is endangered in or extinct from part if its range. The butterfly, its host plants and its natural enemies are an important model system for the study of population and community in ecology in fragmented landscapes (van Nouhuys and Hanski, 2005), and multitrophic level interactions in a spatial context (van Nouhuys and Kraft, 2012). The two specialist parasitoid wasps of *M. cinxia* in Åland (Finland) are *Cotesia melitaearum* (Wilkinson) (Hymenoptera: Braconidae) that

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lays gregarious broods in *M. cinxia* larvae (van Nouhuys and Lei, 2004), and *Hyposoter horticola* (Gravenhorst, 1829) (Hymenoptera: Ichneumonidae) that is solitary and lays eggs in first instar host larvae when still in the eggshell (van Nouhuys and Ehrnsten, 2004). Since the two parasitoids forage for different stages (larvae and eggs), they may rely on different herbivore-induced cues to locate the host. Herbivore-induced VOCs may be responsible for the large attraction of *C. melitaearum* to infested *V. spicata* observed in the field (van Nouhuys and Hanski, 1999), and there is evidence that *H. horticola* responds to volatiles emitted from oviposition-induced VOCs (Castelo et al., 2010). This species has a particularly short time window to parasitize its host and monitors the eggs for weeks (van Nouhuys and Kaartinen, 2008). However characterization of VOC emissions by *V. spicata* has not been accomplished. Studies on agricultural crops have shown variation of herbivore-induced VOCs, but few have compared the volatiles emitted after oviposition and larval feeding (Colazza et al., 2004), and there are few studies of natural rather than agricultural species (for example, Kessler and Baldwin, 2004).

2. Results and discussion

The compounds detected in the headspace of *V. spicata* and their emission rates for each treatment are shown in Table 1. Herbivore-induced VOC blends include emissions of constitutive compounds which may originate from storage pools in the plants and compounds synthesized *de novo* (Paré and Tumlinson, 1997). Results from the PCA showed that 70% of the variation of the data was explained by 3 PCs. 1-penten-3-ol, (*Z*)-3-hexen-1-ol, 1-octen-3-ol, (*Z*)-3-hexenylacetate, hexylacetate, (*E*)-2-hexenyl acetate, and the terpenoids β-pinene, limonene, 1,8-cineole, linalool, (*E*)-DMNT, α-bergamotene/β-farnesene, longicyclene, β-caryophyllene, α-humulene and alloaromadendrene were the compounds most associated with larval feeding, while two ketones 6-methyl-5-hepten-2-one and t-geranylacetone were the most associated with oviposition (PC1, MANOVA, $F_{2,16}$, $P < 0.001$) (Fig. 1). *De novo* biosynthesis of the terpenoids (*E*)-DMNT, α-humulene and alloaromadendrene occurred after larval feeding since these compounds were not detected in the headspace of undamaged plants or in the headspace of plants with egg masses on them. Of these (*E*)-DMNT is one of the most common VOCs emitted after herbivore feeding (Turlings and Wäckers, 2004 for a review). In contrast, plants with egg masses on them emitted significantly less (*Z*)-3-hexen-1-ol, and the emission of (*E*)-2-hexenylacetate was totally suppressed. Suppression of compounds, particularly GLVs appears to be a response to oviposition, and possibly changes in ratios may result in recognition of VOC blends by parasitoids (Bruce et al., 2010).

Removing the eggs masses from the leaves affected the emission rates of (*Z*)-3-hexen-1-ol (Wilcoxon Signed-Rank test, $P = 0.028$), camphene (Wilcoxon Signed-Rank test, $P = 0.043$) and 1-octen-3-ol (Wilcoxon Signed-Rank test, $P = 0.046$), but not other compounds (Table 1). In our study, because plants with eggs were not manipulated before sampling, and because in nature the plants

Table 1
VOC emissions (ng g DW^{-1} h^{-1}) by *Veronica spicata* undamaged, damaged by larval feeding or exposed to eggs depositions by the Glanville Fritillary butterfly (GFB) *M. cinxia* (Mean ± S.E.).

Compound	R.T. (min)	Undamaged ($n = 6$)	GFB-damaged ($n = 7$)	GFB-eggs ($n = 6$)	GFB-eggs removed ($n = 6$)
Aliphatic compounds					
1-Penten-3-ol	8.61	7.84 ± 2.62	23.73 ± 6.68	3.08 ± 0.75	2.05 ± 0.35
Hexanal	12.95	687.08 ± 289.93	687.93 ± 132.41	677.29 ± 178.11	759.78 ± 146.91
(*Z*)-3-hexen-1-ol	15.24	101.17 ± 36.10	399.36 ± 128.63	7.92 ± 3.31	1.32 ± 0.79
(*E*)-2-hexen-1-ol	15.58	1.12 ± 0.51	4.66 ± 1.42	2.11 ± 0.87	2.43 ± 0.70
Hepanal	17.27	61.16 ± 16.21	116.08 ± 25.34	281.80 ± 98.28	371.09 ± 88.28
1-Octen-3-ol	20.58	1.19 ± 0.51	35.06 ± 12.77	1.47 ± 1.14	6.87 ± 3.16
6-Methyl-5-hepten-2-one	20.73	19.44 ± 9.84	38.08 ± 10.41	107.01 ± 23.56	92.44 ± 30.69
(*Z*)-3-hexenylacetate	21.58	199.14 ± 97.41	808.91 ± 370.47	14.82 ± 7.22	2.96[a]
Octanal	21.67	41.56 ± 13.37	76.67 ± 23.07	198.53 ± 45.98	218.96 ± 42.55
Hexylacetate	21.80	6.73 ± 4.30	28.15 ± 12.90	1.51 ± 0.31	1.10 ± 0.53
(*E*)-2-Hexenylacetate	21.88	0.51 ± 0.32	2.87 ± 1.05	–	0.14[a]
Nonanal	25.89	123.87 ± 72.90	252.44 ± 105.91	185.60 ± 75.13	153.66 ± 94.28
Decanal	29.26	192.62 ± 54.72	294.03 ± 133.89	682.55 ± 184.31	480.57 ± 108.25
Bornylacetate	31.75	–	0.049[a]	0.017[a]	0.042[a]
t-Geranylacetone	34.96	34.61 ± 8.33	74.11 ± 21.79	197.61 ± 46.92	188.791 ± 56.299
Terpenoids					
α-Pinene	19.15	10.29 ± 1.89	12.70 ± 3.58	11.34 ± 2.63	11.19 ± 1.52
Camphene	20.00	0.04 ± 0.02	0.04 ± 0.02	0.61 ± 0.37	1.20 ± 0.48
Myrcene	21.06	0.25 ± 0.04	0.26 ± 0.06	0.15 ± 0.05	0.25 ± 0.04
β-Pinene	21.22	2.12 ± 0.78	2.31 ± 0.80	0.46 ± 0.11	0.46 ± 0.06
Carene	22.37	3.61 ± 0.72	5.45 ± 1.87	3.47 ± 0.71	3.36 ± 0.36
p-Cymene	22.98	0.34 ± 0.04	0.45 ± 0.13	0.45 ± 0.12	0.47 ± 0.11
Limonene	23.22	3.03 ± 1.09	3.38 ± 1.55	1.19 ± 0.20	1.68 ± 0.50
1,8-Cineole	23.46	0.09 ± 0.04	0.06 ± 0.03	–	–
(*Z*)-DMNT	25.47	0.02[a]	0.03 ± 0.02	–	–
Linalool	25.73	0.54 ± 0.34	1.26 ± 0.39	0.21 ± 0.17	0.13 ± 0.09
(*E*)-DMNT	26.17	–	0.06 ± 0.03	–	0.02[a]
Longicyclene	34.35	0.04 ± 0.02	0.07 ± 0.03	0.008[a]	0.115 ± 0.085
Iso-longifolene	34.76	–	0.012[a]	–	–
α-Bergamotene/β-farnesene	35.02	0.23 ± 0.21	1.01 ± 0.34	–	–
β-Caryophyllene	35.13	0.45 ± 0.17	78.23 ± 48.95	0.163 ± 0.104	–
α-Humulene	35.82	–	4.76 ± 3.33	–	–
Alloaromadendrene	35.91	–	3.20 ± 1.32	–	–
Phenylpropanoid/bezenoids					
4-Allylanisole	29.34	–	0.011[a]	–	–
Methyl salicylate (MeSA)	29.39	0.023 ± 0.02	0.16 ± 0.10	0.11 ± 0.08	0.463 ± 0.325

[a] These compounds were emitted by a single plant.

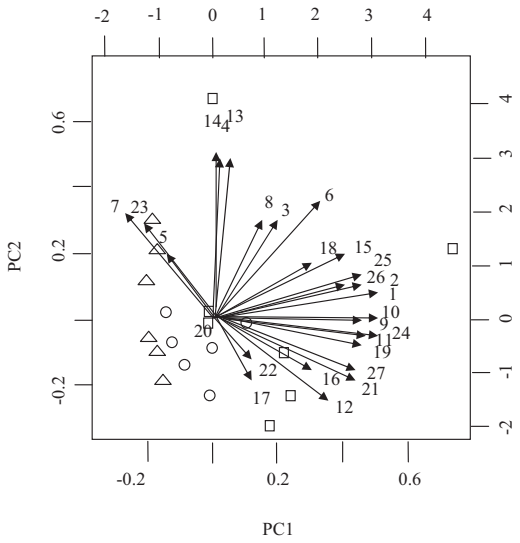

Fig. 1. Score of individual of *Veronica spicata* undamaged (○), damaged by larval feeding (□) or exposed to eggs depositions (△) by the Glanville Fritillary butterfly (GFB) *M. cinxia*, and emitted volatile organic compounds, VOCs, (biplot) projected on the first and second principal components. The vectors are the loadings for each VOC (numbered), and the length of each vector represents the relative magnitudes of its importance in differentiating treatments. VOCs (1) 1-penten-3-ol, (2) (*Z*)-3-hexen-1-ol, (3) (*E*)-2-hexen-1-ol, (4) α-pinene, (5) camphene, (6) 1-octen-3-ol, (7) 6-methyl-5-hepten-2-one, (8) myrcene, (9) β-pinene, (10) (*Z*)-3-hexenylacetate, (11) hexylacetate, (12) (*E*)-2-hexenylacetate, (13) carene, (14) p-cymene, (15) limonene, (16) 1,8-cineole, (17) (*Z*)-DMNT, (18) linalool (19) (*E*)-DMNT, (20) MeSA (21) longicyclene; (22) iso-longifolene, (23) t-geranylacetone, (24) α-bergamotene/β-farnesene, (25) β-caryophyllene, (26) α-humulene, (27) alloaromadendrene.

are found with eggs on them rather than removed from them, we conducted the statistical analyses with the emission rates of the plant–host complex (plant with eggs). However, the fact that most of the compounds remain unaffected suggests that the quantifications reported here resulted from the induction of the plant rather than from the compounds emitted by eggs themselves. Oviposition upon the surface of the leaf has been shown to induce large changes in local accumulation of secondary metabolites (Peñuelas et al., 2006), and more recently to induce changes in gene expression (Kim et al., 2012) and plant volatile emission that influence parasitoid behaviour (Fatouros et al., 2012).

Our results show that *V. spicata* is plastic when deploying indirect defences, exhibiting a different volatile profile when undamaged, damaged by herbivory, and oviposited upon. The induction of several terpenoids and GLVs that are associated with herbivory may contribute to the indirect defences of *V. spicata* against the herbivore *M. cinxia* by attracting the parasitoid *C. melitarearum*. Whereas suppression of GLVs and induction of ketones may be partially responsible for the orientation of *H. horticola* to plants with *M. cinxia* eggs on them. Further studies are needed to assess the bioactivity of these compounds.

3. Experimental

3.1. Living material used in the bioassays

Seeds from *V. spicata* collected from natural habitat patches in the Åland Islands (Southwest Finland, 60°9′0″ N/20°0′0″ E), were sown in a mixture of soil (Kekkilä Oy, Finland) and vermiculite (5:1) in plastic pots (9.5 cm × 9.5 cm × 9 cm) and grown under greenhouse conditions. A few weeks before the experiments, the seedlings were thinned to one plant per pot. At the time of the experiments, plants were 13-weeks old. The larvae and egg clusters of *M. cinxia* were obtained from laboratory colonies maintained in Åland and at the Lammi biological station in Finland.

3.2. Larval feeding

The *M. cinxia* larvae used in the experiments were in their 5th instar, just after winter diapause. Before use they were reared in the laboratory and then kept in diapause for five months at 2 °C under 6:18 h L/D photoperiod until one week before starting the experiments. The diapause was broken by gently spraying the larvae with water. After that, the insects were kept under ambient conditions feeding on *Plantago lanceolata*. Ten gregarious larvae were transferred onto the leaves of each plant and allowed to feed for 48 h. During this period the infested plants were kept enclosed in a net to prevent the larvae from escaping. Larvae and frass were carefully removed from the plants just prior VOC collection. The undamaged plants with no larvae feeding on them were also enclosed in a net as a control.

3.3. Egg deposition on plants

In order to get plants with egg clusters on them, a mated female butterfly was placed in a sleeve cage with a potted *V. spicata* for 6 h. Because behavioural tests have shown that the parasitoid *H. horticola* is strongly attracted to plants with one week old eggs on them (Castelo et al., 2010), we collected VOCs of the infested plants one week after oviposition.

3.4. VOC collection

Each plant pot was carefully wrapped in aluminium foil to minimize the collection of VOCs from the soil and the plastic pot. The potted plant was then placed on a Teflon sheet and covered by a 10-L glass bell-shaped vessel (Simax, Czech Republic), making an effort not to damage the plant. Ambient air was passed through a charcoal filter (PALL Carbon capsule) and pumped into the vessel. Before collecting VOCs, the air was allowed to change twice (20 min). VOCs were collected for 45 min into tubes containing a Tenax-Carbopack-B mix of adsorbent with a vacuum pump (Thomas, model 2107CD20, Thomas, WI, USA). Critical orifices attached to the pump were used to keep the flow constant. The incoming (1 L/min) and outgoing (0.1 L/min) airflows were measured twice or three times per day with a portable device (TSI Incorporated 4100 Series, MN, USA). We collected VOCs from the headspace of the following treatments: undamaged (control) plants ($n = 6$), herbivore-damaged plants ($n = 7$), plants with a one-week old egg cluster on them (plant–host complex) ($n = 6$) and plants after removing the egg cluster ($n = 6$) (Table 1). For the oviposition treatment, the 1-week old *M. cinixa* eggs were carefully removed from the leaves with a fine brush. Then the leaves were washed with Milli-Q water to remove residues and water-soluble compounds from the egg deposition, and gently dried with a paper towel. This procedure was done approximately 30 min before sampling started. The experiments were conducted between 9 am and 4 pm. The glass vessels were cleaned at the end of the day with ethanol, and left to dry overnight. Glass is an inert material, and adsorption of volatiles on the surface can be neglected (Stewart-Jones and Poppy, 2006). Nevertheless, we only collected 2 or 3 samples per treatment per day, and vessels were used for only one treatment. Twice a day samples were taken from the incoming air to monitor the purity of the samples.

3.5. VOC analyses

The samples were analyzed using a thermal desorption instrument (Perkin-Elmer TurboMatrix 650, Waltham, USA)

attached to a gas chromatograph (Perkin-Elmer Clarus 600, Waltham, USA) with a DB-5MS (60 m, 0.25 mm, 1 μm) column and a mass selective detector (Perkin-Elmer Clarus 600T, Waltham, USA). The sample tubes were desorbed at 300 °C for 5 min, cryofocused in a Tenax cold trap (−30 °C) prior to injecting the sample into the column by rapidly heating the cold trap (40 °C min^{-1}) to 300 °C. A five-point calibration was made using liquid standards in methanol solutions. Standard solutions were injected onto adsorbent tubes and flushed with a helium or nitrogen flow (100 ml min^{-1}) for 5 min to remove methanol. The emitted compounds were quantified by comparison with commercially available reference substances or synthesized (*E*)-4,8-dimethyl-1,3,7-nonatriene ((*E*)-DMNT). Hexanal was quantified by proposing the response to be the same as the response of heptanal. Likewise t-geranylacetone and α-bergamotene/β-farnesene were quantified by proposing the responses to be the same as the response of β-caryophyllene. The identification of the compounds was based on mass-spectra and retention times of the authentic standards except for *t*-geranylacetone and α-bergamotene/β-farnesene, which were identified based on mass-spectra alone. Emissions were expressed in nanograms per gram of dry weight (DW) per hour (plants dried at 60 °C for 48 h). The methodology has been described in more detail in Hellén et al. (2012).

3.6. Statistical analyses

Principal components analysis (PCA) was used to compare *V. spicata* that were undamaged with those receiving herbivore damage or egg depositions. Variables were transformed into principal components (PCs), and PCs explaining over 70% of the variance, based on the Broken Stick Distribution, were analyzed using MANOVA. PCA was performed using the R Software package (R Development Core Team, 2007). Prior to the above analyses, we compared the individual compounds in the headspace of *V. spicata* plants before and after egg removal. This was done to identify compounds that may have been emitted by the eggs themselves, rather than by the plants. For this, the data were log transformed (Log 10(x + 2)), and analyzed using a paired-samples *T*-test. Those data that were not normally distributed even after transformation were analyzed using the non-parametric Wilcoxon rank-signed test for related-samples.

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